DMD Fast Forward. Published on April 2, 2008 as doi:10.1124/dmd.107.020016

DMD #20016

Evaluation of HepaRG Cells as an *In Vitro* Model for Human Drug Metabolism Studies

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DMD Fast Forward. Published on April 2, 2008 as DOI: 10.1124/dmd.107.020016
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Running title: Drug metabolism in HepaRG cells

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Number of text pages: 26

Number of tables: 1

Number of figures: 5

Number of references: 20

Number of words in the Abstract: 245

Number of words in the Introduction: 331

Number of words in the Discussion: 1132

Abbreviations: DMSO, dimethyl sulfoxide; P450, cytochrome P450; RT-PCR, reversed transcription-polymerase chain reaction; AoD, Assay-on-Demand Gene Expression assays; HSM, hepatocyte suspension media; CL_{int}, intrinsic clearance; LC/MS, liquid chromatography/mass spectrometry; UGT, UDP-glucuronosyltransferases

Abstract

HepaRG cells, a newly developed human hepatoma cell line, differentiate into hepatocyte-like morphology by treatment with dimethyl sulfoxide (DMSO). The expression of cytochrome P450 (P450) enzymes, transporter proteins and transcription factors were stable in differentiated HepaRG cells over a period of six weeks when cultured with DMSO. Compared with human hepatocytes, expression of P450s in HepaRG cells was in general lower with the exception for a considerably higher expression of CYP3A4 and CYP7A1. The expression of P450s generally decreased when DMSO was removed from the medium, whereas transporters and liver specific factors were unaffected. The relative mRNA content of drug metabolising P450s displayed the highest resemblance between human hepatocytes and differentiated HepaRG cells one day after removal of DMSO from the medium. The metabolism of midazolam, naloxone, and clozapine in HepaRG cells was similar to human hepatocytes, indicating the function of CYP3A4, CYP1A2, and UGT enzymes. However, the metabolism of 7-ethoxycoumarin and dextromethorphan was low, confirming low levels of CYP2E1 and CYP2D6 in HepaRG cells. The P450 probe substrates indicate a decrease in CYP1A2, CYP2B6, CYP2C9 and CYP3A4 activities in HepaRG cells one day after removal of DMSO from the medium. The activities were then relatively stable in DMSO free medium for up to 14 days. Based on the stable expression of liver specific functions over a long period in culture, the relative mRNA content of drug metabolising P450s and metabolic properties, HepaRG cells provide a valuable *in vitro* model for human drug metabolism studies.

Recently a new human hepatoma cell line, HepaRG, was derived from a hepatocellular carcinoma. Seeded at low density HepaRG cells emerge as two cell types when they reach confluence. One is flattened, retains a clear cytoplasm and surrounds the other. The second forms clusters of granular epithelial cells resembling hepatocytes. Addition of 2% dimethyl sulfoxide (DMSO) and 50 µM hydrocortisone hemisuccinate to the culture medium induces differentiation of the hepatocyte-like cells into more granular cells, closely resembling typical adult primary hepatocytes with one or two nuclei and bile canaliculi-like structures. The hepatocyte-like cells represent around 50-55% of the total cell population (Cerec et al., 2007). The HepaRG cells express a large panel of liver specific genes including several cytochrome P450 (P450) enzymes such as CYP1A2, CYP2B6, CYP2C9, CYP2E1 and CYP3A4, which is in contrast to other hepatoma cell lines such as HepG2. The activity of P450s were demonstrated by using several probe substrates (Aninat et al., 2006). The levels of P450s in HepaRG cells are dependent on the duration of confluence and for most of them on the presence of DMSO in the culturing medium. The HepaRG cell line was recently found to be a valuable human relevant *in vitro* model for investigating P450 induction properties of drug compounds (Kanebratt and Andersson, 2008). The expression and function of drug transporters in differentiated HepaRG cells has also been investigated, and was demonstrated to be close to that found in primary human hepatocytes (Le Vee et al., 2006).

The aim of the present study was to characterise and compare the HepaRG cells with primary human hepatocytes regarding the mRNA expression profile and drug metabolism properties. The expression of 44 genes coding for drug metabolising enzymes, transporters, and liver specific factors, were studied during differentiation and over a period of time in differentiated cells. Since DMSO is used to differentiate and keep the hepatocyte like features, we investigated the stability of the differentiated cells during DMSO treatment and after a period without DMSO exposure.

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Materials and methods

Chemicals. Clozapine, dextromethorphan, diclofenac, DMSO, naloxone, propranolol, Williams' medium E without phenol red, and 7-ethoxycoumarin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Paracetamol and phenacetin were purchased from Aldrich Chemical Co. (St Louis, MO, USA). Foetal bovine serum, SuperScript III First-Strand Synthesis System for reversed transcription-polymerase chain reaction (RT-PCR), Trizol, and Williams' medium E with phenol red were obtained from Invitrogen (Carlsbad, CA, USA). 4'-hydroxydiclofenac was obtained from BD Gentest (Woburn, MA, USA) and 1'-hydroxymidazolam was purchased from Ultrafine (Manchester, UK). Midazolam was purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Bupropion was purchased from Kemprotec Ltd. (Middlesbrough, UK) and hydroxybupropion was provided by Toronto Research Chemicals Inc. (North York, ON, Canada). Taqman Assay on Demand, preloaded 384-well cards, and Taqman® Universal Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). Cryopreserved primary human hepatocytes were purchased from In Vitro Technologies (Baltimore, MD, USA). All other chemicals were of analytical grade and highest quality available.

Cell Culture. HepaRG cells were seeded at 50 000 cells per well in 24-well plates and 9000 cells per well in 96-well plates in growth medium composed of Williams' medium E with glutamax-I, supplemented with 10% foetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml bovine insulin, and 50 µM hydrocortisone hemisuccinate. After 2 weeks the cells were shifted to the same medium supplemented with 2% DMSO (differentiation medium). The medium was renewed every 2 to 3 days. For the initial experiment on gene expression over time in culture the cells were cultured at Biopredic International (Rennes, France), and harvested in Trizol reagent, frozen and then shipped to AstraZeneca R&D Mölndal. For subsequent experiments the differentiated HepaRG cells (passages 13 to 19) were purchased from Biopredic International and sent to AZ in 24- or 96-well plates. The cells were cultured in differentiation medium for 3 weeks before shipment to AstraZeneca R&D Mölndal. At

arrival the medium was renewed and the cells were given 24 hours to recover before any experiment. For designated cells the medium was then changed to basal HepaRG medium (Williams' medium E with glutamax-I, supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 µg/ml bovine insulin, and 50 µM hydrocortisone hemisuccinate). The cells were cultured in the basal medium for 1, 5, or 14 days before experiments, and the medium was renewed every 24 to 48 hours.

For mRNA measurements, the cells were harvested in Trizol reagent (0.5 ml per well in 24-well plates) and substrate depletion and metabolite formation were measured directly in 96-well plates.

Human hepatoma HepG2 cells, purchased from ATCC (American Type Culture Collection) were routinely cultured in MEM supplemented with 10% foetal bovine serum, non-essential amino acids, sodium pyruvate (1 mM), penicillin (100 unit/ml), and streptomycin (100 µg/ml). HepG2 cells were harvested when reaching confluence.

RNA Isolation and cDNA Synthesis. Total RNA from HepaRG cells, HepG2 cells, and cryopreserved human hepatocytes was prepared using Trizol reagent according to manufacturers' instructions. Quantity and purity of the RNA were determined spectrophotometrically using a GenQuant pro RNA/DNA calculator (Biochrom, Cambridge, UK). Electrophoretic separation of 0.5 µg total RNA on a 1% agarose gel run in Tris borate-EDTA buffer (0.09 M Tris-borate, 2 mM EDTA, pH 7.8) at 80 mV for 1 hour, allowed integrity assessment of the isolated RNA. Two sharp ribosomal RNA bands and absence of RNA-debris was set as a quality criterion to proceed to cDNA synthesis.

cDNA was prepared from 1 µg of total RNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR with random hexamer primers according to the manufacturer's protocol.

Real-Time PCR. 44 different genes were analysed with quantitative real-time PCR, using an ABI PRISM 7900HT or a 7500 Sequence Detector system (Applied Biosystems, Foster City, CA, USA) and manufacturer designed Assay-on-Demand Gene Expression assays (AoD) (Applied Biosystems). The set of genes were chosen to cover a broad range of drug

metabolising enzymes. The focus was on P450 enzymes, which are the main enzymes responsible for drug metabolism. In addition liver specific transcription factors and nuclear receptors determining the P450 expression were analysed (assay IDs and accession numbers are presented in the supplemental data). Standard AoD were used for detection of CYP2D6, CYP7A1, SULT2A1, OATP2B1, MRP1, PXR, CAR, and AhR. The 7900HT system was used for custom designed 384-well cards loaded with AoD, where the Taqman analysis were performed in 1 µl reaction mixture per gene containing 2 ng of RNA converted to cDNA, Taqman Universal Master Mix, and the AoD mixes containing specific primers and probes, purchased preloaded onto the card. The 7500 system was used for 96-well reaction plates, where the Taqman analysis were performed in 25 µl reaction mixture per gene containing 30 ng of RNA converted to cDNA, Taqman Universal Master Mix, and 1.25 µl AoD. For both detection systems the thermal cycle conditions comprised 2 min at 50 °C, 10 min of polymerase activation at 95 °C, followed by 40 PCR cycles alternating 95 °C for 15 s and 60 °C for 1 min. Amplification curves were analysed using the 7900HT sequence detection software SDS 2.1 (Applied Biosystems) or the 7500 Sequence detector software v1.3.1 (Applied Biosystems). The expression for all genes is normalised against the expression of glyceraldehyde 3-phosphate dehydrogenase in each sample.

Substrate Depletion. *Cryopreserved human hepatocytes*. Cryopreserved human hepatocytes were thawed according to supplier's instructions, and resuspended in hepatocyte suspension media (HSM) consisting of Williams' medium E without phenol red supplemented with 25 mM HEPES and 2 mM L-glutamine, pH 7.4. Viability was determined with the tryphan blue exclusion test and the cell suspension was diluted to 2 million viable cells/ml. Only cell suspensions with viability over 75% were used. 25 µl cell suspension were transferred to wells on a 96-well incubation plate and the plates were preincubated for 5 min in 37 °C. The reaction was started by adding 25 µl substrate solution consisting of midazolam, naloxone, 7-ethoxycoumarin, propranolol, clozapine, or dextromethorphan in HSM. The final concentration of the compounds was 1 µM, which is well below K_m for the enzyme reactions studied. The substrate solutions were prepared by solving the compounds in DMSO, further dilution was

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done in 50% acetonitrile and HSM so the final concentration of DMSO was 0.1% and acetonitrile was 1%. The cells were incubated in 37 °C and the incubation was stopped after 0, 15, 30, 45, 60, and 90 min by addition of 150 µl cold acetonitrile with 0.8% formic acid.

HepaRG cells. Differentiated HepaRG cells in 96-well plates were cultured with DMSO and without DMSO for 1, 5 or 14 days. The cells were washed twice with 100 µl HSM, where after 50 µl substrate solution was added to start the reaction. The substrate solutions were prepared as described for human hepatocytes. The cells were incubated in 37 °C and the incubation was stopped after 0, 15, 30, 45, 60, and 90 min by removing the incubation medium. For clozapine, dextromethorphan, and propranolol, the medium was transferred to another plate at the end of incubation and 150 µl cold acetonitrile with 0.8% formic acid was added to lyse the cells, where after cell suspension and incubation medium was pooled. For midazolam, naloxone, and 7-ethoxycoumarin 150 µl cold acetonitrile with 0.8% formic acid was added to the incubation medium. When only the incubation medium was analysed, the substrate depletion curve described a clear two-phase slope for propranolol, clozapine, and dextromethorphan. When both cell lysate and incubation medium was analysed, the rate of disappearance followed a straight line, indicating that the initial phase represents distribution into the cells and not metabolism for these compounds. The intrinsic clearance (CL_{int}) was therefore calculated from concentration measured in pooled cell lysate and incubation medium for propranolol, clozapine, and dextromethorphan. The rate of disappearance for midazolam, naloxone, and 7-ethoxycoumarin exhibited a linear disappearance from the medium and thus this was used as a measure for the CL_{int} for these compounds.

Metabolite Formation. *Cryopreserved human hepatocytes*. Cryopreserved human hepatocytes were handled in the same way as for substrate depletion until the start of the incubation. The incubations were performed at concentrations representing the K_m for the four substrates. The reaction was started by adding 25 µl substrate solution consisting of bupropion (final concentration 100 µM) or a cocktail of phenacetin (final concentration 26 µM), diclofenac (final concentration 9 µM), and midazolam (final concentration 3 µM) in HSM. The substrate solutions were prepared by solving the compounds in methanol and added to a Falcon tube.

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The methanol was evaporated under nitrogen gas and the compounds dissolved in HSM so that the activity medium did not include any organic solvent. After 15, 30, 45, and 60 min (for bupropion) or 5, 10, 20, 30, 45, 60, and 90 min (for the cocktail) the incubation was stopped by addition of 50 µl cold acetonitrile with 0.8% formic acid.

HepaRG cells. Differentiated HepaRG cells in 96-well plates were cultured with DMSO or without DMSO for 1, 5 and 14 days. The cells were washed twice with 100 µl HSM, where after 50 µl substrate solution was added to start the reaction. The substrate solution was prepared as described for human hepatocytes. After 15, 30, 60, and 120 min (for both bupropion and cocktail) the incubation medium was transferred to another plate and 50 µl cold acetonitrile with 0.8% formic acid was added to lyse the cells, where after cell suspension and incubation medium was pooled.

Metabolite formation samples were analysed for the CYP2B6 metabolite hydroxybupropion or the CYP1A2 metabolite paracetamol, the CYP2C9 metabolite 4'-hydroxydiclofenac, and the CYP3A4 metabolite 1'-hydroxymidazolam. The samples were analysed at separate occasions by means of liquid chromatography/mass spectrometry (LC/MS), see (Kanebratt and Andersson, 2008).

LC/MS analysis. The samples from the substrate depletion assay were analysed at separate occasions by means of LC/MS. The LC system consisted of an HP 1100 series LC pump and column oven (Agilent Technologies, Santa Clara, CA, USA) combined with an HTS PAL injector (CTC Analytics, Zwingen, Switzerland). For midazolam, propranolol, clozapine, and dextromethorphan LC separations were performed on a reversed-phase HyPurity C18 column (2.1x50 mm, 5 µm, ThermoQuest, Runcorn, UK) with a HyPurity C18 precolumn at 40 °C and with a flow rate at 750 µl/min. The mobile phase consisted of (A) 0.1% (v/v) formic acid and (B) 0.1% (v/v) formic acid in acetonitrile. The organic modifier content B was increased linearly from 5 to 90% B over 3 min for midazolam, propranolol, and clozapine. For midazolam and propranolol B was returned back to 5% in 0.1 min directly after reaching 90% and for clozapine B were kept at 90% for 1 min, and then returned back to 5% B in 0.1 min. For dextromethorphan the organic modifier content B was increased linearly from 5 to 90% B over 4.5 min and then

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returned back to 5% in 1 min. For 7-ethoxycoumarin, chromatography was performed on a Zorbax SB-C18 column (4.6x150 mm, 5 µm, Agilent Technologies, Santa Clara, CA, USA) with a HyPurity C18 precolumn, employing the same system and mobile phase. The organic modifier content B was increased linearly from 30 to 90% B over 5 min, back to 30% B in 0.1 min, and continued for 2.9 min. For naloxone, chromatography was performed on a Zorbax Eclipse XDB-C8 column (4.6x150 mm, 5 µm, Agilent Technologies, Santa Clara, CA, USA) with a HyPurity C18 precolumn, employing the same system. The mobile phase consisted of (A) 0.2% (v/v) formic acid in 5% acetonitrile and (B) 0.2% (v/v) formic acid in acetonitrile. The organic modifier content B was increased linearly from 10 to 90% B over 2.8 min, and then back to 10% B in 0.1 min. The retention times of midazolam, propranolol, clozapine, dextromethorphan, 7-ethoxycoumarin, and naloxone were 2.4, 2.2, 2.1, 2.5, 6.2, and 2.6 min, respectively. Detection was performed with a triple quadrupole mass spectrometer, API4000, equipped with electrospray interface (Applied Biosystems/MDS Sciex, Concord, Canada). The MS parameters were optimised using each analyte. The compound dependent parameters were as follows: the collision energy was set at 39, 26, 60, 55, 25, and 50 V for midazolam, propranolol, clozapine, dextromethorphan, 7-ethoxycoumarin, and naloxone, respectively. Collision-activated dissociation gas was at 7, 7, 10, 10, 7, and 7, respectively. The MRM transitions chosen were 326.0>290.7 for midazolam, 260.1>116.3 for propranolol, 327.2>192.0 for clozapine, 272.3>171.2 for dextromethorphan, 191.1>163.1 for 7-ethoxycoumarin, and 328.1>212.1 for naloxone. A dwell time of 200 ms was used. Instrument control, data acquisition and data evaluation were performed using Applied Biosystems/MDS Sciex Analyst 1.4 software.

Data Analysis. The expression levels in HepaRG samples were compared to the expression levels in primary human hepatocytes, which were set to 1 for all genes analysed in Fig. 1 and 2. In Fig. 3A mRNA expression levels has been calculated as $2^{-\Delta C_T}$ (Biosystems, 1997).

Curve fitting for substrate depletion were carried out with XLfit 4.1.1 (ID Business Solutions, Emeryville, CA).

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Results

mRNA expression in HepaRG cells during differentiation and time in culture. The mRNA expression of 44 genes encoding drug metabolising enzymes, transporter proteins, and liver specific factors were determined in undifferentiated HepaRG cells and in HepaRG cells differentiated by addition of DMSO at confluence, and then cultured up to six weeks. The expression levels in HepaRG cells were compared with those in cryopreserved primary human hepatocytes (three individuals, batches 1, 4 and 5, demographic data presented in Table 1). Average mRNA expression levels in human hepatocytes were set to 1 for all genes measured (Fig. 1). The mRNA expression was also measured in HepG2 cells (Fig. 1). In the HepG2 cells very low (<0.2-fold of human hepatocytes) or no detectable mRNA levels of drug metabolising enzymes and transporters were observed, except for of MDR1, MRP1, and BCRP, which were expressed at >0.8-fold of human hepatocytes. High levels of glucose-6-phosphatase (>5-fold of human hepatocytes) and extremely high levels of alpha-fetoprotein (a 10 000-fold difference compared to human hepatocytes) were also detected in HepG2 cells.

In the undifferentiated HepaRG cells the expression of most P450s were low compared with human hepatocytes (<0.2-fold) except for CYP1A1 and CYP7A1, which were comparable to human hepatocytes (0.2-1.2-fold of human hepatocytes). CYP2D6 expression in HepaRG cells was less than 0.1-fold of expression in primary human hepatocytes. The phase II enzyme GSTA1 had higher expression in HepaRG cells than in human hepatocytes (1.2-2-fold), whereas SULT2A1 and UGT2B7 were detected at lower levels (0.01-0.2-fold). The genes coding for efflux transporter proteins MDR1, MDR3, MRP1, MRP2, MRP3, and BCRP were expressed at levels comparable or higher than in human hepatocytes (0.2 to >5-fold), whereas BSEP levels were lower than in human hepatocytes (0.01-0.2-fold). For the uptake transporters OATP2B1, OATP1B1, OATP1B3, SLC22A7, SLC22A1, SLC10A1, and SLC15A1 the expression levels were lower than in primary human hepatocytes (<0.2-fold). The expression of the nuclear receptors PXR and CAR in undifferentiated cells was low compared to human hepatocytes (<0.01-fold), whereas AhR, FXR, and RXR levels were almost similar to primary

human hepatocytes (0.2-0.8-fold). The expression of the liver enriched transcription factors HNF4α, CEBPα, and CEBPβ, D-site binding protein, and transcription factor GATA-4 was diverse, stretching from 0.01-0.2-fold to 2-5-fold of human hepatocytes. Alpha-fetoprotein is a marker for foetal liver and was expressed at high levels (2-5-fold of human hepatocytes) in undifferentiated HepaRG cells, whereas albumin and transthyretin, two plasma proteins, were expressed at lower levels than in human hepatocytes (0.01-0.2-fold).

Addition of DMSO to the culture medium is used to differentiate the HepaRG cells into more hepatocyte-like cells. When DMSO was added, the expression of most of the P450s, especially CYP2C9, CYP3A4, and CYP7A1 was increased. Among the phase II enzymes measured, the high levels of GSTA1 were further increased in the HepaRG cells. Expression of the uptake and efflux transporters was in general increased by the addition of DMSO except for OATP1B3 and BSEP, which were not affected. The expression of the nuclear receptors was increased after addition of DMSO, the exception being RXRα, which was unaffected. Some of the liver specific factors and the liver enriched transcription factors such as HNF4α, albumin, transcription factor GATA-4, and transthyretin were increased by addition of DMSO, while no major changes in expression were seen for CEBPα, CEBPβ, alpha-fetoprotein, and D-site binding protein.

The effect of DMSO removal on mRNA expression in differentiated HepaRG cells. The mRNA expression was investigated in differentiated HepaRG cells cultured without DMSO for 1, 5 or 14 days (Fig. 2). The expression of several P450s decreased considerably already after one day without DMSO in the medium, the most distinct being CYP3A4, going from 2-5-fold to 0.01-0.2-fold of human hepatocyte levels. Between 1 and 14 days without DMSO in the medium the expression of most of the P450s, e.g. CYP1A, CYP2C, CYP2E1, and CYP3A4, was relatively stable. The expression of transporters was in general not affected when DMSO was removed from the medium in differentiated HepaRG cells. Also the expression of the nuclear receptors and liver specific factors were in general stable after removal of DMSO except for albumin and transthyretin. Albumin increased from 0.2-0.8-fold to 2-5-fold of human hepatocytes after 5 days without DMSO, whereas transthyretin decreased from 2-5-fold to 0.8-1.2-fold of human hepatocytes after 14 days without DMSO.

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Analysis of the relative mRNA content of drug metabolising P450s in differentiated HepaRG cells. The mRNA expression of the major drug metabolising P450s in primary human hepatocytes, differentiated HepaRG cells cultured with 2% DMSO and differentiated HepaRG cells cultured without DMSO for 1 day has been depicted in Fig. 3A. In Fig. 3A only results from preloaded 384-well cards are included. CYP2D6 was not detectable in HepaRG samples using the preloaded 384-well cards. Nevertheless, CYP2D6 was detected at very low levels using AoD analysis in 96-well plates, shown in Fig. 1 and 2. In primary human hepatocytes CYP3A4 and CYP2B6 exhibits a large variation in expression but are the dominant forms followed by CYP2C9. In HepaRG cells cultured in 2% DMSO all investigated P450s are expressed at lower levels than in human hepatocytes except for the high expression of CYP3A4 mRNA, which becomes the dominating P450. When DMSO is withdrawn from the culture medium the expression of CYP1A1, CYP1A2, CYP2A6, CYP2C9, and especially CYP3A4 mRNA decreases during the first day. Overall the levels of mRNA expression for drug metabolising P450s is lower in HepaRG cells cultured without DMSO for 1 day than in human hepatocytes, the exception being CYP2C19.

In Fig 3B the percentage of total mRNA expression of the drug metabolising P450s in human hepatocytes and HepaRG cells is depicted. CYP3A4 is the dominant form in HepaRG cells cultured in 2% DMSO. Also the relative CYP2C19 mRNA content is high in HepaRG cells, which result in lower percentage of all the other P450s when compared with the relative mRNA expression of P450s in human hepatocytes. In HepaRG cells cultured without DMSO for 1 day the relative mRNA content of the various drug metabolising P450s reflects the relative mRNA content of drug metabolising P450s in human hepatocytes, except for a relatively high CYP2C19 mRNA content and a relatively low CYP1A2 mRNA content.

Substrate depletion in differentiated HepaRG cells cultured with and without DMSO. The CL_{int} was calculated from substrate depletion of midazolam, naloxone, 7-ethoxycoumarin, propranolol, clozapine, and dextromethorphan in differentiated HepaRG cells cultured with 2% DMSO and in differentiated HepaRG cells cultured without DMSO for 1, 5 and 14 days. Midazolam is metabolised by CYP3A (Gorski et al., 1994), and when compared with primary

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human hepatocytes (batches 6,7,8; Table 1) a considerably higher midazolam CL_{int} was found in HepaRG cells cultured in 2% DMSO. The CL_{int} in HepaRG cells decreased to values comparable with human hepatocytes when cultured without DMSO (Fig. 4). Naloxone is mainly metabolised by UDP-glucuronosyltransferases (UGTs) (Di Marco et al., 2005). The CL_{int} for naloxone in HepaRG cells cultured in 2% DMSO was at the same level as in human hepatocytes and decreased slightly when DMSO was removed from the culture medium. The CL_{int} for 7-ethoxycoumarin, which is metabolised by CYP2E1>CYP2B6>CYP1A2 (Yamazaki et al., 1996; Waxman and Chang, 2006) was considerably lower in HepaRG cells at all culture conditions as compared with human hepatocytes. Propranolol is metabolised by CYP2D6>CYP1A2>CYP2C19, and UGT1A9 and UGT2B7 (McGinnity et al., 2000; Sten et al., 2006). Propranolol CL_{int} in HepaRG cells was 30-50% of CL_{int} in primary human hepatocytes and was not affected by removal of DMSO from the culture medium. Clozapine is metabolised by CYP1A2, CYP2C19, CYP3A4, and UGT1A4 (Olesen and Linnet, 2001; Mori et al., 2005). Clozapine CL_{int} was similar in primary human hepatocytes and HepaRG cells at all culture conditions. Dextromethorphan is mainly metabolised by CYP2D6, but also by CYP3A4 and CYP2C9 (von Moltke et al., 1998). Dextromethorphan metabolism was very low in HepaRG cells as compared with human hepatocytes and in HepaRG cells cultured without DMSO for 14 days no CL_{int} for dextromethorphan could be determined.

P450 specific activities in differentiated HepaRG cells cultured with and without DMSO. The P450 probe substrates phenacetin (CYP1A2), bupropion (CYP2B6), diclofenac (CYP2C9) and midazolam (CYP3A) were used as sensitive measures for the activity of the respective P450 enzymes. The phenacetin *O*-dealkylase and bupropion hydroxylase activities were low in HepaRG cells at all culture conditions as compared with human hepatocytes (Fig. 5; human hepatocyte batches 1, 2, and 3; Table 1). Bupropion hydroxylase activity in HepaRG cells cultured with DMSO was only 1% of the average activity in human hepatocytes, which was greatly affected by one individual displaying markedly higher activity than in the two other hepatocyte batches. The very high bupropion hydroxylase activity in this hepatocyte batch could not be linked to any specific factor from the demographic data. High variation for this enzyme

has also been seen in human liver microsomes and human hepatocytes (Gervot et al., 1999; Madan et al., 2003). Compared with primary human hepatocytes the diclofenac 4'-hydroxylase and midazolam 1'-hydroxylase activities were 30 and 70%, respectively, in HepaRG cells cultured with DMSO. The midazolam 1'-hydroxylase activity exhibited great variation in human hepatocytes since one individual displayed considerably lower activity compared with the two other (Fig 5). Diclofenac 4'-hydroxylase and midazolam 1'-hydroxylase activities decreased when DMSO was removed from the culture medium.

Discussion

The present study shows that the differentiated HepaRG cells express several liver specific factors, drug metabolising enzymes, transporters, and nuclear transcription factors over a long culture period. The stable expression over a long time in culture and the activity of several of the drug metabolising enzymes indicate the value of the HepaRG cell line as a *in vitro* model for human drug metabolism and disposition studies.

The differentiated HepaRG cells exhibit significant expression of drug metabolising enzymes and drug transporters. This is in contrast to the well-known HepG2 cells, where mRNA for several drug metabolising enzymes was not detected at all. Differentiated HepaRG cells cultured in DMSO exhibit only minor changes in mRNA expression for drug metabolising enzymes, transporters, nuclear receptors, and liver specific factors over a period of six weeks. A tendency towards lower expression of CYP1A1, CYP2C9, CYP3A4, alpha-fetoprotein, and transthyretin six weeks after addition of DMSO could be a sign that the cell function starts to deteriorate at this time point. However, the expression profiles of the investigated genes in the HepaRG cells is reassuring and indicate that HepaRG cells under these conditions could be used in drug metabolism studies during the time period investigated.

The high expression of the efflux transporters MDR1 and MRP1, and glucose 6-phosphatase in HepaRG cells at all culture conditions as compared with human hepatocytes were the most prominent difference between the two cell systems. The low levels of CYP2D6 mRNA detected in HepaRG cells is consistent with the suggestion that the cell line is derived from an individual that is a CYP2D6 poor metaboliser (Guillouzo et al., 2007).

DMSO has been used as a differentiation-inducing agent for many tumour cell lines (Yu and Quinn, 1994). However, the mechanism by which DMSO induces the differentiation of tumour cell lines and certain other cell types is poorly understood. In rat hepatocytes it has been shown that DMSO helped maintain normal expression levels of nuclear receptors and liver specific transcription factors (Su and Waxman, 2004). DMSO has also been shown to increase the expression of CYP3A4 in primary human hepatocytes (Nishimura et al., 2003). This suggests

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that DMSO has a dual effect on the cells, affecting the differentiation of cells but also the gene expression in differentiated cells. Therefore, the effect on the gene expression in differentiated HepaRG cells was investigated when DMSO was withdrawn from the culture medium. The greatest effect when DMSO was removed from the medium seems to be on the P450s, especially on CYP3A4 expression and activity, whereas expression of transporters, nuclear receptors and liver specific factors overall were unaffected. The decrease was seen already after one day without DMSO in the medium and after that only minor alterations were detected.

The relatively high mRNA expression of CYP3A4 in HepaRG cells cultured in 2% DMSO resulted in high midazolam 1'-hydroxylase activity, which was on the same level as in human hepatocytes. When DMSO was withdrawn from the medium, the CYP3A4 mRNA expression and CYP3A4 activity rapidly decreased and after one day in DMSO free culture medium the CYP3A4 mRNA content and the CYP3A4 specific activity were considerably lower than the mean value in human hepatocytes. Phenacetin *O*-dealkylase, bupropion hydroxylase, and diclofenac 4'-hydroxylase activities also decreased when DMSO was removed from the medium, which is in agreement with the decrease in mRNA content of the P450 enzymes responsible for their metabolism. The changes in mRNA expression thus reflect the changes of enzyme activities in the HepaRG cells.

When comparing the relative mRNA content of P450 enzymes, differentiated HepaRG cells cultured without DMSO for 1 day is more similar to human hepatocytes than the relative mRNA content in HepaRG cells cultured with DMSO. The relative content of drug metabolising enzymes in the cell system is an important property when the cells are used to investigate the metabolic pattern of drug molecules. The decline in the specific CYP3A4 midazolam 1'-hydroxylase activity in HepaRG cells when DMSO was removed could also, as expected, be seen for the CL_{int} for midazolam, which mainly is a CYP3A4 substrate. Although the midazolam CL_{int} in HepaRG cells cultured with 2% DMSO was considerably higher as compared with human hepatocytes, the midazolam 1'-hydroxylase activity in HepaRG cells cultured with 2% DMSO was at the same level as in human hepatocytes. It should be kept in mind that the CL_{int} studies and the specific P450 activity measurements were performed with hepatocyte

preparations from different individuals and results could thus describe the interindividual variation in humans. The metabolism of propranolol, 7-ethoxycoumarin, and dextromethorphan is lower in HepaRG cells as compared with human hepatocytes, which is in accordance with the lower mRNA expression for the drug metabolising P450s responsible for the metabolism of these compounds. Clozapine is metabolised by several enzymes and displays similar CL_{int} in HepaRG cells as in human hepatocytes. The metabolism of naloxone was comparable in human hepatocytes and in HepaRG cells, indicating that the responsible UGTs have similar activities in the two *in vitro* models. UGT dependent metabolism of propranolol could also contribute to the CL_{int} for propranolol in HepaRG cells, which still is 50% of CL_{int} in hepatocytes although HepaRG cells at all culture conditions have a low expression of CYP2D6 and CYP1A2, the major P450s responsible for propranolol metabolism.

Aninat et al. (2006) reported that addition of the potent CYP3A inducer rifampicin did not increase CYP3A activity in HepaRG cells seeded at high density and cultured in 2% DMSO (Aninat et al., 2006), which indicates that the enzyme may be maximally induced in DMSO treated cells. HepaRG cells cultured in DMSO therefore overestimate the importance of CYP3A metabolism of drug compounds but also underestimate the induction potential of compounds via nuclear receptors. Recently we demonstrated that differentiated HepaRG cells cultured without DMSO for 5 days responded to prototypical P450 inducers in a similar way as human hepatocytes, and the cell system could be used as a valuable model to make quantitative predictions of CYP3A induction *in vivo* by drug compounds (Kanebratt and Andersson, 2008).

In conclusion, the HepaRG cells have a stable expression of P450 enzymes, phase II enzymes, transporters, and nuclear transcription factors over a time period of six weeks in culture. The results presented here on transporter gene expression and previous functional transporter studies (Le Vee et al., 2006) indicate that the HepaRG cells also could be used for investigations of drug transporters. Although the metabolism of some of the tested compounds and the formation of measured metabolites is lower in HepaRG cells compared to cryopreserved human hepatocytes, the relative content of drug metabolising P450s were similar to primary human hepatocytes. Based on the relative content of drug metabolising P450s and

metabolic properties, differentiated HepaRG cells cultured without DMSO for one day could be used as a valuable *in vitro* model for human drug metabolism studies.

Acknowledgments

The authors wish to thank Britta Bonn and Dr. Xue-Qing Li for development of analytical LC/MS methods.

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Footnotes

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Legend for figures

Fig. 1. Expression of mRNA for 44 genes in cryopreserved human hepatocytes (n=3, batches 1, 4, and 5), HepG2 cells, and HepaRG cells at different weeks of culture (n=1). The expression is set to 1 in human hepatocytes for all genes measured. N.D.=not detectable.

Fig. 2. Expression of mRNA for 44 genes in cryopreserved human hepatocytes and in differentiated HepaRG cells cultured with and without DMSO (n=3, human hepatocyte batches 1, 4, and 5). The expression is set to 1 in human hepatocytes for all genes measured. N.D.=not detectable.

Fig 3. mRNA expression levels of drug metabolising P450s calculated as $2^{-\Delta C_T}$ (A) and relative expression calculated as % of total expression of drug metabolising P450s (B) in primary human hepatocytes (batches 1, 4, and 5), differentiated HepaRG cells cultured with 2% DMSO, and differentiated HepaRG cells cultured without DMSO for 1 day. Results in A is mean + S.D., n=3.

Fig 4. Measurement of CL_{int} in primary human hepatocytes (batches 6, 7, and 8) and differentiated HepaRG cells cultured with DMSO and without DMSO for 1, 5, or 14 days. Results are mean +S.D., human hepatocyte results are mean for three individuals, n=5 for HepaRG results.

Fig. 5. P450 selective activities using phenacetin, bupropion, diclofenac, and midazolam in primary human hepatocytes (individual values of batches 1, 2, and 3, average is marked by a line), differentiated HepaRG cells cultured with DMSO and without DMSO for 1, 5, or 14 days. Results for HepaRG cells are mean +S.D., n=6.

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Tables

Table 1. Demographic data of human hepatocytes used in the study.

Hepatocyte batch	Sex	Age	Ethnicity	Smoker	Alcohol	Medical history / medications	Use in present study
1	M	42	AA	Yes	Yes	None	Metabolite formation, mRNA
2	M	31	C	No	Yes	Depression, antidepressants	Metabolite formation
3	F	56	AA	Yes	Yes	Diabetes, hypertension, vascular disease	Metabolite formation
4	F	45	C	Yes	Yes	Hypertension, kidney stones	mRNA
5	F	54	C	No	No	Diabetes, hypertension, arthritis, kidney disease	mRNA
6	M	47	C	Yes	N.I.*	Diabetes	Substrate depletion
7	F	60	C	No	Yes	Antidepressants	Substrate depletion
8	M	55	AA	No	N.I.	Hypertension	Substrate depletion

*N.I. = no information

Fig. 1

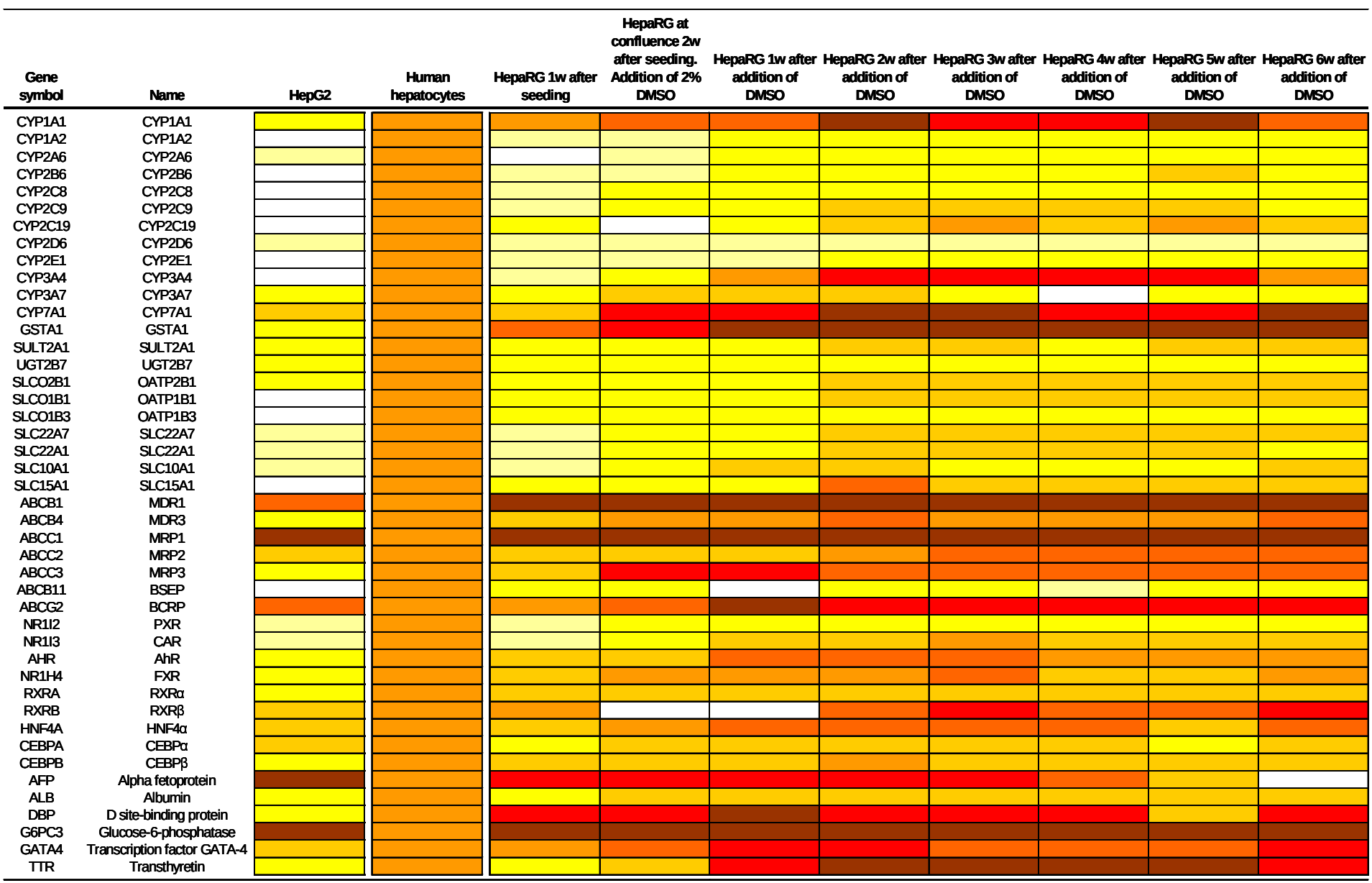


Fig. 2

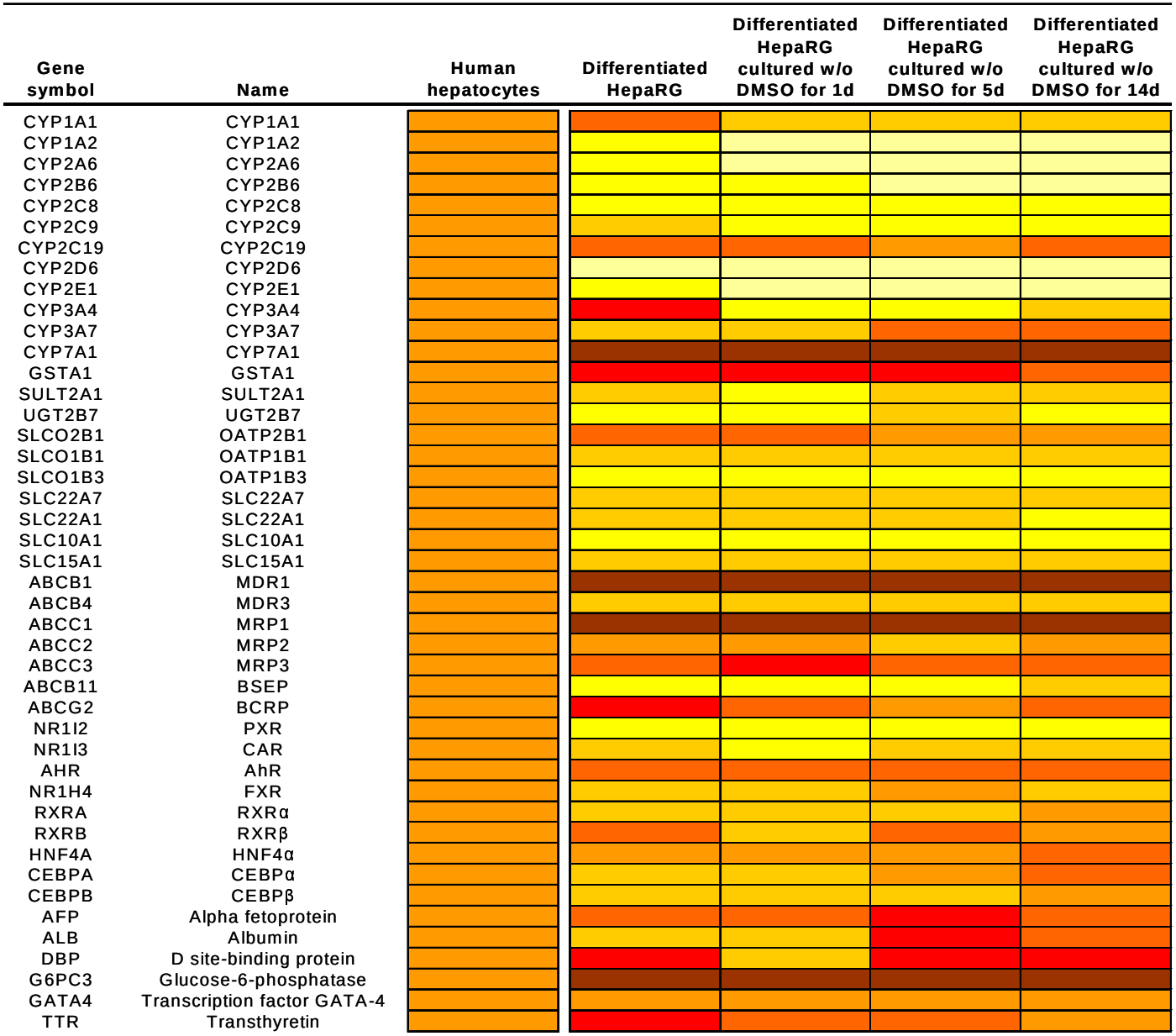
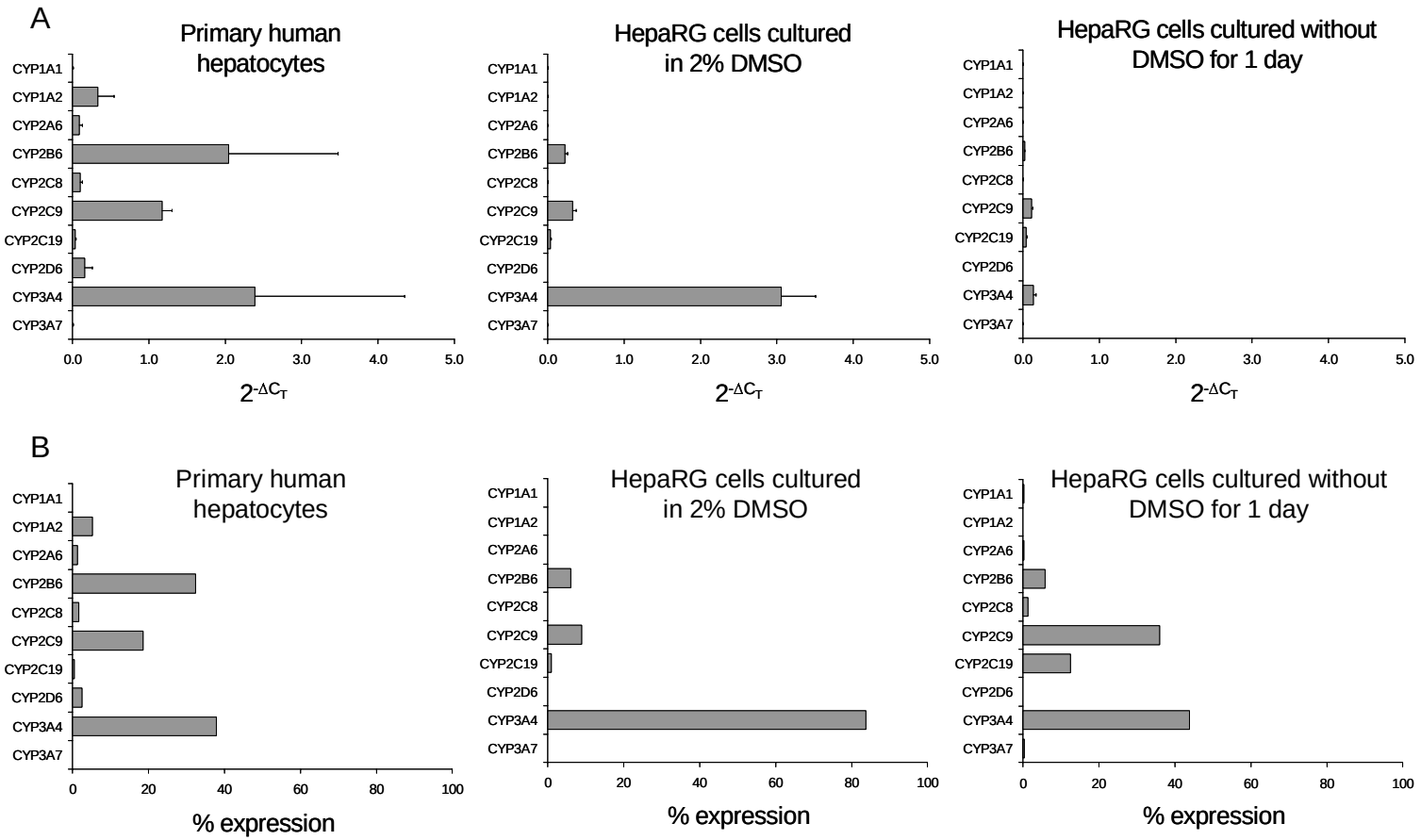


Fig. 3



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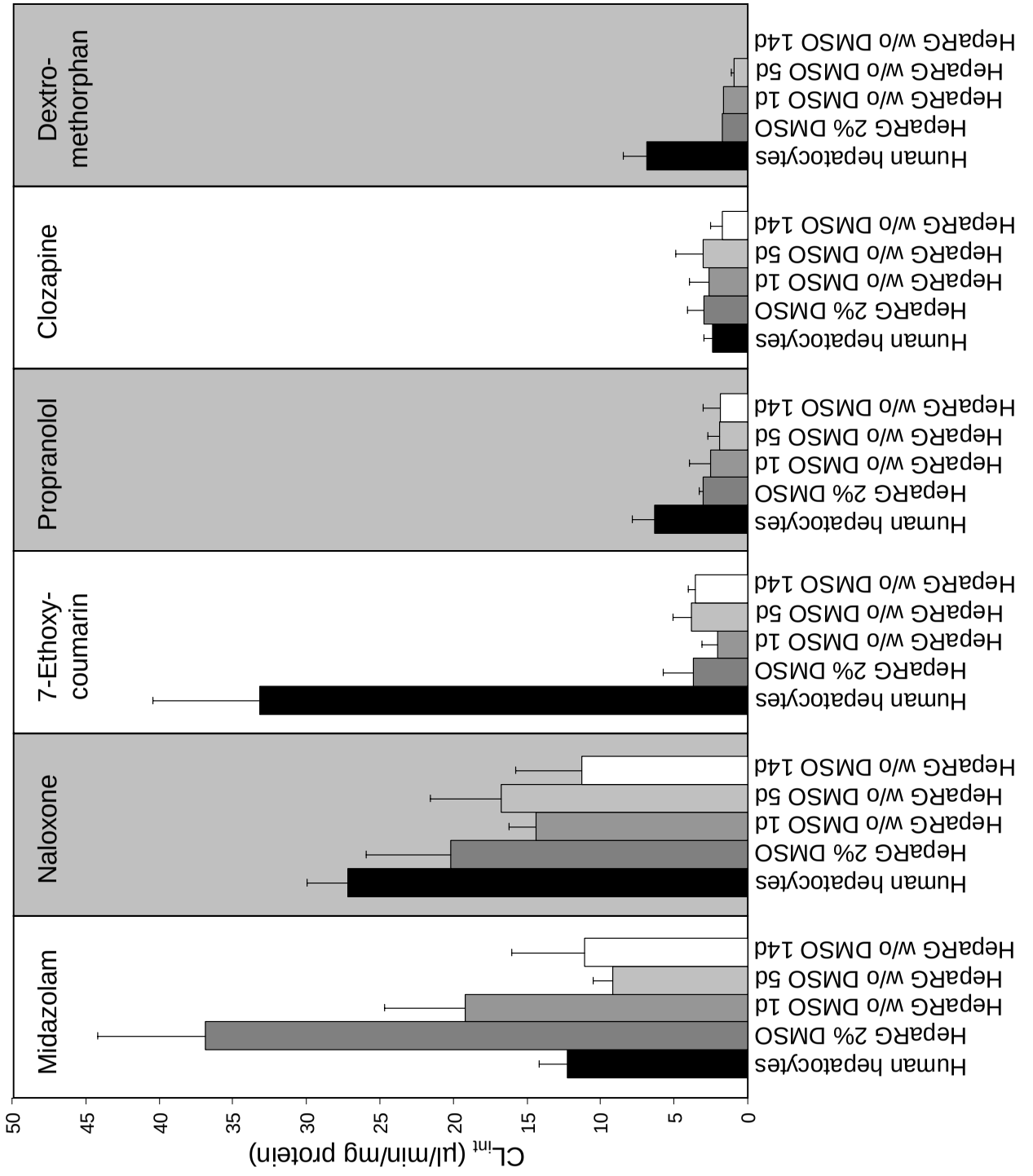

Fig. 4

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Fig. 5