STABLE EXPRESSION, ACTIVITY AND INDUCIBILITY OF CYTOCHROMES P450 IN DIFFERENTIATED HepaRG CELLS

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The abbreviations used are:
AhR, aryl hydrocarbon receptor; BSEP, bile salt export pump; BCRP, breast cancer resistance protein; CAR, constitutive androstane receptor; CDFDA, carboxydichlorofluorescein diacetate; CYP, cytochrome P450; DMSO, dimethylsulfoxide; FCS, fetal calf serum; FIH, freshly isolated hepatocytes; GST, glutathione transferase; HD, high density; LD, low density; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; NTCP, Na⁺-dependent taurocholic cotransporting polypeptide; OME, omeprazole; PB, phenobarbital; PBS, phosphate-buffered saline; PXR, pregnane X receptor; RIF, rifampicin; RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction; UGT, UDP-glucuronosyl transferase.
HepaRG cells possess the unique property to differentiate \textit{in vitro} and to express various functions of mature hepatocytes, including the major CYPs. In the present study we carefully analyzed mRNA expression and activity of the major CYPs and their responsiveness to three prototypical inducers, phenobarbital, rifampicin and omeprazole, in differentiated HepaRG cell cultures over a 4-week period after low and high seeding. Only minor differences were observed in CYP activities when measured by two cocktails of probe substrates, likely related to the choice and/or concentration of substrates. Similar results were obtained from the two cell seeding conditions. Expression and activities of several CYPs were DMSO-dependent. However, basal CYP expression and activities as well as their responsiveness to the prototypical inducers were well maintained over the 4-week period and a good correlation was observed between transcript levels and corresponding activities. Thus, CYP1A2, CYP2B6, and CYP3A4 were found to accurately respond to their respective prototypical inducers, i.e. omeprazole, phenobarbital and rifampicin. Similarly, basal expression of several phase II enzymes, transporters and nuclear receptors and response to inducers were also well preserved. More genes were found to be induced in HepaRG cells than in primary human hepatocytes and no marked variation was noticed between the different passages. Taken altogether, these data support the conclusion that HepaRG cells represent a promising surrogate to primary human hepatocytes for xenobiotic metabolism and toxicity studies.
INTRODUCTION

Drug-induced hepatotoxicity represents a major clinical problem accounting for around 50% of all cases of acute liver failure and is a major cause of attrition in drug development. Estimation of cytochrome P450 (CYP) induction and inhibition and prediction of drug-drug interactions are an important consideration for the development of novel therapeutic agents (Park and Miller, 1996; Pelkonen et al., 2008). Predicting the ability of a drug to modulate CYP expression at an early stage of its discovery and development should reduce the risk of failure in the clinic and, more importantly, permit the identification of alternative non-inducing/non-inhibiting chemical structures. Over the past decades, various in vitro and/or ex vivo models have been developed to investigate drug metabolism. In vitro liver cell systems represent a good experimental approach to screen potential hepatotoxic compounds and to investigate mechanisms by which chemicals induce liver lesions (Guillouzo, 1998). Primary human hepatocytes are considered the most pertinent model for in vitro testing of the induction/inhibition potential of drug candidates (Guillouzo, 1998; Gomez-Lechon et al., 2004; Hewitt et al., 2007). However, their use is limited by scarce availability of donor organs, large interdonor functional variability and early phenotypic changes in vitro. Most hepatocyte cell lines, mainly originated from tumors, have indefinite proliferative capacity, but they are considered inappropriate for prediction of hepatotoxicity in preclinical drug development due to the low levels, if any, of major CYP enzymes and several transporters. An exception is represented by the HepaRG cell line derived from a human liver carcinoma (Gripon et al., 2002). When seeded at low density (LD), HepaRG cells transdifferentiate into bipotent hepatic progenitors and actively divide before acquiring typical morphological and functional characteristics of adult human hepatocytes in primary culture under appropriate culture conditions (Cerec et al., 2007). Indeed, after two weeks at confluence in the presence of 2% dimethylsulfoxide (DMSO) and corticosteroids they appear as hepatocyte-like colonies surrounded by biliary-like cells. Transdifferentiation of differentiated HepaRG cells can be avoided by seeding at high density (HD) (Aninat et al., 2006; Cerec et al., 2007). Differentiated HepaRG cells express various liver functions, including CYPs, phase II enzymes, transporters and nuclear receptors at levels comparable to those found in primary hepatocytes and are responsive to prototypical inducers, suggesting that they could represent a
surrogate to the latter in drug metabolism and toxicity studies (Aninat et al., 2006; Le Vee et al., 2006; Guillouzo et al., 2007; Josse et al., 2008; Kanebratt and Andersson, 2008b; Turpeinen et al., 2009). Moreover, some evidence has been provided that LD-seeded HepaRG cells can retain relatively stable expression and activities of CYPs for several weeks at confluence (Josse et al., 2008; Kanebratt and Andersson, 2008a). However, long-term maintenance of CYP activities and responsiveness to inducers has not been fully characterized, especially in HD-seeded cultures. The present study was undertaken within the EU-framework LIINTOP project to compare transcript and activity levels of the major CYPs and their responsiveness to the prototypical inducers, phenobarbital (PB), rifampicin (RIF), and omeprazole (OME), over a 4-week period in differentiated HepaRG cells. For this purpose CYP activities were simultaneously estimated using two different cocktail substrate settings. Such CYP-substrate cocktails are increasingly used for the determination of basal and induced CYP activities (Zhou et al., 2004). Indeed the use of a variety of substrate combinations, numbers of substrates, or analytical methods has revealed no significant differences between the results obtained from individual or cocktail analyses (Dierks et al., 2001; Kim et al., 2005; Lahoz et al., 2008a). In addition expression of various other genes related to xenobiotic metabolism and transport were also measured for comparison purposes. We show that basal mRNA and activity levels of major CYPs, expression of several phase II enzymes and transporters as well as inducibility by RIF, PB, and OME were well-maintained in differentiated HepaRG cells during the 4-week period tested, whether the cells were seeded at LD or HD.
MATERIALS AND METHODS

Chemicals. DMSO, RIF, PB, OME and insulin were purchased from Sigma (St. Quentin Fallavier, France). Williams' E medium was obtained from Eurobio Laboratories (Les Ulis, France). Fetal calf serum (FCS) was supplied by Perbio (Brebières, France). Penicillin and streptomycin were from Invitrogen (Cergy Pontoise, France). Hydrocortisone hemisuccinate was obtained from Upjohn Pharmacia (Guyancourt, France), carboxydichlorofluorescein diacetate (CDFDA) by Molecular Probes (Interchim, Montluçon, France) and BODIPY FL vinblastine, a fluorescent analog of the anticancer drug vinblastine from Invitrogen. Substrates and metabolite standards used for the Unidad Mixta Fundación Hospital La Fe-Advancell (UAM) cocktail: 4'-hydroxydiclofenac, 6-hydroxychlorzoxazone, hydroxybufuralol, midazolam, 1'-hydroxymidazolam, phenacetin, bufuralol, mephenytoin, hydroxymephenytoin, and acetaminophen were supplied by BD Biosciences Discovery Labware (Bedford, MA). Substrates used for the Novamass (NM) cocktail: bupropion, midazolam, and OME were purchased from Sequoia Research Products (Pangbourne, UK), testosterone from Fluka (Buchs, Switzerland), and phenacetin from ICN Biomedicals (Costa Mesa, CA, USA). Metabolite standards O-desmethyldextromethorphan, 6-hydroxychlorzoxazone, desethylamodiaquine, hydroxytolbutamide, and 6β-hydroxytestosterone were purchased from BD Biosciences Discovery Labware (Bedford, MA, USA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cell cultures. HepaRG cells were cultured at a density of either 2.6 x 10^4 cells/cm² (LD) or 0.45 x 10^6 differentiated cells/cm² (HD) as previously described (Gripon et al., 2002; Aninat et al., 2006). LD-seeded HepaRG cells were first incubated in the Williams' E medium supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 2 mM glutamine, and 5 x 10^-5 M hydrocortisone hemisuccinate for two weeks. Maximum liver-specific activities were attained after two additional weeks in the same medium added with 2% DMSO (Gripon et al., 2002). HD-seeded HepaRG cells were directly incubated in the DMSO-containing medium; they did not transdifferentiate and retained their morphological and functional characteristics. The medium of both LD and HD cultures was renewed every 2 or 3 days.
Human hepatocytes from five adult donors undergoing resection for primary and secondary tumors were obtained by perfusion of histologically normal liver fragments (Guguen-Guillouzo et al., 1982). Briefly, hepatocytes were seeded at a density of 190,000 cells/cm\(^2\) in 24-well plates in Williams' E medium supplemented with 10% FCS, 100 units/ml penicillin, 100µg/ml streptomycin, 1µg/ml insulin, 2 mM glutamine, and 1µg/ml bovine serum albumin. Cells were allowed to attach for 12 h, at which time medium was replaced with the same medium deprived of FCS and supplemented with 10\(^{-7}\) M dexamethasone. The medium was renewed every day.

**Induction treatments.** For induction studies in HepaRG cells, experimental conditions were based on the observations that CYP induction by DMSO was lost 24h after its depletion and that no marked changes occurred in CYP transcript levels after two more days (Aninat et al., 2006). HepaRG cells (from passages 14, 17, and 20) were seeded either at LD and cultured for up to 56 days or at HD and cultured for up to 28 days (Figure 1). Three days before inducer addition the medium was changed to a DMSO-free medium containing 2% SVF and after that period the cells were exposed to the prototypical inducers (10 µM RIF, 1 mM PB or 50 µM OME) or their vehicle, i.e. DMSO (final concentration of 0.5%) for RIF and OME and water for PB, for 48h (mRNA quantification) or 72h (measurements of CYP activities). Human hepatocytes were exposed to the prototypical inducers or their vehicle 1 to 3 days after seeding. Treatments also lasted either 48h (mRNA quantification) or 72h (measurements of CYP activities).

**Isolation of RNA and reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA was extracted from 10\(^6\) HepaRG cells or 10\(^6\) human hepatocytes with the SV total RNA isolation system (Promega, Madison, WI), which directly included a DNase treatment step. RNAs were reverse-transcribed into cDNA using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed by the fluorescent dye SYBR Green methodology using the SYBR Green PCR Master Mix (Applied Biosystems) and the ABI Prism 7000 (Applied Biosystems). Table 1 (Supplemental data) shows primer pairs for each transcript chosen with Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), except for CYP2A6, CYP2C8, CYP2C19 and CYP2D6 which were provided by Biopredic International (Rennes, France) (Girault et al., 2005). The amplification curves were read with the ABI Prism 7000 SDS software using the comparative cycle threshold method. The relative quantification of the
steady-state mRNA levels was calculated after normalization of the total amount of cDNA tested using 18S RNA as a reference. Furthermore, a dissociation curve was performed after the PCR analysis to verify the specificity of the amplification. Results were expressed as percentage of mRNA levels measured in freshly isolated hepatocytes (arbitrarily set at 100%) for basal expression studies, or as percentage of mRNA levels measured in the corresponding control samples exposed to the vehicle (arbitrarily set at 100%) for induction studies.

**Evaluation of CYP activities**

Two substrate cocktails were used: the Unidad Mixta Fundacion Hospital La Fe-Advancell cocktail (UAM cocktail) and the Novamass cocktail (NM cocktail).

- **The UAM cocktail**: Activity assays utilized direct incubation of cell monolayers with a cocktail of 8 substrates as previously described (Lahoz et al., 2008b). The substrate mixture stock solutions were prepared in DMSO, and then methodically diluted in the incubation medium to obtain the following final concentrations: 10μM phenacetin (CYP1A2), 5μM coumarin (CYP2A6), 10μM bupropion (CYP2B6), 10μM diclofenac (CYP2C9), 50μM mephenytoin (CYP2C19), 10μM bufuralol (CYP2D6), 50μM chlorzoxazone (CYP2E1), and 5μM midazolam (CYP3A4). The final concentration of DMSO during incubation was 0.5% (v/v). Incubations with the cocktail of substrates lasted 2h. Metabolism of all compounds was linear during this incubation period. At the end, aliquots of media (300 μl) were collected and stored at –80°C, until delivery and analysis in UAM laboratories. Samples were subsequently extracted twice with ethyl acetate 1:1 (v/v), and dextrometorphan was used as recovery standard. The organic phase was transferred to a clean tube, evaporated under vacuum, dissolved in 100 μl HEPES (5% ACN) and submitted to analysis. Metabolites formed and released into the culture medium were quantified by high performance liquid chromatography tandem mass spectrometry (LC/MS/MS). This system comprised a Micromass Quattro Micro (Waters, Milford MA, USA) triple quadrupole mass spectrometer in electrospray ionization mode, interfaced with an Alliance 2795 HPLC (Waters Chromatography). Chromatography was performed at 35°C and an aliquot (20 μl) was injected into a Teknokroma C18 column (100 mm × 2.1 mm, 3μm particle size) at a flow rate of 0.4 ml/min. The mobile phase was 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B). The proportion of acetonitrile was increased linearly from 0 to 90% in 6 min, and then the column was allowed to re-equilibrate at the initial conditions for 4 min. The column eluent was directed without splitting to an electrospray ionization interface, operating at 320°C and using nitrogen as cone gas (50 l/h). The MS/MS experiments were carried out with a triple quadrupole analyzer.
operating in multiple reaction monitoring mode. Enzymatic activities were expressed as pmol of metabolites formed/hour/mg total protein.

The NM cocktail: Activity assays were performed by direct incubation of cell monolayers with a cocktail of 10 substrates as previously described (Turpeinen et al., 2005; Tolonen et al., 2007). The substrate mixture stock solutions were prepared in DMSO and diluted in incubation media to obtain the following final concentrations: 5µM melatonin (CYP1A2), 2µM coumarin (CYP2A6), 2µM bupropion (CYP2B6), 5µM amodiaquine (CYP2C8), 8µM tolbutamide (CYP2C9), 5µM OME (CYP2C19), 1µM dextromethorphan (CYP2D6), 10µM chlorzoxazone (CYP2E1), 1µM midazolam (CYP3A4) and 5µM testosterone (CYP3A4). The final concentration of DMSO during incubation was 0.5% (v/v). After a 4h incubation with the cocktail of substrates, aliquots (300 µL) were collected and stored at –80°C until delivery and analysis. Frozen samples were thawed at room temperature and centrifuged for 10 min at 13200 g, then 20 µl of the supernatant was injected into the LC/MS/MS. A Waters 2695 Alliance HPLC system (Waters Corp., Milford, USA) was used together with a Waters Sunfire RP18 column (2.1 × 100 mm column with 5 µm particle size) and a Luna C18 pre-column (2.0 × 4.0 mm) (Phenomenex, Torrance, USA), at 30 °C. The HPLC eluents were aqueous 1% formic acid + 10 mM ammonium acetate, pH 2.4 (A) and methanol (B). The gradient elution with 5–50–80% B was applied in 0–1.0–4.0 min, followed by 0.5 min isocratic elution with 80% B and column equilibration, resulting in total time of 8 min/injection. The eluent flow rate was 0.5 ml/min. The flow was split post-column with an Accurate Post-Column Stream Splitter (LC Packings, Amsterdam, The Netherlands) with the MS ion source-to-waste ratio of 1:3. Data were acquired using a Micromass Quattro Micro triple quadrupole mass spectrometer (Altrincham, UK), equipped with a Z-spray electrospray ion source. Multiple reaction monitoring mode using a polarity switching between positive and negative ion mode was applied. The capillary voltage used was 4200 V and extraction cone voltage 2 V for all compounds. The dissolution temperature and source temperature were 280 °C and 150 °C, respectively. Nitrogen was used as a drying gas with a flow rate of 700 l/h and as a nebulizer gas with a full flow rate. The collision cell argon pressure was set to 3.8 × 10–3 mbar. The dwell times for monitoring each reaction were 100 msec and the delay between positive and negative polarities during hydroxychlorzoxazone detection (2.7–3.8 min) was 300 msec. Enzyme activities were expressed as pmol of metabolites formed/hour/mg total protein.
**Efflux Transport Assays.** Analysis of efflux transport was performed using two fluorescent substrates: CDFDA, a substrate of multidrug resistance-associated protein 2 (MRP2) and BODIPY FL vinblastine, a substrate of bile salt export pump (BSEP) and multidrug resistance protein 1 (MDR1). After washing with the uptake buffer (136mM NaCl, 5.3mM KCl, 1.1mM KH2PO4, 0.8mM MgSO4, 1.8mM CaCl2, 11mM D-glucose, 10mM Hepes, pH7.4), HepaRG cells were incubated for 30 min at 37°C with fluorescent substrates (3μM CDFDA or 2μM vinblastine), then washed with cold PBS and observed under fluorescence microscopy.

**Statistical analysis.** Each value corresponded to the mean ± SD of three independent experiments. The Kruskal-Wallis non-parametric test was used to compare mRNA levels and CYP activities between different time points. The Mann-Whitney U test was applied to compare mRNA levels and CYP activities between inducer-treated cultures and corresponding control samples. Data were considered significantly different when p < 0.05.
RESULTS

1. Basal mRNA levels of phase I and II metabolizing enzymes, membrane transporters and nuclear receptors

Basal mRNA levels of twenty genes were analyzed by RT-qPCR in freshly isolated (FIH) and 1- and 3-day cultured primary human hepatocytes and LD- and HD-seeded HepaRG cells at different times for up to 4 weeks after differentiation. These genes were the following: ten CYPs (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4), two phase II enzymes [glutathione transferase A1/A2 (GSTA1/A2) and UDP-glucuronosyl transferase 1A1 (UGT1A1)], five membrane transporters [breast cancer resistance protein (BCRP), bile salt export pump (BSEP), multidrug resistance protein-1 (MDR1), multidrug resistance-associated protein-2 (MRP2), and Na⁺-dependent taurocholic cotransporting polypeptide (NTCP)] and three nuclear receptors [aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR)]. All expression values were compared with those measured in 1-day human hepatocyte cultures set at 100%. Differential changes were observed in basal gene expression in primary human hepatocytes (Figure 2) as a function of time in culture; they could be classified in 5 groups characterized by either a decrease followed by an increase at day 3 (CYP2C8, CYP2C9, CYP3A4, UGT1A1, CAR, NTCP), a continuous and strong decrease (CYP1A2, CYP2B6, CYP2E1, BSEP), a decrease followed by a relative stability (CYP1A1, CYP2A6, CYP2A6, CYP2B6, PXR), a continuous increase (MDR1, MRP2) or a relative stability (CYP2A6, CYP2D6, AhR, PXR). When compared to 1-day primary hepatocyte cultures, all the genes tested were expressed in DMSO-exposed HepaRG cultures; the levels of transcripts were either markedly lower (CYP1A2, CYP2C8, CYP2D6, CYP2E1, BSEP, NTCP), at the 30-50% level (CYP1A1, CYP2A6, CYP2B6, PXR), or comparable or even higher (CYP2C9, CYP2C19, CYP3A4, GSTA1/2, UGT1A1, MDR1, MRP2, AhR, CAR) (Figure 3 A and C). However, when HepaRG cells were shifted to a DMSO-free medium for 72h before mRNA quantification some major differences were evidenced (Figure 3 B and D). Transcripts of CYP1A1, CYP2A6, CYP2B6, CYP3A4, and UGT1A1 were decreased whereas those of CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, GSTA1-A2, AhR, CAR, and
transporters were only slightly altered. Moreover, most genes were much less expressed in both proliferative and poorly differentiated HepaRG cells cultured for 5 or 15 days respectively in the absence of DMSO after LD seeding; the only exception was CYP1A1 which was highly expressed (Figure 3A and B). All the data showed a long-term, relatively stable gene expression of all tested drug metabolizing enzymes and transporters in differentiated HepaRG cells from both LD- and HD-seeded cultures maintained at confluence for up to 4 weeks.

2- Basal activities of various CYPs

The UAM and NM cocktails were used for the determination of CYP activities in HepaRG cells and primary human hepatocytes. In addition to the use of several different substrates the major differences between the two assay procedures were the use of lower substrate concentrations and longer incubation times in the NM assay. Concentrations of the four common substrates were 5-fold (bupropion, chlorzoxazone, midazolam) and 2.5-fold (coumarin) higher in the UAM than that in the NM assay. Lower values were frequently obtained with the NM cocktail (Figures 4 and 5). Compared to the values measured in freshly isolated hepatocyte suspensions, only CYP2A6, CYP2C9, and CYP2E1 activities were markedly decreased after 24h of culture using the UAM probe cocktail (Figure 3A). Notably, our results showed important inter-donor variability between human hepatocyte populations for some activities, especially for CYP2C19 (from 0.98 to 10.04 pmol/hour/mg protein; n=5) and CYP2B6 (from 1.04 to 31.62 pmol/hour/mg protein; n= 5).

In DMSO-exposed HepaRG cultures, (Figure 4B) CYP activities were found to be either lower (CYP1A2, CYP2A6, CYP2C9, CYP2E1) or relatively close (CYP2B6, CYP2C19, CYP2D6, CYP3A4) to those of 1-day primary hepatocyte cultures. In HepaRG cells maintained in DMSO-free medium, (Figures 4C and 5B) most CYP activities were lower, especially CYP2B6 and CYP3A4. As expected CYP activities were all quite low in 5- and 15-day LD-seeded cultures, corresponding respectively to proliferating and poorly differentiated HepaRG cells.

All activities were relatively well-maintained over the 28 day period of confluence. Using the NM cocktail, CYP activity values were either comparable to (CYP1A2, CYP2B6, CYP2C19, CYP2D6) or lower (CYP2C9, CYP3A4) than those estimated with the UAM one. Furthermore low CYP2A6 and CYP2E1 activities were found with the UAM cocktail while they were undetectable with the NM cocktail. However, other CYP activities were found to be
relatively well-maintained by using the NM cocktail, and CYP2C8 activity was demonstrated (amodiaquine deethylation).

3- Functional activities of membrane transporters

To verify whether canalicular efflux transporters remained functional in hepatocyte-like HepaRG cells after several weeks at confluence, transport of specific fluorescent substrates was analysed on day 56 after LD seeding. First, no morphological modification of bile canalicular structures was observed when HepaRG cells were cultured in DMSO-free medium for 5 days (Figure 6A). Moreover, accumulation of CDFDA, a fluorescent substrate of MRP2, was restricted to bile canaliculi of HepaRG hepatocytes (Figure 6B). In addition, specific accumulation of BODIPY FL vinblastine, a fluorescent substrate of BSEP/MDR1, was also evidenced in canalicular spaces of HepaRG cells. Similar observations were made in both LD- and HD-seeded HepaRG cell cultures (data not shown).

4. Induction of CYPs by prototypical inducers at mRNA and activity levels

To further estimate the functional capacity of HepaRG cells maintained at confluence for several weeks, their responsiveness to three prototypical inducers (RIF, PB and OME) was assessed over a 4-week period. At appropriate time-points HepaRG cultures were shifted to a DMSO-free medium supplemented with 2% FCS for 72 h and then treated with the prototypical inducers for 48 and 72 h for mRNA and activity measurements, respectively. Comparable results were obtained in LD- and HD-seeded HepaRG cultures and the levels of CYP induction were relatively consistent for 4 weeks in differentiated HepaRG cells in both culture conditions (non-significant variations, Kruskal-Wallis test).

The main results are summarized in Table 1. As expected CYP1A2, CYP2B6, and CYP3A4 transcripts and activities were strongly increased by their prototypical inducers. Thus, CYP1A2 mRNA and activity were induced by OME (Figures 7C and 8C) and CYP2B6 and CYP3A4 mRNA and corresponding activities were induced by both PB and RIF (Figures 7A and B and 8A and B). In addition, various other CYPs were modulated by the three inducers (Supplemental data: Tables 2, 3 and 4). CYP1A1 and 1A2 transcripts were augmented in both HepaRG cells and human hepatocytes by OME. CYP1A1 expression was weakly increased by PB (3- to 6-fold) and very strongly by OME (450- to 850-fold) in HepaRG cells. CYP1A2 activity was induced by the three compounds in HepaRG cells. CYP1A1 activity was not
Specifically measured by the probe substrates. Expression and activity of CYP2B6 and CYP3A4 were also induced by three compounds in both LD- and HD-seeded HepaRG cells. However, induction of CYP3A4 was demonstrated only by 3-hydroxylation of omeprazole (NM) and 1-hydroxylation of midazolam (UAM). No induction of 1-hydroxylation of midazolam or 6β-hydroxylation of testosterone was evidenced with the NM cocktail. Expression of CYP2A6 was induced by PB in HepaRG cells while it was increased by the three prototypical inducers in human hepatocytes. On the other hand, CYP2E1 expression was decreased by all 3 inducers in both human hepatocytes and HepaRG cells. However, CYP2A6 and CYP2E1 activities were not detected with the cocktails in DMSO-free cultured HepaRG cells whatever the inducer.

RIF and OME decreased CYP2D6 expression in HepaRG cells but not in primary hepatocyte cultures. CYP2D6 activity was increased by RIF and PB in both LD and HD-seeded HepaRG cells by using the UAM cocktail (with bufuralol as a substrate), while this activity was decreased by the 3 inducers using the NM cocktail (with dextromethorphan as a substrate). However, these variations of activities were low compared to the fold inductions of other CYPs.

Induction of CYP2C (CYP2C8, CYP2C9 and CYP2C19) expression by PB and more weakly by RIF was observed in HepaRG cells, whereas only RIF induced CYP2C8 and CYP2C19 expression in human hepatocytes. CYP2C9 activity was weakly increased by OME as well as CYP2C8 by RIF and PB in HepaRG cells using the NM cocktail. Conflicting results were found for CYP2C19 activity with the two cocktails: a strong decrease of activity by OME and an increase by RIF were observed with the UAM cocktail (mephenytoin as a substrate), while this activity was strongly induced by PB and RIF and more weakly by OME with the NM cocktail (5-hydroxylation of omeprazole as the model reaction).

It is noteworthy that induction rates much lower in human hepatocytes than in HepaRG cells were observed (Figure 7). CYP1A1, CYP2C8, CYP2C9, CYP2C19, and CYP3A4 were found to be induced by PB in HepaRG cells but not in human hepatocytes (Supplemental data: Table 2).

5. Induction of phase II enzymes, transporters and nuclear receptors expression

Expression of phase II enzymes, transporters and nuclear receptors was investigated in both HepaRG cells and primary human hepatocytes in response to prototypical inducers (Supplemental data: Table 5). Overall effects were relatively well-maintained in differentiated...
HepaRG cells whether they were seeded at LD or HD over the 4-week period (Supplemental data: Table 5). GSTA1/2 and UGT1A1 expression was induced by the three inducers in LD and HD-seeded HepaRG cells, and only by RIF and OME in primary human hepatocytes. Similarly, expression of efflux transporters MDR1 and MRP2 was increased by the three inducers in HepaRG cell cultures and only by RIF and OME in primary hepatocyte cultures. An induction of BCRP was observed after PB and OME exposure in HepaRG cells, and only after OME treatment in human hepatocytes. In addition, an inhibition of the uptake-transporter NTCP expression by OME and a decreased expression of the efflux transporter BSEP by RIF were evidenced in both differentiated HepaRG cells and human hepatocytes. Some responses of nuclear receptors to inducers were also observed. A RIF-dependent decrease and a PB-dependent induction of CAR expression were observed in HepaRG cells.
Because human hepatocytes are a precious, limited resource and show an extensive inter-donor variability in response to inducers, alternatives are required for use in drug discovery. Previous studies have shown that human hepatoma HepaRG cells express most of the major drug metabolizing enzymes when they are differentiated in vitro. The present results confirm and extend these observations; they show that expression and activities of the major CYPs as well as their responsiveness to prototypical inducers were well maintained over a 4-week period in differentiated HepaRG cells obtained from either LD- or HD- seeded cultures. Only random and limited differences were noticed between these two seeding conditions. Contrary to LD seeding that requires one month before HepaRG cells can be used as fully differentiated (Cerec et al., 2007) HD seeding prevents transdifferentiation (Aninat et al., 2006; Cerec et al., 2007) and is associated with only transient and limited decrease in certain liver-specific functions. Consequently, HD- seeded cultures are of potentially great value for high volume, convenient use of the HepaRG cell line.

An extensive analysis of CYP activities was performed using substrate cocktails. This approach requires much fewer sample numbers as the same culture is incubated with all the substrates. Most results obtained with the UAM and NM cocktails employed in the present study were found to be comparable. However, some differences were observed, which could be due to differences in the experimental conditions and/or the choice of probe substrates. Indeed, UAM and NM cocktails were composed of 8 and 10 substrates, respectively (Turpeinen et al., 2005; Lahoz et al., 2008b) and only 4 substrates were in common: bupropion for CYP2B6, coumarin for CYP2A6, chlorzoxazone for CYP2E1, and midazolam for CYP3A4. Interestingly, CYP activities were higher with the UAM cocktail and detectable CYP2A6 and CYP2E1 activities were demonstrated only in the presence of DMSO and with this cocktail. This could be explained by the fact that substrate concentrations in the UAM cocktail are close to or higher than their $K_m$ values whereas lower substrate concentrations are used in the NM cocktail that has been primarily designed for drug-drug interactions studies. No evidence of any kind of interaction or interference between the substrates has been observed in the latter (Turpeinen et al., 2005). Since testosterone is rapidly metabolized in HepaRG cells (Turpeinen et al., 2009) it is likely that the absence of detectable 6ß-OH-testosterone metabolites with the NM cocktail was due to use of an inadequate concentration of testosterone and the conjugation of all the metabolites formed during the incubation period.
However, two major differences were observed after OME exposure: CYP1A2 induction was much higher using the UAM cocktail and CYP2C19 activity was increased using the NM cocktail and decreased using the UAM cocktail. In both cases such effects could be attributed to the choice of different substrates. In the case of CYP1A2, phenacetin and melatonin were used in UAM and NM assays, respectively. Melatonin has been proven to be a less selective substrate (FDA-Guide, 2001), than phenacetin for CYP1A2 induction studies. Regarding CYP2C19, UAM and NM cocktails contained mephenytoin and omeprazole as a substrate, respectively. CYP2C19 inhibition has been described to occur when OME was used at high concentrations (Ko et al., 1997). As OME is a substrate for CYP2C19, its potential intracellular accumulation during induction studies could inhibit mephenytoin metabolism by CYP2C19.

In support of previous observations (Aninat et al., 2006) using the two substrate cocktails, we showed that several CYPs which were strongly increased in the presence of DMSO recovered their basal levels after DMSO withdrawal. Activities of CYP3A4 and CYP2B6 were the most sensitive to DMSO and those of CYP2A6 and CYP2E1 became undetectable in cells cultured for 3 days in a DMSO-free medium, indicating that the DMSO-free conditions were not suitable for their expression. The cause of this loss of CYP2E1 activity was unclear. Its inhibition by insulin has been reported in a rat hepatoma cell line (Moncion et al., 2002). However, medium depletion or lower concentrations of insulin did not prevent CYP2E1 activity loss in HepaRG cells (not shown).

A good correlation was observed between transcripts and corresponding activities. However, fold-inductions of mRNA levels were much higher especially for CYP1A2 (800-fold for transcripts vs 30-fold for activity with the UAM cocktail), CYP2B6 (50-fold for transcripts vs 30-fold for activity), and CYP3A4 (80-fold for transcripts vs 10-fold for activity). As previously emphasized (Aninat et al., 2006), maintenance of HepaRG cells in conditions in which basal CYP activities were low, resulted in strong increased levels after treatment with prototypical inducers. Fold-inductions of CYP transcripts and activities were much higher than in primary hepatocytes, especially for CYP3A4, demonstrating than HepaRG cells were still highly responsive when treatments with inducers were started 3 days after DMSO withdrawal.

While basal expression and activity of CYPs and responsiveness to prototypical inducers are variable in human hepatocyte populations (Morel et al., 1990; Madan et al., 2003) they were always found at comparable levels in differentiated HepaRG cells when comparing different passages. Interestingly several genes, including CYPs, which have been found to be induced
in vitro only in a fraction of human hepatocyte populations (Goyak et al., 2008) were responsive in differentiated HepaRG cells. Accordingly, important inter-donor variation was found for basal CYP2B6 and CYP2C19 activities in primary human hepatocytes and expression of CYP1A1, CYP2C8, CYP2C9, CYP2C19 and CYP3A4 was induced by PB in only HepaRG cells (Table 1). Recently, induction of CYP1A1/2 activities in HepaRG cells, but not in primary human hepatocytes, was also reported after PB treatment (Turpeinen et al., 2009). A major difference between HepaRG cells and primary hepatocytes was the continuous expression of CYP1A1 in the former; this could be related to their transformed state rather to the presence of biliary cells. RIF is another well established inducer and our data (Table 1) reproduced well the previous findings in primary hepatocytes (Morel et al., 1990; Lahoz et al., 2008b) and HepaRG cells (Aninat et al., 2006; Kanebratt and Andersson, 2008b). Therefore HepaRG cells appear to be representative of a large fraction of human hepatocyte populations.

Phase II enzymes and plasma transporters are also targets of microsomal enzyme inducers. However only limited studies still exist on the regulation of UGTs in human hepatocytes (Soars et al., 2004; Nishimura et al., 2008). We show that in addition to the major CYPs, several phase II enzymes and transporters were modulated by PB, RIF, and/or OME. The three inducers increased both UGT1A1 and GSTA1/A2 transcripts in differentiated HepaRG cells; this is in agreement with previous observations in primary hepatocytes. Indeed, it has been reported that PB is an inducer of GSTA1/2 (Morel et al., 1993) and that OME and RIF are inducers of UGT1A1 (Nishimura et al., 2008). Notably, the fold-inductions of these two genes with the 3 inducers were much lower than those observed with CYPs, ranging mostly between 1.5- and 5-fold with the exception of UGT1A1 induction by PB and OME that reached 7-10-fold (Table 1). The low mean increase of UGT1A1 (1.5-fold) and the absence of modulation of GSTA1/A2 in 3-day hepatocytes exposed to PB in the present study can be explained by inter-donor variability in response to this compound (Morel et al., 1993; Soars et al., 2004).

Various transporters were also analysed at the transcriptional level and found to be modulated in HepaRG cells (Table 1). MDR1 and MRP2 were increased by the three inducers and BCRP by OME and PB. NTCP was inhibited mainly by OME and BSEP by RIF and slightly by OME. Our results fully agree with previous reports showing induction of MDR1 and MRP2 by PB in HepaRG cells (Le Vee et al., 2006; Lambert et al., 2009). Our results replicate data
previously obtained with human hepatocytes, demonstrating consistent and wide inter-donor variability in response to identical inducers (Jigorel et al., 2006).

In summary, although HepaRG cells are originated from only one donor and are the product of in-vivo transformation, they exhibit a drug metabolism capacity, including responsiveness to chemical modulators, that largely reflects that observed with the majority of human hepatocyte populations in primary culture and in addition, offer several unique advantages including: 1- the data are reproducible during several passages; 2- the functional activities are well-maintained for several weeks at confluence; 3- the levels of activities can be modulated by selecting appropriate culture conditions, especially the composition of the culture medium. Therefore we conclude that HepaRG cells represent not only a promising surrogate to primary human hepatocytes for investigating induction of xenobiotic metabolizing enzymes and transporters and drug-drug interactions but also a unique metabolically competent cell model for in vitro chronic toxicity studies.
REFERENCES


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Footnotes

We thank Dr David Steen for critical reading of the manuscript. This work was supported by EEC contracts: LIINTOP-STREP-037499 and Predict-IV-contract 202222 (to AG), the Ministerio Ciencia e Inovación /Instituto de Salud Carlos III for a Miguel Server contract (CP08/00125 to AL).
Figure 1: Seeding and culture of HepaRG cells. HepaRG cells were cultured at low density (LD) or high density (HD). At LD seeding HepaRG cells transdifferentiated into bipotent progenitors and actively divided until confluence (15 days); then they differentiated into both hepatocyte-like and biliary-like cells (around 50% each). Maximum liver-specific activities were attained after 2 weeks in the growth medium supplemented with 2% DMSO (28 days). When seeded at HD, HepaRG cells were directly incubated in the DMSO-containing medium; they did not transdifferentiate and retained their morphological and functional characteristics. For induction studies, HepaRG cells were seeded either at LD and cultured for up to 56 days or at HD and cultured for up to 28 days.

Figure 2: Basal mRNA levels of phase I and II metabolizing enzymes, membrane transporters and nuclear receptors in human hepatocytes. Human hepatocytes were used either freshly isolated, or 1 to 3 days after seeding. Transcripts of CYPs (A), phase II metabolizing enzymes, membrane transporters and nuclear receptors (B) were then analyzed using RT-qPCR assays. Results are the mean ± SD of 3 independent experiments and are expressed as percentage compared to 1-day cultured human hepatocytes, arbitrarily set at 100%. The Mann-Whitney U test was applied to compare mRNA levels ( * : p<0.05, ns: no significant).

Figure 3: Basal mRNA levels of phase I and II metabolizing enzymes, membrane transporters and nuclear receptors in HepaRG cells. HepaRG cells were seeded at LD and cultured for 5-56 days or at HD and cultured for 5-28 days. Three days before expression analysis they were shifted to DMSO-free medium (B and D) or maintained in presence of DMSO (A and C). Transcripts of CYPs (A and B), phase II metabolizing enzymes, membrane transporters and nuclear receptors (C and D) were then analyzed using RT-qPCR assays. Results are the mean ± SD of 3 independent experiments (for DMSO-free cultured HepaRG cells) and are expressed as percentages compared to 1-day cultured human hepatocytes, arbitrarily set at 100%. The Kruskal-Wallis non-parametric test was applied to compare mRNA levels between different time points in differentiated HepaRG cells (LD-D28 to LD-D56 and HD-D5 to HD-D28, §: p<0.05, ns: no significant).
Figure 4: Comparative basal activity levels of CYPs in primary human hepatocytes and HepaRG cells using the UAM cocktail of substrates assay. Human hepatocytes were used either freshly isolated or 1-day after seeding (A). HepaRG cells were seeded at LD and cultured for 5-56 days or at HD and cultured for 5-28 days. Three days before CYP activity analysis they were shifted to DMSO-free medium (C) or maintained in presence of DMSO (B). CYPs activities were then analyzed using the UAM cocktail of substrates assay. Results are the mean ± SD of 3 independent experiments and are expressed in pmoles/min/mg protein. The Mann-Whitney U test was applied to compare CYP activities in FIH and in 1 day-cultured-human hepatocytes (*: p<0.05), and the Kruskal-Wallis non-parametric test was applied to compare CYP activities between different time points in differentiated HepaRG cells (LD-D28 to LD-D56 and HD-D5 to HD-D28, §: p<0.05, ns: no significant).

Figure 5: Basal activity levels of CYPs in HepaRG cells using the NM cocktail of substrates assay. HepaRG cells were seeded at LD and cultured for 5-56 days or at HD and cultured for 5-28 days. Three days before CYP activity analysis they were shifted to DMSO-free medium (B) or maintained in presence of DMSO (A). CYPs activities were then analyzed using the NM cocktail of substrates assay. Results are the mean ± SD of 3 independent experiments and are expressed in pmoles/min/mg protein. The Kruskal-Wallis non-parametric test was applied to compare CYP activities between different time points in differentiated HepaRG cells (LD-D28 to LD-D56 and HD-D5 to HD-D28, §: p<0.05, ns: no significant).

Figure 6: Light microscopic appearance of HepaRG cells after DMSO withdrawal and activity of efflux transporters (BSEP/MDR1 and MRP2) in differentiated HepaRG cells. HD-seeded HepaRG cells were cultured for 56 days and then used 5 days after DMSO withdrawal (A). The cells were incubated with fluorescent substrates (CDFDA, fluorescent substrate of MRP2 and BODIPY FL vinblastine, fluorescent substrate of BSEP and MDR1) for 30 min at 37°C, and then observed under phase-contrast and fluorescence microscopy (B). The pictures are representative of three independent experiments. BC, bile canaliculus. Original magnification 150x.
Figure 7: Effects of RIF, PB and OME on expression of CYP3A4 (A) CYP2B6 (B) and CYP1A2 (C) respectively, in HepaRG cells and human hepatocytes. Human hepatocytes were used 1 to 4 days after seeding. HepaRG cells were seeded at LD and cultured for 15-56 days or at HD and cultured for 5-28 days. Three days before inducer addition they were cultured in DMSO-free and 2% SVF medium and then exposed to 3 prototypical inducers (10 μM RIF (Rif), 1 mM PB (PB) or 50 μM OME (OME)) or their vehicles for 48h in DMSO-free and 2% SVF medium. Transcripts were analyzed using RT-qPCR assays. Results are the mean ± SD of 3 independent experiments and are expressed as percentage of mRNA levels measured in the vehicle (arbitrarily set at 100%). The Kruskal-Wallis non-parametric test was used to compare mRNA levels between different time points in differentiated HepaRG cells (LD-D28 to LD-D56 and HD-D5 to HD-D28, §: p<0.05, ns: no significant) and the Mann-Whitney U test was applied to compare mRNA levels between treated cultures with inducers and corresponding control samples ( * : p<0.05).

Figure 8: Effects of RIF, PB and OME on activity of CYP3A4 (A) CYP2B6 (B) and CYP1A2 (C) respectively, in HepaRG cells. HepaRG cells were seeded at LD and cultured for 15-56 days or at HD and cultured for 5-28 days. Three days before inducer addition they were cultured in DMSO-free and 2% SVF medium and then exposed to 3 prototypical inducers (10 μM RIF (Rif), 1 mM PB (PB) or 50 μM OME (OME)) or their vehicles for 72h in DMSO-free and 2% SVF medium. CYPs activities were then analyzed using the two cocktail of substrates assays (UAM and NM). Results are the mean ± SD of 3 independent experiments and are expressed as percentage of activity levels measured in the vehicle (arbitrarily set at 100%). The Kruskal-Wallis non-parametric test was used to compare CYP activities between different time points (§: p<0.05, ns: no significant), and the Mann-Whitney U test was applied to compare CYP activities between treated with inducers and corresponding control samples ( * : p<0.05).
Table 1: Fold-induction of phases I and II enzymes, transporters and nuclear receptors by RIF, PB and OME in differentiated HepaRG cells (A: transcripts; B: activities)

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Figure 1
Figure 2

(A) Expression (% of HH 1 day)

(B) Expression (% of HH 1 day)
Figure 4

(A) 

(B) + DMSO
- LD - 28 days
- LD - 42 days
- LD - 56 days
- HD - 3 days
- HD - 15 days
- HD - 28 days

(C) DMSO-free
- LD - 5 days
- LD - 10 days
- LD - 20 days
- LD - 40 days
- LD - 56 days
- HD - 3 days
- HD - 15 days
- HD - 28 days

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Figure 6

(A) Fluorescence phase contrast

+ DMSO  DMSO-free

BC

(B) Phase contrast  Fluorescence

CDFDA (MRP2)

Vinblastine (BSEP/MDR1)
Figure 7

(A) HepaRG HD

(B) HepaRG LD

(C) HH
Figure 8

(A) UAM vs NM for HepaRG LD and HD.

(B) UAM vs NM for HepaRG LD and HD.

(C) UAM vs NM for HepaRG LD and HD.