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# EXPRESSION OF CYTOCHROMES P450, CONJUGATING ENZYMES and NUCLEAR RECEPTORS IN HUMAN HEPATOMA HepaRG CELLS

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**D. Abbreviations**

CYP, cytochrome P450; AhR, aryl hydrocarbon receptor; PXR, pregnane X receptor; CAR, constitutive androstane receptor; PPARα, peroxisome proliferator-activated receptor α; RXR, 9-cis retinoid X receptor ; UGT, UDP-glucuronosyl transferase ; GST, Glutathione S-transferase; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction ; FCS, fetal calf serum; $IC_{50,}$ inhibitory concentration 50; $AFB_1$, Aflatoxin $B_1$; EROD, Ethoxyresorufin-O-deethylase; DMSO, Dimethylsulfoxide; MTT, methylthiazoletetrazolium; GCS, gamma glutamylcysteine synthase.

## ABSTRACT

Most human hepatocyte cell lines lack a substantial set of liver-specific functions, especially major cytochrome P450 (CYP)-related enzyme activities, making them unrepresentative of *in vivo* hepatocytes. We have used the HepaRG cells derived from a human hepatocellular carcinoma, that exhibit a high differentiation pattern after 2 weeks at confluency to determine whether they could mimic human hepatocytes for drug metabolism and toxicity studies. We show that when passaged at low density these cells reversed to an undifferentiated morphology, actively divided and after having reached confluency formed typical hepatocyte-like colonies surrounded by biliary epithelial-like cells. By contrast, when seeded at high density, hepatocyte-like clusters retained their typical differentiated morphology. Transcripts of various nuclear receptors (AhR, PXR, CAR, PPARα), CYPs (1A2, 2C9, 2D6, 2E1, 3A4), phase 2 enzymes (UGT1A1, GSTA1, GSTA4, GSTM1) and other liver-specific functions were estimated by RT-qPCR and found to be expressed for most of them at comparable levels in both confluent differentiated and high density differentiated HepaRG cells and in cultured primary human hepatocytes. For several transcripts the levels were strongly increased in presence of 2% dimethylsulfoxide. Measurement of basal activities of several CYPs and their response to prototypical inducers as well as analysis of metabolic profiles and cytotoxicity of several compounds confirmed the functional resemblance of HepaRG cells to primary cultured human hepatocytes. In conclusion, HepaRG cells constitute the first human hepatoma cell line expressing high levels of the major CYPs involved in xenobiotic metabolism and represent a reliable surrogate to human hepatocytes for drug metabolism and toxicity studies.

## INTRODUCTION

Human primary hepatocytes and immortalized hepatocytes are widely used for xenobiotic metabolism, toxicity studies and the design of bioartificial liver devices; however, both systems have limitations (Guillouzo et al., 1993; Maurel, 1996; Guillouzo, 1998; LeCluyse, 2001). Primary hepatocytes have scarce and unpredictable availability, limited growth activity and life-span, and undergo early phenotypic alterations. Huge variations in functional activities, especially CYP levels as well as in magnitude of CYP induction after treatment with prototypical inducers have been reported from one human hepatocyte population to another (Guillouzo and Chesne, 1996; Madan et al., 2003). Moreover, all CYPs are not similarly maintained with time in culture. Although various culture conditions, such as the use of sophisticated media, extracellular matrices as supports or sandwich systems, addition of DMSO or co-culturing with other cell types, may improve to some extent maintenance of CYPs and other liver-specific functions, these are usually already markedly and differently decreased early after hepatocyte seeding. Nevertheless, primary hepatocytes have proved to be the most suitable model for investigating induction of CYPs by chemical inducers and metabolic profiles of new drugs (Guillouzo et al., 1993; Maurel, 1996; Guillouzo, 1998). Hepatocyte cell lines, mainly originated from tumors, have undefinite proliferative capacity but they lack a variable and substantial set of liver-specific functions, making them unsuitable as representative of *in vivo* liver parenchymal cells. In particular, CYP expression is usually very low or undetectable in human hepatoma cells. Even the human hepatoma HepG2 cells that have retained several liver-specific functions, contain little CYP with the exception of the isoforms expressed in fetal cells such as CYP1A1 and CYP3A7 (Sassa et al., 1987; Ogino et al., 2002). Stably transfected liver cell lines have been established by using eukaryotic CYP expression vectors under the control of inducible/constitutive promoters (Pfeifer et al., 1993) and reexpression of CYPs has been

obtained in human hepatoma cells by transfection of liver-specific transcription factors such as c/EBP alpha (Jover et al., 1998). However, these cell lines do not mimic regulation of gene expression observed in normal hepatocytes (e.g. CYP induction by phenobarbital) (Zelko and Negishi, 2000).

Recently, we have obtained a new human hepatoma cell line derived from an hepatocellular carcinoma, named HepaRG, that exhibits extensive differentiation after 2 weeks at confluency and has the unique property to be susceptible to hepatitis B virus infection (Gripon et al., 2002). In the present study we have analyzed expression of the main drug metabolizing enzymes in HepaRG cells under different culture conditions. Our results demonstrate that, in conditions in which cells attain a differentiated hepatocyte-like morphology, they retain a unique set of drug metabolizing enzymes at levels comparable to those measured in normal human hepatocytes in primary culture.

## METHODS

### Chemicals

Aflatoxin $B_1$ ($AFB_1$), aflatoxin $M_1$ ($AFM_1$), aflatoxin $P_1$ ($AFP_1$), chlorpromazine, amiodarone hydrochloride, acetaminophen, testosterone, 6β-hydroxytestosterone, chlorzoxazone, 3-methylcholantrene, chlopropamide, resorufin, 7-ethoxyresorufin, methylthiazoletetrazolium (MTT), dimethylsulfoxide (DMSO), rifampicin, salicylamide, isoniazid, dexamethasone and insulin were purchased from Sigma (St. Quentin Fallavier, France). Metabolites of acetaminophen were kindly provided by Biopredic (Rennes, France). Aflatoxin-glutathione conjugate ($AFB_1$-GSH) and aflatoxin $B_1$-dialcohol were kindly supplied by Pr Guengerich. 6-Hydroxychlorzoxazone was from Ultrafine chemicals (Manchester, UK). William's E medium was purchased from Eurobio (Les Ulis, France). Fetal calf serum (FCS) was from Perbio (Brebières, France). Penicillin, streptomycin, miminum essential medium (MEM) alpha and non essential amino acids were from Invitrogen Corporation (Gibco). Hydrocortisone hemisuccinate was from Upjhon Pharmacia (Guyancourt, France). All other chemicals were of the highest quality available.

### Cell cultures

HepaRG cells were obtained from a liver tumor of a female patient suffering from hepatocarcinoma (Gripon et al., 2002). Briefly, small tumor pieces were digested with 0.025% collagenase D diluted in Hepes buffer supplemented with 0.075% $CaCl_2$. The cell population was suspended in William's E medium added with 10% FCS, 5µg/ml insulin and $5x10^{-7}$ M hydrocortisone hemisuccinate and distributed in several dishes. The most hepatocyte-like populations were selected and passaged by gentle trypsinisation. After three passages cell aliquotes were cryopreserved. Induction of differentiation was obtained by treating confluent

cells with the culture medium containing 2% DMSO and $5x10^{-5}$M hydrocortisone hemisuccinate.

For the present studies, HepaRG[1] cells were detached by gentle trypsinization and seeded at a density of either $2.6 \times 10^4$ cells/cm$^2$ (low density) or $0.45 \times 10^6$ differentiated cells/cm$^2$ (high density). Low density cells were first seeded in the growth medium composed of William's E medium supplemented with 10% FCS, 100 units/mL penicillin, 100µg/mL streptomycin, 5µg/mL insulin, 2mM glutamine and $5 \times 10^{-5}$ M hydrocortisone hemisuccinate. After 2 weeks they were shifted to the same culture medium supplemented with 2% DMSO (differentiation medium) for 2 more weeks (confluent DMSO-treated cells) (Gripon et al., 2002). The medium was renewed every 2-3 days. High density differentiated HepaRG cells were directly seeded in the differentiation medium.

Human hepatocytes from 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 110,000 cells/cm$^2$ in 24-well plates in William's E medium supplemented with 10% FCS, 100 units/mL penicillin, 100µg/mL streptomycin, 1µg/mL insulin, 2mM glutamine and 1µg/mL bovine serum albumin. The medium was discarded 12 hours after seeding and cells were thereafter maintained in serum-free medium supplemented with $10^{-7}$M dexamethasone. The medium was renewed every day. Human hepatocytes were used either freshly isolated or 3 to 5 days after seeding. HepG2 cells were seeded at a density of 100,000 cells/cm$^2$ in 24-well plates and were used at the time they reached confluency. The growth medium was composed of MEM alpha supplemented with 10% FCS, non essential amino acids, 100 units/mL penicillin and 100µg/mL streptomycin**.**

**Isolation of RNA and RT-qPCR analysis.**

Total RNA was extracted from $10^6$ HepaRG cells, $10^6$ HepG2 cells or $10^6$ human hepatocytes with the SV total RNA isolation system (Promega, Madison, WI, USA) which directly included a DNAase treatment step. RNAs were reversed-transcribed into cDNA using High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR for all genes except for PPARα 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 shows primer pairs for each transcript chosen with Primers 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), except for PPARα (Assay-on-demand, Applied Biosystems) and CYP2D6 (gift from Biopredic, Rennes, France). For PPARα, we detected FAM fluorescence of the Taqman probe using the TaqMan® Universal PCR Master Mix kit (Applied Biosystem). 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 by an active reference, 18S RNA. Furthermore, a dissociation curve was performed after the PCR reaction to verify the specificity of the amplification. Results were expressed as a percentage of mRNA levels measured in freshly isolated hepatocytes arbitrarily set at 100%.

**Determination of drug metabolizing enzyme activities and metabolic profiles**

For the determination of CYP-related activities, HepaRG cells and primary human hepatocytes were incubated with specific substrates in phenol red-free medium deprived of either both FCS and 2% DMSO or only FCS. The substrate concentrations were 200µM testosterone, 300µM chlorzoxazone, 0.2mM tolbutamide and 5µM ethoxyresofurin. For induction studies, HepaRG cells were first exposed to either 25 or 50µM rifampicin or 50µM

isoniazid for 72h, or to 5µM 3-methylcholantrene for 24h before incubation with the specific substrate. These concentrations of substrates and inducers are those usually used with primary human hepatocyte cultures (Langouet et al, 1995, Guillouzo and Chesne, 1996; Madan et al, 2003). 6β-Hydroxylation of testosterone, tolbutamide 4-hydroxylation and chorzoxazone 6-hydoxylation were estimated by HPLC analysis (Guillouzo and Chesne, 1996). Resorufin formation by 7-ethoxyresorufin O-deethylation (EROD) was quantified by fluorimetry with a fluorescence plate reader (SpectraMAX Gemini; Molecular Devices®) after a 30min incubation with ethoxyresorufin in the presence of 3µM salicylamide in order to inhibit phase II enzyme activities (Burke and Mayer, 1983). Fluorescence was determined at $\lambda_{ex}$ 544 nm and $\lambda_{em}$ 584 nm.

Acetaminophen and $AFB_1$ metabolites were analysed by HPLC. Metabolites of acetaminophen were determined as previously described (Guillouzo and Chesne, 1996). Acetaminophen was incubated at 2mM for 10 hours before medium harvesting. $AFB_1$ metabolism was analysed as follows: after a 8 hours incubation with the mycotoxin at 5 µM (Langouet et al., 1995), aliquots of media were collected and centrifugated 2 minutes at 10,000 rpm. 90µL of sample were injected in the HPLC system. An Interchrom $C_{18}$ column (250 × 4.6mm, 5µm) was used for the chromatographic separation. The mobile phase consisted of two solvents, A (ammonium acetate 0.01M pH 6.5) and B (acetonitrile) with the following gradient : 0 min 15 % B – 23 min 23% B – 43 min 53%B – 45 min 100% B – 47 min 100% B – 47.5 min 15%B – 52 min 15% B. All the gradients were linear and the flow rate was set at 1 mL/min. Fluorescence detection was monitored at excitation and emission wavelengths of 430 nm and 370 nm respectively.

The HPLC apparatus consisted of an Agilent 1100 series HPLC equiped with an autosampler and Agilent 1100 Series fluorescence and UV detectors. A computer running Agilent 1100 software (ChemStation®) was used to integrate and calculate the separated peak

areas and to plot metabolite patterns. Metabolites tested were identified by comparison of retention times and quantifications were estimated from calibration curves.

**Evaluation of drug cytotoxicity**

The effects of four reference hepatotoxic compounds, namely acetaminophen, chlorpromazine, amiodarone and $AFB_{1,}$ were evaluated on HepaRG and HepG2 cells after either 24 or 72 hours of exposure. Incubations were performed with medium deprived of 2% DMSO and FCS. At the end of the incubation time, cultures were observed by phase-contrast microscopy using an Olympus 1X70. Then the medium was discarded and replaced with FCS-free medium containing 1mg MTT/mL. After 2 hours formozan cristals were dissolved in DMSO and the intensity of color was determined at 540 nm using a microplate reader (iEMS Reader MF, Labs systems®).

**Statistical analysis**

Data are presented as means +/- SD. Each value corresponded to a different cell culture. Student's t test was applied to samples for statistical analysis. Data were considered significantly different when $p<0.05$.

## RESULTS

### Behavior of HepaRG cells in culture (Figure 1).

When seeded at low density onto plastic, HepaRG cells took a morphology of elongated undifferentiated cells characterized by a clear cytoplasm and actively divided until they reached confluency, i.e. around day 10. Then they progressively formed typical colonies of granular epithelial cells surrounding by more flattenned and clearer epithelial cells. Addition of DMSO was followed by appearance of a denser cytoplasm and a morphology resembling that of typical normal hepatocytes in primary culture with the formation of bile canaliculus-like structures. At this stage, hepatocyte-like cells represent 30 to 40% of the population. When seeded at high density after differentiation, HepaRG cells retained their hepatocyte-like morphology. This particular condition allowed to concentrate the hepatocyte-like population.

### Expression of specific genes analyzed by RT-qPCR

Transcripts of 6 CYPs (1A2, 2B6, 2C9, 2D6, 2E1, 3A4), 4 nuclear receptors (AhR, PXR, CAR, PPARα), 4 phase 2 enzymes (UGT1A1, GSTA1, GSTA4, GSTM1), 3 liver-specific proteins (albumin, haptoglobin, aldolase B) as well as alpha-fetoprotein (AFP), glutathione-related enzymes (GCS regulatory subunit, GCS catalytic subunit, glutathione synthase, glutathione reductase) and thioredoxin were analyzed by RT-qPCR in HepaRG cells at different times after seeding and in the different culture conditions described in Figure 2. For comparison, the same transcripts were analyzed in HepG2 cells, in freshly isolated human hepatocytes and in addition for several major genes in primary human hepatocytes at various times of culture. As shown in Tables 2 and 3, all the mRNAs analysed, including CYP ones, were expressed in HepaRG cells. However, transcripts of CYPs and the CAR nuclear receptor were detectable in low density seeded cells only after 8 days. mRNA levels were usually

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slightly higher in HepaRG cells seeded at high density than in those seeded at low density and attaining differentiation after 2 weeks at confluency, probably due to a greater proportion of differentiated hepatocyte-like cells in the former. Furthermore, the highest values were frequently observed in differentiated cells exposed to 2% DMSO. However, some differences were evidenced: mRNA levels of CYP2B6, CYP2E1, CYP3A4 and to a lower extent CAR, UGT1A1, GSTA4, GSTM1 and GSTA1/A2, were markedly increased in the presence of DMSO while those of CYP1A2, CYP2C9, CYP2D6, PXR, PPARα, AhR, albumin, aldolase B and haptoglobin were only slightly modulated. Compared to those measured in primary human hepatocytes (Table 4), with the exception of CYP2D6 that remained low whatever the culture conditions, a trend towards either close or higher (CYP2B6 and CYP3A4) levels of other transcripts was observed in HepaRG cells. As expected, the greatest values found in human hepatocytes were obtained after 3-5 days of culture. Compared to HepG2 cells major differences were noticed: indeed some transcripts were not detected in these cells (CYP2C9, CYP2E1, CYP3A4, aldolase B) while the levels of others, with the exception of CYP2D6 and especially alpha-fetoprotein, showed a trend towards a decrease (CYP1A2, CYP2B6, CAR, UGT1A1, GSTA1/2, GSTA4, GSTM1, haptoglobin and albumin) or were close (AhR, PXR, PPARα) compared to HepaRG cells. Whatever the cell type and the culture conditions, no variations were observed in thioredoxin mRNAs as well as in transcripts of glutathione-related enzymes involved in glutathione metabolism (data not shown).

**Drug metabolizing enzyme activities**

Four enzyme activities, namely 7-ethoxyresorufin O-deethylation, 6β-hydroxylation of testosterone, tolbutamide 4-hydroxylation and chorzoxazone 6-hydroxylation, were analysed in HepaRG cells cultured in five different experimental conditions (Table 5). Incubations with specific inducers were performed in media free of FCS and in the presence or absence of

DMSO for either 24h (ethoxyresorufin ± 3-methylcholantrene) or 72h (other substrates ± inducers). With the four substrates the lowest values were obtained with 15 days cultures from low density cell seeding. Whatever the culture condition only traces of 7-ethoxyresorufin O-deethylation was detected in the absence of inducer. By contrast after a 24h treatment with 5µM 3-methylcholantrene, a strong induction of this activity was observed. Furthermore, the highest values were found in DMSO-exposed cultures (more than a 2-fold increase) compared to corresponding DMSO-free cultures. Tolbutamide 4-hydroxylase activity (CYP2C9) was detected in all culture conditions. However, in basal conditions, this activity was increased around 6-10 fold in differentiated HepaRG cells whether seeded at low or high density and exposed or not to DMSO compared with 15 days cultures. A 72h treatment with 25µM rifampicin resulted in a non significant increase in 15 days cultures and no more than around 2-fold increase in differentiated cells exposed or not to DMSO. Chlorzoxazone 6-hydroxylase activity (CYP2E1) was also much greater in differentiated than in 15 days HepaRG cell cultures. The highest values were obtained with cells seeded at high density. A 3 days exposure to 50µM isoniazid had limited effects if any. As tolbutamide 4-hydroxylase and chlorzoxazone 6-hydroxylase activities, 6β-hydroxytestosterone formation (CYP3A4) was augmented in DMSO-free differentiated HepaRG cell cultures compared with 15 days cultures (3.5 -5.0 fold) and was further strongly increased in differentiated cells when maintained in the presence of DMSO (around 14-fold more) whether the cells were seeded at low or high density. These activities represented around 10 and 500-800 pmoles/mg/min in 15 days HepaRG cells and DMSO-treated differentiated HepaRG cells respectively. A 72h rifampicin treatment also resulted in a huge significant increase both in 15 days cells and in differentiated cells seeded at low and high density and exposed in the absence of DMSO (11.8, 5.3 and 10-fold respectively). By contrast, when differentiated cells seeded at high

density were treated with rifampicin in the presence of DMSO no significant increase of testosterone 6β-hydroxylase activity was evidenced

**Metabolic profiles**

$AFB_1$ is the most powerful hepatocarcinogen compound in human. Its action is mediated by the formation of $AFB_1$-8,9-epoxide which can bind covalently DNA to form $AFB_1$-DNA adducts. CYP1A2 and CYP3A4 are involved in its biotransformation (Langouet et al., 1996). Metabolism of AFB1 leads to the formation of several metabolites : $AFM_1$ and $AFP_1$ which are hydroxylated products of $AFB_1$ and aflatoxin $B_1$-dialcohol and $AFB_1$-GSH which are detoxified products of $AFB_1$-8,9-epoxyde. The metabolite profiles of $AFB_1$ after incubation with differentiated HepaRG cells and 3 days primary human hepatocytes are displayed in Figure 3.A. $AFM_1$, $AFP_1$ and aflatoxin $B_1$-dialcohol were identified and the same range of values was obtained in both cell cultures ($AFM_1$: 0.27 and 0.4 ; $AFP_1$: 66 and 92 and $AFB_1$-diol: 0.3 and 0.3 pmol/mg/min in HepaRG cells and human hepatocytes respectively). $AFB_1$-GSH conjugates were detected only after a 3-methylcholantrene induction in HepaR cells and were found in higher amounts in human hepatocyte cultures (<0.1 and 0.32 pmol/mg/min respectively).

Acetaminophen is one of the most highly used non-prescription analgesic and antipyretic drugs. At therapeutic doses, this compound is detoxified through the formation of glucuronides and sulfoconjugates that are rapidly excreted in urine. At toxic doses, a depletion in glutathione occurred and an electrophilic metabolite, the N-acetyl-p-benzoquinone imine, is formed by CYP2E1, CYP3A4 and CYP1A2. Figure 3.B shows that differentiated HepaRG cells seeded at low density were able to metabolize acetaminophen in sulfate and glucuronide metabolites. The same profile was observed when acetaminophen was incubated with human hepatocytes after 5 days in primary culture. The values were 280.2±37.0 and 347.9±70.2

pmol/mg/min for glucuronides and 51.5±6.4 and 132.6±11.7 pmol/mg/min for sulfoconjugates in HepaRG cells and human hepatocytes respectively.

**Evaluation of xenobiotic cytotoxicity**

Toxicity of four compounds, namely acetaminophen, $AFB_1$, amiodarone and chlorpromazine, was estimated in differentiated HepaRG cells from low or high density cultures and in HepG2 cells using the MTT test (Figure 4). Whatever the compound similar data were obtained with differentiated HepaRG cells whether they were seeded at low or high density and differences between HepaRG and HepG2 cells were observed only for the two compounds, acetaminophen and $AFB_1$, which toxicity is mediated by formation of toxic metabolites by CYPs. Indeed, as expected, their toxicity, especially that of the most toxic one, $AFB_1$, was much greater in HepaRG than in HepG2 cells after either 24 or 72h of treatment. No toxicity of $AFB_1$ was even observed in HepG2 cells after a 24 hours exposure to the highest dose tested (100 µM) while the $IC_{50}$ was around 5µM in HepaRG cell cultures at the same time. At 72h, no viable HepaRG cells were observed with $AFB_1$ concentrations greater than 5µM while around 50% of HepG2 cells were still alive with a 100µM concentration. Similar but much less marked differences were observed with acetaminophen. The slightly more intense toxicity observed in HepaRG cells seeded at high density than in HepaRG cells seeded at low density after a 24 or 72h incubation with $AFB_1$ or acetaminophen was probably due to a higher proportion of hepatocyte-like cells after seeding at high density.

For the two other compounds, amiodarone and chlorpromazine, that do not require metabolism to exhibit toxic effects, the dose-curve responses were quite similar in the two cell lines. Amiodarone, an antiarythmic drug, induced phospholipidosis characterized by cytoplasmic accumulation of vesicles in hepatocytes (Poucell et al., 1984). Such vesicles were easily observed in HepaRG cells after a 24h treatment by phase-contrast microscopy (Figure

5); they also appeared in HepG2 cells (not shown). Similar dose-curves responses were observed in HepaRG and HepG2 cells. $IC_{50}$ were around 50µM and 20µM after respectively 24 and 72h of treatment. Chlorpromazine, a cholestatic agent, exhibited $IC_{50}$ close to those calculated in amiodarone-treated HepaRG and HepG2 cell cultures.

**DISCUSSION**

Primary human hepatocytes represent the most pertinent model for *in vitro* drug metabolism and toxicity studies. However, they are not functionally stable with time in culture. Indeed, in agreement with previous observations (Morel et al., 1990; Abdel-Razzak et al., 1993; Morel et al., 1993) and as shown in this study, compared to those expressed in freshly human hepatocytes, a rapid and marked decrease of transcripts was observed for some genes, especially those encoding CYPs, after cell plating although significant levels of various phase 1 and phase 2 xenobiotic metabolizing enzyme activities were still observed for at least a few days in standard culture conditions. Transcript levels expressed in freshly human hepatocytes are comparable to those found in human liver tissue (Rodriguez-Antona et al., 2000) and a pool of three cell populations was used to reduce interindividual variations. Several approaches have been reported to improve preservation of liver-specific functions in primary hepatocyte cultures, e.g. co-culture with rat liver bilary epithelial cells (Guguen-Guillouzo et al., 1983), plating cells at different densities and using sandwich configuration by additional layer of extracellular matrix (Hamilton et al., 2001); however in all cases marked phenotypic changes have been observed resulting particularly in reduced expression of several major CYPs. By contrast, most human and animal hepatoma cell lines have retained little drug metabolism capacity. We show here that HepaRG cells exhibited a unique behavior in culture characterized by their ability to express various liver-specific functions after having reached confluency.

Six CYPs were analysed : three of them, CYP2C9, CYP3A4 and CYP2D6 are responsible for the metabolism of around 90% of the drugs presently in use while the three others, CYP1A2, CYP2B6 and CYP2E1 also metabolize various drugs and other chemicals including a number of carcinogens. When comparison was performed with human

hepatocytes in primary culture maintained 3 to 5 days without DMSO, transcript levels of these CYPs were in the same range as those found in differentiated HepaRG cells except for CYP3A4 which is particulary enhanced in DMSO-treated HepaRG cells. Furthermore, levels of CYP activities in differentiated HepaRG cells were also comparable to those usually found in primary human hepatocyte cultures (Guillouzo and Chesne, 1996; Gomez-Lechon et al., 2001). By contrast, the levels of CYPs expressed in HepG2 cells (Sassa et al., 1987; Sumida et al., 2000; Ogino et al., 2002) and other transformed hepatocytes of tumoral origin (Iwahori et al., 2003) or obtained by oncogenic immortalization (Pfeifer et al., 1993; Mills et al., 2004) are quite low if detectable. Recently, Mills et al. (Mills et al., 2004) measured conversion of testosterone to 6β–hydroxytestosterone catalysed by CYP3A4 in an immortalized human hepatocyte cell line and found that, although responsive to the prototypical inducer rifampicin, this activity was several hundred fold lower than that estimated in HepaRG cells. Even when compared to the levels of CYP activities determined in human hepatoma B16A2 cells (Gomez-Lechon et al., 2001), another cell line obtained in our laboratory and found to express various liver-specific functions, those found in HepaRG cells were much higher (formation of the 6β-hydroxytestosterone was around 60 pmol/mg/min in B16A2 cells and around 500 pmol/mg/min in differentiated HepaRG cells in presence of 2% DMSO).

Most of the genes belonging to CYP1-4 families can be transcriptionally induced by xenobiotics and specific receptor proteins are playing a key role in gene induction stimulated by each of the four mechanistically distinct classes of P450-inducing xenochemicals (Rushmore and Kong, 2002; Dickins, 2004). The first identified, the Ah receptor, belongs to the bHLH/PAS (basic helix-loop-helix/Per-Arnt-Sim) family of transcription factors and stimulates transcription of CYP1A genes and various other genes by a well understood mechanism. This receptor, which is expressed in various cell types, including undifferentiated ones (van Grevenynghe et al., 2005), was found at comparable levels in HepaRG cells

whatever their stage of differentiation and in primary human hepatocytes. The three other known xenobiotic induction mechanisms include three distinct orphan receptors, CAR, PXR and PPARα, that are members of hormone nuclear receptor family. They form heterodimers with RXR and for CAR and PXR regulate expression of CYP 2 and 3 families and several GSTs and UGT enzymes; they also interact with nuclear receptors controlling various pathways of endogenous metabolism. PPARα regulates expression of CYP4A in rodents and plays a role in the regulation of lipid and carbohydrate metabolism. These receptors that are most highly expressed in the liver were also found in high levels in differentiated HepaRG cells. Interestingly, a high expression of CAR has never been reported before in any hepatoma cell line (Zelko and Negishi, 2000). Accordingly, CYPs 1A2, 2C9 and 3A4 known to be transcriptionally increased by prototypical inducers in normal hepatocytes were similarly induced in HepaRG cells.

For some functions, the highest values were found in HepaRG cells maintained in the presence of DMSO. This agent is recognized as a differentiation inducing compound in many tumor cell lines. DMSO also increases albumin production in immortalized hepatocytes (Higgins and Borenfreund, 1980) and favors cell survival and maintenance of differentiated functions, such as production of plasma proteins, in primary rat hepatocytes (Isom et al., 1985; Mitaka et al., 1993). The mechanism(s) by which DMSO induces cellular differentiation is still poorly understood; it has been shown to act as a reactive oxygen species scavenger and an antiapoptotic agent (Villa et al., 1991; Gilot et al., 2002). The results reported here clearly show that DMSO induced a more differentiated state and enhanced expression of several metabolizing enzymes in HepaRG cells. However, it was uneffective on some CYPs and the liver-specific proteins studied, including albumin. Interestingly, certain CYP inducers were ineffective in DMSO-exposed HepaRG cells, especially when cells were seeding at high density. Indeed, the levels of activities reached in the presence of DMSO was

comparable to those observed in DMSO-free cells treated with the prototypical inducer. It may be postulated that by enhancing expression of certain nuclear receptors and liver transcription factors, DMSO allowed certain CYP genes to reach maximum transcription activity as already observed by others (Nishimura et al., 2003; Su and Waxman, 2004).

In agreement with the active expression of phase 1 and phase 2 xenobiotic metabolizing enzymes the suitability of HepaRG cells for the determination of chemical metabolism profiles was supported by analyzing metabolic profiles of acetaminophen and $AFB_1$ and the cytotoxicity effects of several hepatotoxicants. The same metabolites as those obtained with primary human hepatocyte cultures were observed with the two compounds indicating that HepaRG cells expressed the different enzymes involved in their biotransformation at suitable levels. This conclusion is supported by the toxicity of reference hepatotoxicants. Their cytotoxicity was dependent on the compound, dose and duration of exposure. As expected, $AFB_1$ and acetaminophen that are hepatotoxic via the formation of electrophilic metabolites by CYP-dependent reactions were more cytotoxic to HepaRG than to HepG2 cells. By contrast, amiodarone and chlorpromazine induced similar toxicity in both cells. Characteristic accumulation of cytoplasmic vesicles corresponding to secondary lysosomes loaded with phospholipids (Poucell et al., 1984) was observed under light microscopy after treatment with amiodarone in both types of HepaRG cells and in HepG2 cells.

Why are HepaRG cells much more differentiated than any other human hepatocyte cell line already described ? It may be noticed that they are derived from an hepatocarcinoma and not, like HepG2 cells, from an hepatoblastoma. Second, they show only limited chromosomal rearrangements (Gripon et al., 2002; Parent et al., 2004). Third, and certainly the most original feature, when passaged at low density, they recover characteristic features of

progenitor cells able to differentiate in both hepatocytes and biliary epithelial cells (Parent et al., 2004) and to form a co-culture system that strongly resembles that we have a long time ago described by associating primary normal hepatocytes with rat liver epithelial cells and resulting in long-term maintenance of liver-specific functions at high levels (Guguen-Guillouzo et al., 1983). Indeed, several studies have demonstrated that various functions, including phase 1 and phase 2 xenobiotic enzyme activities were better and longer preserved in cocultures versus pure cultures of human and animal hepatocytes (Begue et al., 1983; Fraslin et al., 1985; Niemann et al., 1991). Such a model should represent a unique tool to distinguish the liver epithelial cell type target of hepatotoxicants.

Human hepatoma cell lines have also been used for the design of bioartificial livers although their detoxifying capacity was limited even when cultured in a 3-dimentional perfusion system that led to an increased expression of CYP3A4 and inducibility by rifampicin (Iwahori et al., 2003). HepaRG cells could represent a very good alternative to either primary hepatocytes or other human hepatoma cell lines; not only they exhibit a large set of liver-specific functions but in addition, although they derive from an hepatitis C virus-infected liver they do not express viral markers.

In conclusion, for the first time we report that a human hepatoma cell line is able to express the major CYP-related activities as well as other liver-specific functions. The fact that when seeded at high density hepatocyte-like clusters retain their differentiated state and can immediately be used after seeding makes them suitable for high throughput screening as well as studies on metabolism and hepatotoxicity of chemicals.

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**REFERENCES**

Abdel-Razzak Z, Loyer P, Fautrel A, Gautier JC, Corcos L, Turlin B, Beaune P and Guillouzo A (1993) Cytokines down-regulate expression of major cytochrome P-450 enzymes in adult human hepatocytes in primary culture. *Mol Pharmacol* **44:**707-715.

Begue JM, Le Bigot JF, Guguen-Guillouzo C, Kiechel JR and Guillouzo A (1983) Cultured human adult hepatocytes: a new model for drug metabolism studies. *Biochem Pharmacol* **32:**1643-1646.

Burke MD and Mayer RT (1983) Differential effects of phenobarbitone and 3-methylcholanthrene induction on the hepatic microsomal metabolism and cytochrome P-450-binding of phenoxazone and a homologous series of its n-alkyl ethers (alkoxyresorufins). *Chem Biol Interact* **45:**243-258.

Dickins M (2004) Induction of cytochromes P450. *Curr Top Med Chem* **4:**1745-1766.

Fraslin JM, Kneip B, Vaulont S, Glaise D, Munnich A and Guguen-Guillouzo C (1985) Dependence of hepatocyte-specific gene expression on cell-cell interactions in primary culture. *Embo J* **4:**2487-2491.

Gilot D, Loyer P, Corlu A, Glaise D, Lagadic-Gossmann D, Atfi A, Morel F, Ichijo H and Guguen-Guillouzo C (2002) Liver protection from apoptosis requires both blockage of initiator caspase activities and inhibition of ASK1/JNK pathway via glutathione S-transferase regulation. *J Biol Chem* **277:**49220-49229.

Gomez-Lechon MJ, Donato T, Jover R, Rodriguez C, Ponsoda X, Glaise D, Castell JV and Guguen-Guillouzo C (2001) Expression and induction of a large set of drug-metabolizing enzymes by the highly differentiated human hepatoma cell line BC2. *Eur J Biochem* **268:**1448-1459.

Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, Guyomard C, Lucas J, Trepo C and Guguen-Guillouzo C (2002) Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci U S A* **99:**15655-15660.

Guguen-Guillouzo C, Campion JP, Brissot P, Glaise D, Launois B, Bourel M and Guillouzo A (1982) High yield preparation of isolated human adult hepatocytes by enzymatic perfusion of the liver. *Cell Biol Int Rep* **6:**625-628.

Guguen-Guillouzo C, Clement B, Baffet G, Beaumont C, Morel-Chany E, Glaise D and Guillouzo A (1983) Maintenance and reversibility of active albumin secretion by adult rat hepatocytes co-cultured with another liver epithelial cell type. *Exp Cell Res* **143:**47-54.

Guillouzo A (1998) Liver cell models in in vitro toxicology. *Environ Health Perspect* **106 Suppl 2:**511-532.

Guillouzo A and Chesne C (1996) Xenobiotic metabolism in epithelial cell cultures. *Epithelial Cell Culture, A Practical Approach, Ed. A.J. Shaw, Oxford University Press, p67-85*.

Guillouzo A, Morel F, Fardel O and Meunier B (1993) Use of human hepatocyte cultures for drug metabolism studies. *Toxicology* **82:**209-219.

Hamilton GA, Jolley SL, Gilbert D, Coon DJ, Barros S and LeCluyse EL (2001) Regulation of cell morphology and cytochrome P450 expression in human hepatocytes by extracellular matrix and cell-cell interactions. *Cell Tissue Res* **306:**85-99.

Higgins PJ and Borenfreund E (1980) Enhanced albumin production by malignantly transformed hepatocytes during in vitro exposure to dimethylsulfoxide. *Biochim Biophys Acta* **610:**174-180.

Isom HC, Secott T, Georgoff I, Woodworth C and Mummaw J (1985) Maintenance of differentiated rat hepatocytes in primary culture. *Proc Natl Acad Sci U S A* **82:**3252-3256.

Iwahori T, Matsuura T, Maehashi H, Sugo K, Saito M, Hosokawa M, Chiba K, Masaki T, Aizaki H, Ohkawa K and Suzuki T (2003) CYP3A4 inducible model for in vitro analysis of human drug metabolism using a bioartificial liver. *Hepatology* **37:**665-673.

Jover R, Bort R, Gomez-Lechon MJ and Castell JV (1998) Re-expression of C/EBP alpha induces CYP2B6, CYP2C9 and CYP2D6 genes in HepG2 cells. *FEBS Lett* **431:**227-230.

Langouet S, Coles B, Morel F, Becquemont L, Beaune P, Guengerich FP, Ketterer B and Guillouzo A (1995) Inhibition of CYP1A2 and CYP3A4 by oltipraz results in reduction of aflatoxin B1 metabolism in human hepatocytes in primary culture. *Cancer Res* **55:**5574-5579.

Langouet S, Coles B, Morel F, Maheo K, Ketterer B and Guillouzo A (1996) Metabolism of aflatoxin B1 by human hepatocytes in primary culture. *Adv Exp Med Biol* **387:**439-442.

LeCluyse EL (2001) Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. *Eur J Pharm Sci* **13:**343-368.

Madan A, Graham RA, Carroll KM, Mudra DR, Burton LA, Krueger LA, Downey AD, Czerwinski M, Forster J, Ribadeneira MD, Gan LS, LeCluyse EL, Zech K, Robertson P, Jr., Koch P, Antonian L, Wagner G, Yu L and Parkinson A (2003) Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. *Drug Metab Dispos* **31:**421-431.

Maurel P (1996) The use of adult human hepatocytes in primary culture and other in vitro systems to investigate drug metabolism in man. *Adv. Drug Deliv. Rev.* **22:**105-132.

Mills JB, Rose KA, Sadagopan N, Sahi J and de Morais SM (2004) Induction of drug metabolism enzymes and MDR1 using a novel human hepatocyte cell line. *J Pharmacol Exp Ther* **309:**303-309.

Mitaka T, Norioka K and Mochizuki Y (1993) Redifferentiation of proliferated rat hepatocytes cultured in L15 medium supplemented with EGF and DMSO. *In Vitro Cell Dev Biol Anim* **29A:**714-722.

Morel F, Beaune PH, Ratanasavanh D, Flinois JP, Yang CS, Guengerich FP and Guillouzo A (1990) Expression of cytochrome P-450 enzymes in cultured human hepatocytes. *Eur J Biochem* **191:**437-444.

Morel F, Fardel O, Meyer DJ, Langouet S, Gilmore KS, Meunier B, Tu CP, Kensler TW, Ketterer B and Guillouzo A (1993) Preferential increase of glutathione S-transferase class alpha transcripts in cultured human hepatocytes by phenobarbital, 3-methylcholanthrene, and dithiolethiones. *Cancer Res* **53:**231-234.

Niemann C, Gauthier JC, Richert L, Ivanov MA, Melcion C and Cordier A (1991) Rat adult hepatocytes in primary pure and mixed monolayer culture. Comparison of the maintenance of mixed function oxidase and conjugation pathways of drug metabolism. *Biochem Pharmacol* **42:**373-379.

Nishimura M, Ueda N and Naito S (2003) Effects of dimethyl sulfoxide on the gene induction of cytochrome P450 isoforms, UGT-dependent glucuronosyl transferase isoforms, and ABCB1 in primary culture of human hepatocytes. *Biol Pharm Bull* **26:**1052-1056.

Ogino M, Nagata K and Yamazoe Y (2002) Selective suppressions of human CYP3A forms, CYP3A5 and CYP3A7, by troglitazone in HepG2 cells. *Drug Metab Pharmacokinet* **17:**42-46.

Parent R, Marion MJ, Furio L, Trepo C and Petit MA (2004) Origin and characterization of a human bipotent liver progenitor cell line. *Gastroenterology* **126:**1147-1156.

Pfeifer AM, Cole KE, Smoot DT, Weston A, Groopman JD, Shields PG, Vignaud JM, Juillerat M, Lipsky MM, Trump BF and et al. (1993) Simian virus 40 large tumor antigen-immortalized normal human liver epithelial cells express hepatocyte characteristics and metabolize chemical carcinogens. *Proc Natl Acad Sci U S A* **90:**5123-5127.

Poucell S, Ireton J, Valencia-Mayoral P, Downar E, Larratt L, Patterson J, Blendis L and Phillips MJ (1984) Amiodarone-associated phospholipidosis and fibrosis of the liver. Light, immunohistochemical, and electron microscopic studies. *Gastroenterology* **86:**926-936.

Rodriguez-Antona C, Jover R, Gomez-Lechon MJ and Castell JV (2000) Quantitative RT-PCR measurement of human cytochrome P-450s: application to drug induction studies. *Arch Biochem Biophys* **376:**109-116.

Rushmore TH and Kong AN (2002) Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes. *Curr Drug Metab* **3:**481-490.

Sassa S, Sugita O, Galbraith RA and Kappas A (1987) Drug metabolism by the human hepatoma cell, Hep G2. *Biochem Biophys Res Commun* **143:**52-57.

Su T and Waxman DJ (2004) Impact of dimethyl sulfoxide on expression of nuclear receptors and drug-inducible cytochromes P450 in primary rat hepatocytes. *Arch Biochem Biophys* **424:**226-234.

Sumida A, Fukuen S, Yamamoto I, Matsuda H, Naohara M and Azuma J (2000) Quantitative analysis of constitutive and inducible CYPs mRNA expression in the HepG2 cell line using reverse transcription-competitive PCR. *Biochem Biophys Res Commun* **267:**756-760.

van Grevenynghe J, Bernard M, Langouet S, Le Berre C, Fest T and Fardel O (2005) Human CD34-positive hematopoietic stem cells constitute targets for carcinogenic polycyclic aromatic hydrocarbons. *J Pharmacol Exp Ther*.

Villa P, Arioli P and Guaitani A (1991) Mechanism of maintenance of liver-specific functions by DMSO in cultured rat hepatocytes. *Exp Cell Res* **194:**157-160.

Zelko I and Negishi M (2000) Phenobarbital-elicited activation of nuclear receptor CAR in induction of cytochrome P450 genes. *Biochem Biophys Res Commun* **277:**1-6.

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## LEGENDS FOR FIGURES

**Figure 1. Phase-contrast micrographs of HepaRG cells.** Cells seeded at low density and cultured for either 10 days without DMSO (the cells exhibited variable shapes) (A), 30 days without DMSO (B) or 15 days without DMSO then exposed to 2% DMSO for 15 days (C), differentiated HepaRG cells seeded at high density and maintained in culture for 72h in a medium deprived of 2% DMSO (D), or supplemented with 2% DMSO (E). Hepatocyte-like (H) and epithelial-like (EP) cells ; bile canaliculus (BC). (Original magnification x 150)

**Figure 2. mRNA analysis by RT-qPCR** (A) HepaRG cells seeded at low density and cultured for 5, 8, 15 and 30 days in the absence or presence of 2% DMSO between days 15 and 30. (B) HepaRG cells cultured for 35 or 37 days. At day 15, 2% DMSO was added to the culture medium until day 34. Then the cells were cultured the last 24h (day 35) or 72h (day 37) without 2% DMSO. (C) HepaRG cells seeded at high density and cultured for 24h or 72h with or without 2% DMSO.

**Figure 3. HPLC metabolic profiles of $AFB_1$ and acetaminophen in primary human hepatocyte cultures and 30 days differentiated DMSO-treated HepaRG cells.** (A-1) aflatoxin $B_1$-dialcohol (A-2) Aflatoxin $M_1$ ($AFM_1$), (A-3) Aflatoxin $P_1$ ($AFP_1$) and (A-4) $AFB_1$, (B-1) glucuronide acetaminophen, (B-2) sulfate acetaminophen and (B-3) acetaminophen. Incubations lasted 8h with 5µM $AFB_1$ and 10h with 2mM acetaminophen; human hepatocyte primary cultures were used 5 and 3 days after seeding respectively. Detection was performed at $\lambda$=250nm for acetaminophen and by fluorescence ($\lambda_{ex}$ 430nm and $\lambda_{em}$ 370nm) for $AFB_1$.

**Figure 4. Comparative cytotoxic effects of chlorpromazine, amiodarone, $AFB_1$ and acetaminophen on HepaRG and HepG2 cells.** Differentiated HepaRG cells from low density seeding (●) and high density seeding (□) and confluent HepG2 cells (▲) were exposed to the chemicals for 24h (A) or 72h (B). Cell viability was assayed using a standard MTT test. The results were normalized to control cells and expressed as means ± S.D. (n=3 cultures).

**Figure 5 – Effects of amiodarone on HepaRG cells.** Phase-contrast micrographs of confluent DMSO-treated HepaRG cells cultured for 24h in a FCS-free medium containing either only the vehicle (0.2% DMSO) (A) or 50µM amiodarone (B). Numerous intracytoplasmic vesicles are visible in amiodarone-treated HepaRG cells (arrow). (Original magnification x 300).

**Table 1. Primer sequences used for RT-qPCR**

| *Accession number* | *Genes* | *Foward Primer* | *Reverse Primer* |
|---|---|---|---|
| A06977 | Albumin | TGCTTGAATGTGCTGATGACAGG | AAGGCAAGTCAGCAGGCATCTCATC |
| X02747 | Aldolase B | GCATCTGTCAGCAGAATGGA | TAGACAGCAGCCAGGACCTT |
| V01514 | Alpha-fetoprotein | TGCAGCCAAAGTGAAGAGGGAAGA | CATAGCGAGCAGCCCAAAGAAGAA |
| X00637 | Haptoglobin | CGGTTCGCTACCAGTGTAAGAAC | CCACTGCTTCTTATCATTTAAGGTGTA |
| L19872 | AhR | ACATCACCTACGCCAGTCGC | TCTATGCCGCTTGGAAGGAT |
| BC069626 | CAR | TGATCAGCTGCAAGAGGAGA | AGGCCTAGCAACTTCGCATA |
| BC017304 | PXR | CCAGGACATACACCCCTTTG | CTACCTGTGATGCCGAACAA |
| Z00036 | CYP 1A2 | TGGAGACCTTCCGACACTCCT | CGTTGTGTCCCTTGTTGTGC |
| M29874 | CYP 2B6 | TTCCTACTGCTTCCGTCTATCAAA | GTGCAGAATCCCACAGCTCA |
| S46963 | CYP 2C9 | GGACAGAGACGACAAGCACA | AATGGACATGAACAACCCTCA |
| J02625 | CYP 2E1 | TTGAAGCCTCTCGTTGACCC | CGTGGTGGGATACAGCCAA |
| X12387 | CYP 3A4 | CTTCATCCAATGGACTGCATAAAT | TCCCAAGTATAACACTCTACACAGACAA |
| M16594 | GSTA1/2 | TGCAACAATTAAGTGCTTTACCTAAGTG | TTAACTAAGTGGGTGAATAGGAGTTGTATT |
| Y13047 | GSTA4 | GGATGGTAACCACCTGCTG | TGCCAAAGAGATTGTGCTT |
| X08020 | GSTM1 | ATGGTTGTCCAGGTCTGG | CGCCATCTTGTGCTACATT |
| M57899 | UDP-glucuronosyl transferase 1A1 | TGACGCCTCGTTGTACATCAG | CCTCCCTTTGGAATGGCAC |

**Table 2 – Comparative expression of CYPs and nuclear receptors mRNA in HepaRG cells, freshly isolated human hepatocytes and HepG2 cells.** Results are expressed as percentage compared to freshly isolated hepatocytes (FIH) arbitrarily set at 100%. FIH correspond to a pool of 3 different cell populations. Results are the mean of two independent experiments in duplicate.

| | CYP1A2 | CYP2B6 | CYP2C9 | CYP2D6 | CYP2E1 | CYP3A4 | PXR | CAR | PPARα | AhR |
|---|---|---|---|---|---|---|---|---|---|---|
| **FIH** | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **HepG2 cells** | 0.3 | 0.5 | 0 | 2.3 | 0 | 0 | 49.5 | 2.4 | 208.5 | 35.5 |
| **HepaRG cells seeded at low density (days)** | | | | | | | | | | |
| **5** | 0.1 | 0 | 0 | 1 | 0 | 0 | 6.8 | 0 | 56.5 | 94.5 |
| **8** | 0.1 | 0.1 | 0.1 | 1.2 | 0 | 0 | 8 | 0.1 | 126.8 | 134.3 |
| **15** | 0.3 | 3.2 | 3.3 | 0.9 | 0.1 | 4.5 | 51.3 | 4.1 | 175.6 | 121.7 |
| **30** | 2.3 | 3.5 | 9.5 | 1.2 | 0.2 | 8.1 | 95.7 | 9.1 | 187.2 | 64.2 |
| **30 (DMSO)** | 9.1 | 34.6 | 34.7 | 0.8 | 3.5 | 176 | 142.9 | 22.2 | 346.5 | 169.7 |
| **35 (-DMSO 24h)** | 5.5 | 3.5 | 26.5 | 1 | 0.8 | 1.6 | 99.9 | 8.3 | 136.7 | 98.8 |
| **37 (-DMSO 72h)** | 3.9 | 1.6 | 16.7 | 0.9 | 0.8 | 2.3 | 122.6 | 7.1 | 135.1 | 47.5 |
| **HepaRG cells seeded at high density after differentiation** | | | | | | | | | | |
| **+ DMSO 24h** | 17.8 | 72.4 | 38.1 | 1.1 | 13.3 | 243 | 176.8 | 26.4 | 330.1 | 92.5 |
| **- DMSO 24h** | 7.5 | 4.9 | 22.1 | 0.8 | 0.7 | 6.8 | 106.4 | 5.3 | 173.1 | 72.9 |
| **+ DMSO 72h** | 3.7 | 73.1 | 43.8 | 1 | 7 | 255.6 | 175.3 | 30.2 | 561.1 | 66.4 |
| **- DMSO 72h** | 5.4 | 11.9 | 22 | 0.8 | 1 | 2.8 | 151.1 | 6.4 | 222.9 | 73.4 |

**Table 3 – Comparative expression of phase 2 enzymes and liver specific-proteins mRNA in HepaRG cells, freshly isolated human hepatocytes and HepG2 cells.** Results are expressed as percentage compared to freshly isolated human hepatocytes (FIH) arbitrarily set at 100%. FIH correspond to a pool of 3 different cell populations. Results are the mean of two independent experiments in duplicate.

| | GSTA1/2 | GSTA4 | GSTM1 | UGT1A1 | Aldolase B | Haptoglobin | Albumin | AFP | Thioredoxin |
|---|---|---|---|---|---|---|---|---|---|
| **FIH** | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **HepG2 cells** | 0.2 | 2.1 | 2.4 | 5.2 | 0 | 5.1 | 12.3 | 89011 | 115.8 |
| **HepaRG cells seeded at low density (days)** | | | | | | | | | |
| **5** | 0.4 | 6.7 | 8.5 | 7.4 | 0 | 5.1 | 1.8 | 0.6 | 1250.6 |
| **8** | 8 | 5.9 | 7.6 | 9 | 0.2 | 76.4 | 14.1 | 21 | 877 |
| **15** | 37.3 | 9 | 9.8 | 10.5 | 7.8 | 199.9 | 85.6 | 75.6 | 422 |
| **30** | 36.8 | 13.1 | 15.5 | 17.1 | 20.9 | 256.6 | 82.9 | 79.4 | 303.4 |
| **30 (DMSO)** | 108.6 | 22.6 | 26.3 | 186.3 | 13.5 | 216.4 | 70.7 | 45.8 | 304.4 |
| **35 (-DMSO 24h)** | 35.7 | 3.9 | 5 | 43 | 8.7 | 294.1 | 63.1 | 71.3 | 610.2 |
| **37 (-DMSO 72h)** | 18.3 | 5.8 | 6.8 | 35.4 | 9.6 | 344.7 | 77.9 | 64.8 | 479.7 |
| **HepaRG cells seeded at high density after differentiation** | | | | | | | | | |
| **+ DMSO 24h** | 258.2 | 26.4 | 33.6 | 240.6 | 16.3 | 197.5 | 57.1 | 40.2 | 429.8 |
| **- DMSO 24h** | 50 | 4.2 | 5.8 | 82.4 | 7.3 | 344.8 | 47.2 | 54 | 790.9 |
| **+ DMSO 72h** | 160.3 | 36.7 | 45.1 | 221.1 | 14.9 | 196.6 | 60.4 | 36.9 | 280.9 |
| **- DMSO 72h** | 31.3 | 9.7 | 7.5 | 47.3 | 9.4 | 479.8 | 65.2 | 114 | 460.9 |

**Table 4 – Expression of CYPs and nuclear receptors mRNA in primary human hepatocytes.** RT-qPCR analysis of hepatocyte RNA samples prepared from primary hepatocytes from day 0 (Freshly isolated hepatocytes (FIH)) to day 5 of culture in absence of DMSO. Experiment was performed on one cell population. Results are expressed as % compared to freshly isolated hepatocytes.arbitrarily set at 100%.

| Days | CYP1A2 | CYP2B6 | CYP2C9 | CYP2D6 | CYP2E1 | CYP3A4 | PXR | CAR |
|---|---|---|---|---|---|---|---|---|
| **0** | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **1** | 15 | 29 | 18 | 20 | 14 | 22 | 95 | 7 |
| **3** | 2 | 8 | 48 | 9 | 2 | 6 | 160 | 14 |
| **5** | 12 | 4 | 58 | 60 | 11 | 30 | 121 | 29 |

**Table 5 – Drug metabolizing enzyme activities in HepaRG cells**. Determinations of testosterone 6β-hydroxylation (CYP3A4), O-dealkylation of 7-ethoxyresorufin (CYP1A2), chlorzoxazone 6-hydroxylation (CYP2E1) and tolbutamide 4-hydroxylation (CYP2C9) activities were performed on HepaRG cells seeded at low density and cultured for either 15 days or 30 days with addition of 2% DMSO between days 15 and 30, and on differentiated HepaRG cells seeded at high density. Pretreatment with inducers or their vehicle was performed using FCS-free medium added or not with 2% DMSO. The cultures were exposed either 24h (3-methylcholantrene : 3-MC) or 72h (rifampicin : RIF, isoniazid : ISO) to the inducers or their vehicle (UT). Results are expressed as pmoles/mg/min. Student's t test was applied between UT cells and cells treated with inducer for statistical analysis :* $p< 0.05$, ** $p<0.01$ and *** $p<0.001$ (GraphPad Prism software).

| | DMSO | EROD (CYP1A1/2) | | Tolbutamide 4-hydroxylase activity (CYP2C9) | | Chlorzoxazone 6-hydroxylase activity (CYP2E1) | | Testosterone 6β-hydroxylase activity (CYP3A4) | |
|---|---|---|---|---|---|---|---|---|---|
| | | UT | 3MC (5µM) | UT | RIF (25µM) | UT | ISO ( 50µM) | UT | RIF (50µM) |
| 15 days culture HepaRG cells seeded at low density | - | Traces | 48.2 ± 3 (n=4) | 1.0 ± 0.6 (n=5) | 4.5 ± 3.8 (n=6) | 31.9 ± 13.2 (n=6) | 33.4 ± 11.9 (n=6) | 10.9 ± 4.7 (n=5) | 129.3 ± 22*** (n=6) |
| Differentiated HepaRG cells seeded at low density | - | Traces | 68 ± 5.2 (n=6) | 6 ± 1.4 (n=3) | 7.8 ± 1.8 (n=3) | 131.3 ± 11.2 (n=3) | 96 ± 12 * (n=3) | 39.7 ± 15 (n=3) | 212.9 ± 48 ** (n=3) |
| | + | Traces | 154.6 ± 13.7 (n=6) | 7.5 ± 0.3 (n=3) | 10.15 ± 0.5** (n=3) | 140.9 ± 56.5 (n=3) | 221.5 ± 10 (n=3) | 576 ± 3 (n=3) | 806.8 ± 104 (n=3) |
| Differentiated HepaRG cells seeded at high density | - | Traces | 63.1 ± 0.7 (n=6) | 6.2 ± 1.6 (n=3) | 13.1 ± 2.6* (n=3) | 278 ± 8.4 (n=3) | 197.8 ± 103 (n=3) | 54.4 ± 10 (n=3) | 540.1 ± 68 *** (n=3) |
| | + | Traces | 129 ± 16.3 (n=6) | 9.8 ± 1.5 (n=3) | 11.5 ± 3.5 (n=3) | 483.1 ± 30.3 (n=3) | 455.2 ± 65 (n=3) | 781.8 ± 85 (n=3) | 820.5 ± 210 (n=3) |

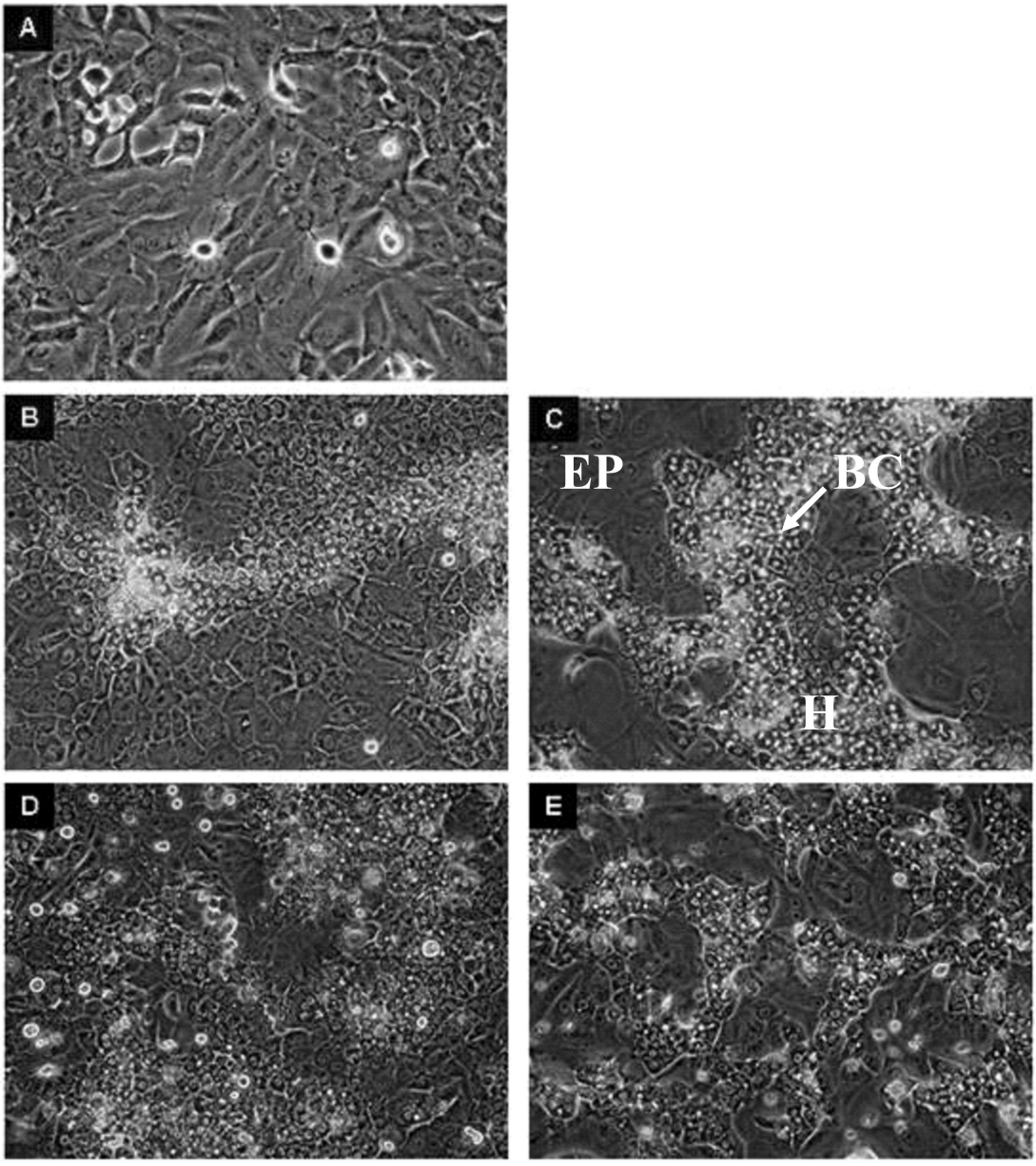


Figure 1

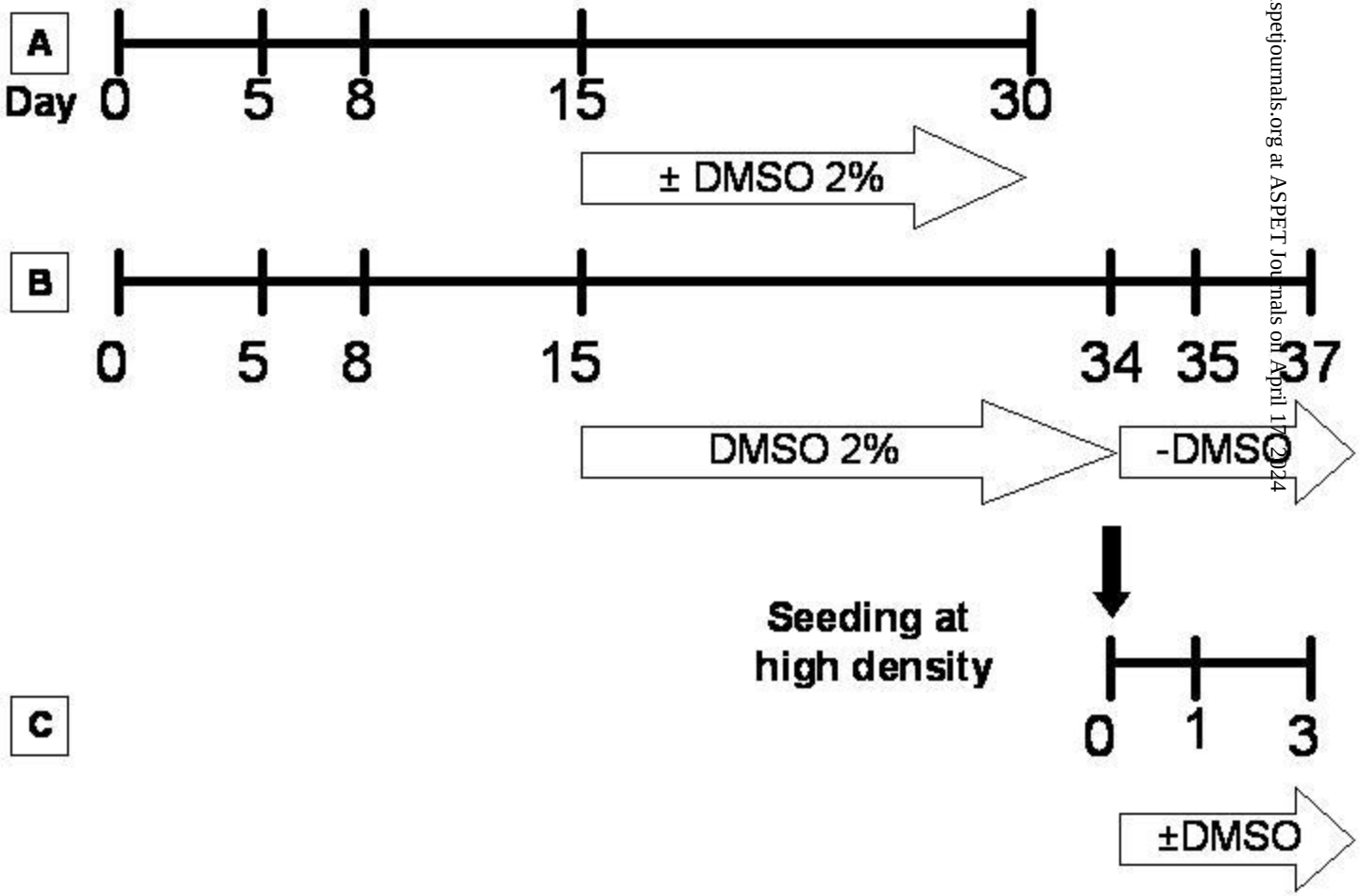


Figure 2

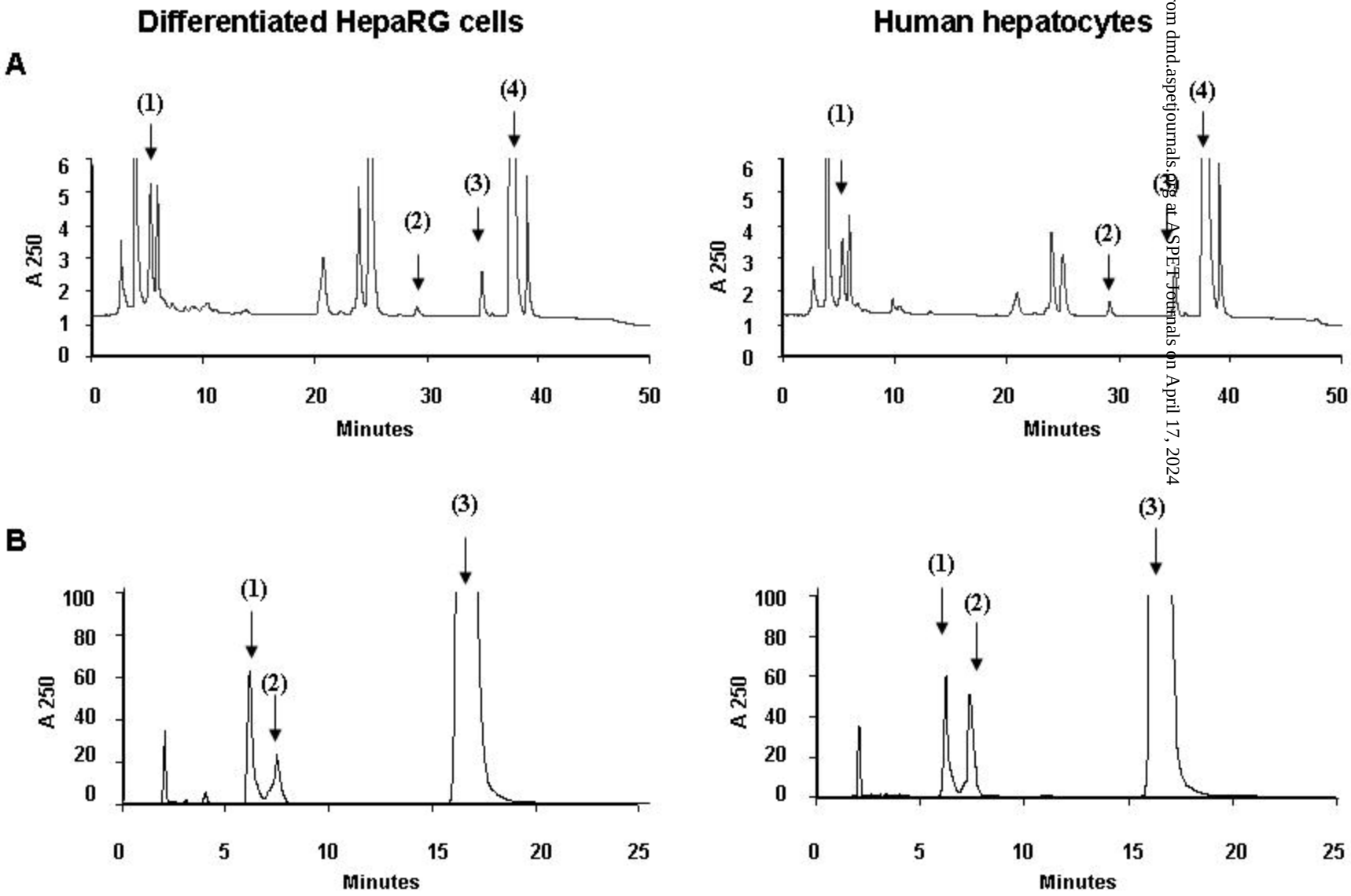


Figure 3

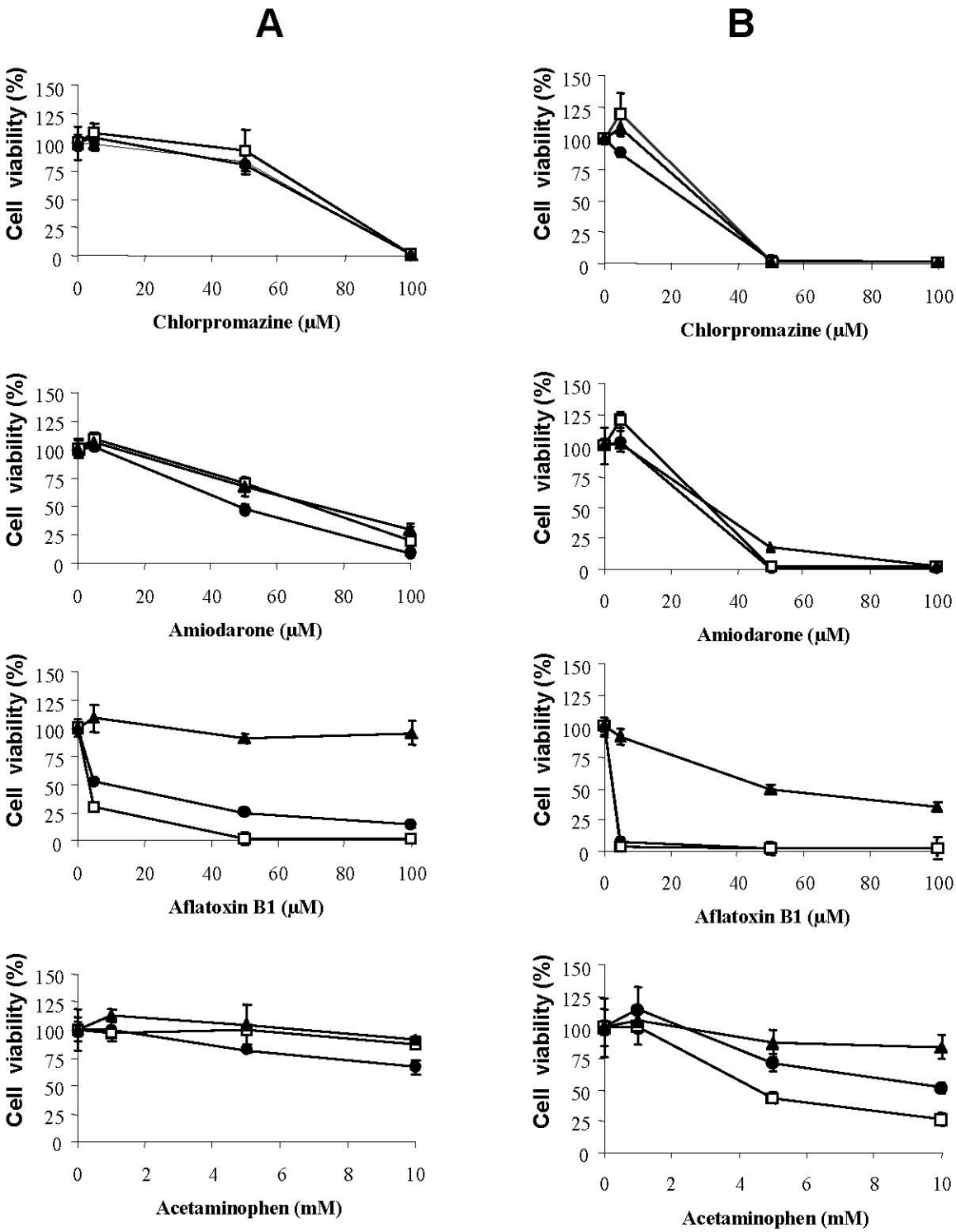
A
B
Cell viability (%)
150
125
100
75
50
25
0
0
20
40
60
80
100
Chlorpromazine (μM)
Amiodarone (μM)
Aflatoxin B1 (μM)
2
4
6
8
10
Acetaminophen (mM)

Figure 4

**Figure 5**