DMD Fast Forward. Published on March 17, 2008 as doi:10.1124/dmd.107.019901

Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies

Rozenn Jossé, Caroline Aninat , Denise Glaise, Julie Dumont, Valérie Fessard, Fabrice Morel, Jean-Michel Poul, Christiane Guguen-Guillouzo and André Guillouzo[1].

INSERM U620 ; Université de Rennes 1, Rennes, France (R.J., C.A., JD, F.M., A.G.), INSERM U522, Hôpital Pontchaillou, Rennes, France (D.G., C.G.) and AFSSA, Laboratoire d'Etudes et de Recherches sur les Médicaments Vétérinaires et les Désinfectants, Fougères, France (VF., JMP.)

Running title : Stability of liver-specific functions in HepaRG cells

A. Corresponding author :

GUILLOUZO André

INSERM U620, Détoxication et Réparation Tissulaire, Faculté des Sciences Pharmaceutiques et Biologiques, Université de Rennes I, 35043 Rennes Cedex, France

Tel (33) 2 23 23 47 91

Fax 33) 2 23 23 47 94

Andre.Guillouzo@univ-rennes1.fr

C. Text : 18

Tables : 3

Figures : 6

References : 25

Abstract : 249

Introduction : 690

Discussion : 1434

D. Abbreviations

CYP, cytochrome P450; PXR, pregnane X receptor; CAR, constitutive androstane receptor; UGT, UDP-glucuronosyl transferase ; GST, Glutathione transferase; mEH, microsomal epoxide hydrolase; MnSOD, manganese superoxide dismutase; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; OCT1, organic cation transporter; BSEP, bile salt export pump; AFB1, aflatoxin B1; MMS, methyl methane sulfonate; 3-MC, 3-methylcholanthrene; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction ; FCS, fetal calf serum; DMSO, dimethylsulfoxide; OTM, Olive tail moment.

ABSTRACT

The human hepatoma HepaRG cells are able to differentiate *in vitro* into hepatocyte-like cells and to express various liver-specific functions, including the major cytochromes P450. This study was aimed to determine whether differentiated HepaRG cells retained their specific functional capacities for a long time period at confluence. We show that expression of transcripts encoding CYP1A2, 2B6, 3A4 and 2E1, several phase II and antioxidant enzymes, membrane transporters, including OCT1 and BSEP, the nuclear receptors CAR and PXR and aldolase B remained relatively stable for at least the 4-week confluence period tested. Similarly, activities of CYP3A4 and CYP1A2 and their responsiveness to prototypical inducers were well preserved. Aflatoxin B1, a potent hepatotoxicant and carcinogen, induced a dose–dependent and cumulative cytotoxicity. Furthermore, at a concentration as low as 0.1µM, this mycotoxin caused a decrease in both CYP3A4 activity and intracellular ATP associated with morphological alterations, after 14 days following every two days exposure. Moreover, using the comet assay a dose-dependent DNA damage was observed after a 3h treatment of differentiated HepaRG cells with 1 to 5µM aflatoxin B1, in the absence of any cell damage and this DNA damaging effect was strongly reduced in the presence of ketoconazole, a CYP3A4 inhibitor. These results bring the first demonstration of long-term stable expression of liver-specific markers in HepaRG hepatocyte cultures maintained at confluence and show that these cells represent a suitable *in vitro* liver cell model for analysis of acute and chronic toxicity as well as genotoxicity of chemicals in human liver.

INTRODUCTION

Drug-induced liver injury is the most frequent cause cited for the withdrawal from the market of an approved drug. Thus, 6 out of 16 drugs withdrawn in the US during the 1975-1999 period were for their hepatic toxicity (Lasser et al., 2002). It is also a major cause of attrition in drug development, indicating that preclinical evaluation of new drugs is of critical importance. A number of drugs and other xenobiotics are potentially hepatotoxic either directly or more frequently after bioactivation leading to the formation of chemically reactive metabolites or generation of reactive oxygen species. However, it must be borne in mind that around half of the drugs found to be responsible for liver injury during clinical trials did not cause any liver damage in animal experiments (Olson et al., 2000). Similarly, while there are known to be hepatocarcinogens in animals especially in rodents, a number of chemicals, with few exceptions such as aflatoxin B1 (AFB1), are only suspected to induce liver tumors in humans. Indeed, their carcinogenic activity is usually based on epidemiological studies showing a higher frequency of cancers with occupational exposure or certain alimentary habits. Such species differences can be explained, at least in many cases, by the frequent major variations in the routes and the rates of chemical metabolism between laboratory animals and humans. Xenobiotic biotransformation is also one of the major determinants of genotoxic effects of chemicals. In *in vitro* mutagenicity assays, tested compounds are routinely bioactivated using exogenous rodent subcellular systems (generally the liver S9 fraction) containing drug-metabolizing enzymes (Paolini et al., 1997). However, doubts persist regarding the real validity of these *in vitro* models in representing both *in vivo* conditions and species differences.

Obviously, there is a need for more accurate prediction of chemical hepatic cytotoxicity and genotoxicity in humans. *In vitro* human liver preparations appear to be the most pertinent

models; they include both cellular (tissue slices, suspensions and primary cultures of hepatocytes, hepatic cell lines) and subcellular (liver microsomes, recombinant enzymes) systems (Guillouzo 1998). Only living cells are potentially able to express the whole metabolic equipment and consequently to mimic the diverse mechanisms of toxicity occurring in the liver. Primary human hepatocytes and human immortalized liver cell lines have been widely used but both models have limitations (Guillouzo 1998). Indeed, primary hepatocytes have scarce and unpredictable availability, limited growth activity and life-span and undergo early and variable changes in various functions, including the major cytochromes P450 (CYP) involved in drug metabolism. However, this model remains the gold standard model for xenobiotic metabolism and toxicity studies (Guillouzo 1998; Hewitt et al., 2007). Indeed, liver cell lines obtained either from tumors or by oncogenic immortalization, lack a variable and substantial set of liver-specific functions, making them unsuitable for mimicking *in vivo* hepatocytes (Sassa et al., 1987; Wilkening et al., 2003; Majer et al., 2004). In particular, they exhibit only quite low, if any, CYP activities. However, we have recently reported that a new human hepatoma cell line, named HepaRG, has retained the major CYP activities, as well as various liver-specific functions, at levels comparable to those found in primary human hepatocytes (Aninat et al., 2006; Aninat et al., 2008).

Various mechanisms can contribute to drug-induced liver injury and only a few compounds induce hepatotoxicity within a few days. Moreover, most drugs cause liver injury infrequently and only a few exert dose-dependent effects (Lee 2003). In most cases, liver injury is related to long-term treatments, thereby supporting the idea that there is a need to predict not only acute toxicity and genotoxic effects but also chronic toxicity due to repeated exposures to low doses or even a mixture of compounds in human liver (Guillouzo et al., 2006). This led us to question as to whether HepaRG cells could remain functionally stable at confluence for several weeks or more and consequently could represent a suitable tool for such purposes. We

demonstrate that differentiated HepaRG cells retained various liver-specific markers at relatively stable levels for several weeks at confluence and that AFB1, a potent hepatotoxicant and carcinogen, induced in these cells dose-dependent and cumulative cytotoxicity as well as DNA damage as evidenced by the comet assay.

Materials and Methods

Chemicals

Aflatoxin B_1 (AFB_1), testosterone, 6β-hydroxytestosterone, 3-methylcholantrene (3-MC), dimethylsulfoxide (DMSO), ketoconazole, phenacetin, rifampicin, dexamethasone and insulin were purchased from Sigma (St. Quentin-Fallavier, France). Methyl methane sulfonate (MMS) was supplied by Acros Organics (Geel, Belgium). Williams E medium was purchased from Eurobio (Les Ulis, France). Fetal calf serum (FCS) was from Perbio (Brebières, France). Penicillin and streptomycin 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 and cultured as previously described (Gripon et al., 2002). Briefly, liver pieces were digested with 0.025% collagenase D in Hepes buffer supplemented with 0.075% $CaCl_2$ and the resulting cell population was suspended in Williams E medium and then maintained in culture by serial passages. The cells reached maximum differentiation when maintained at confluency for two weeks in the presence of 2% DMSO and $5x10^{-5}$M

hydrocortisone hemisuccinate. For the present studies HepaRG cells were seeded at a density of 2.6×10^4 cells/cm^2 in the growth medium composed of Williams E medium supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, 100µg/mL streptomycin, 5µg/mL insulin, 2mM glutamine and 5×10^{-5} M hydrocortisone hemisuccinate. At such a low density seeding HepaRG cells took a morphology of elongated undifferentiated cells characterized by a clear cytoplasm and actively divided until they reached confluence. After 2 weeks they were shifted to the same culture medium supplemented with 2% DMSO for 2 more weeks to obtain differentiated HepaRG cell cultures (Aninat et al. 2006; Cerec et al., 2007).

Human hepatocytes from adult donors undergoing resection for primary and secondary tumors were obtained by collagenase perfusion of histologically normal liver fragments (Guguen-Guillouzo et al., 1982) and were used for the preparation of RNA samples and primary cultures. Hepatocyte cultures were obtained by seeding 170.000 cells/cm² in 96-well plates in a Williams E medium supplemented with 10% FCS, 100 units/µL penicillin, 100µg/mL streptomycin, 1µg/mL insulin, 2mM glutamine and 1µg/mL bovine serum albumin. The medium was discarded 12h after seeding and cells were thereafter maintained in serum-free medium supplemented with 5×10^{-5} M hydrocortisone hemisuccinate. The medium was renewed every day. Human hepatocytes were used 24h after seeding.

HepG2 cells obtained from ATCC (HB-8065, ATCC, Rockville MD, USA), were passaged using a split ratio of 1/3 at 5-day intervals and were used at the time of confluency. The growth medium was composed of minimum essential medium α supplemented with 10% FCS, non-essential amino acids, 100 units/mL penicillin and 100µg/mL streptomycin.

All experimental procedures complied with French laws and were approved by the National Ethics Committee.

Isolation of RNA and RT-qPCR analysis.

DMD Fast Forward. Published on March 17, 2008 as DOI: 10.1124/dmd.107.019901
This article has not been copyedited and formatted. The final version may differ from this version.

Total RNA was extracted from 10^6 HepaRG 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 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). 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 percentages of mRNA levels measured at day 30 after seeding, arbitrarily set at 100%. For induction studies HepaRG cells were first exposed to either 50µM rifampicin or 5µM 3-MC for 24h before cell harvesting for RNA extraction.

Determination of drug metabolizing enzyme activities

For the determination of CYP3A4- and 1A2-related activities, HepaRG cells were incubated with 200µM testosterone for 2 hours and 200µM phenacetin for 20 hours respectively, in phenol red-free medium deprived of both FCS and DMSO. For induction studies, the cells were first exposed to either 50µM rifampicin or 5µM 3-MC for 48h before incubation with the specific substrates. Culture media were then collected and centrifuged for 2min at 10,000 rpm; 50µl of sample were injected in the HPLC system for quantification of the specific metabolites, 6β-hydroxytestosterone and acetaminophen, resulting from 6β-hydroxylation of testosterone and phenacetin deethylation activities, respectively. A nucleosil C18 column (150

x 4.6mm, 5µm) was used for chromatographic separation of testosterone metabolites at 254nm. was detected The mobile phase consisted of two solvents, A (0.1% acetic acid) and B (acetonitrile), with the following gradient : 0min, 20% B; 20min, 33% B. Chromatographic separation of phenacetin metabolites was carried out with an Interchrom C18 column (250 x 4.0mm, 5µm) at 250nm. The mobile phase consisted of two solvents, A (2mM ammonium acetate pH 2.6) and B (acetonitrile) with a linear gradient (20min, 3% B).

The HPLC apparatus consisted of an Agilent 1100 Series high performance liquid chromatograph equipped 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 area and to plot metabolite patterns. Metabolites were identified by comparison of retention times, and quantifications were estimated from calibration.

Evaluation of AFB1 cytotoxicity

The effects of varying concentrations of AFB_1 were evaluated on primary human hepatocytes, HepaRG and HepG2 cells after various times of exposure. Incubations were performed with a medium supplemented with both 2% DMSO and 10% FCS. For long time treatments of HepaRG cells the mycotoxin was added every two days with each medium renewal The cultures were examined every day by phase-contrast microscopy using an Olympus 1X70. Cytotoxicity was estimated by intracellular ATP measurements using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Charbonnières, France). The test was performed according to the manufacturer's instructions. ATP concentrations were expressed as chemoluminescence units and converted into percentages by the ratio of the values measured in treated and corresponding control samples.

In addition, functional capacity of remaining living hepatocytes was selectively evaluated by determination of CYP3A4 activity at different time points.

Comet assay

Undifferentiated (5 days after seeding) and differentiated (30 days after seeding) HepaRG cells were exposed to 1, 2.5 or 5 µM AFB1 dissolved in DMSO (final concentration 0.2%) or 5µg/ml MMS (positive control) in a FCS-free medium for 3h. Then, they were trypsinized, centrifuged (2min, 1000rpm) and cell pellets were suspended in a prewarmed low melting point agarose (0.5% in PBS) and deposited on conventional microscope slides (initially dipped in 1% agarose and dried) precoated with normal agarose (0.8% in PBS). The slides were then put in a lysis solution composed of 2.5M NaCl, 0.1M EDTA, 10mM Tris-HCl at pH 10, extemporarily added with 10% DMSO and 1% Triton X-100, for 1h at around 5°C. DNA was allowed to unwind for 40min in fresh electrophoresis buffer (0.3M NaOH, 1 mM EDTA, pH 13) and the electrophoretic migration was then performed at room temperature (24min, 0.7V cm-1, 300mA). The slides were bathed twice for 5 min in neutralizing solution (0.4M Tris-HCl, pH7.5) and dried for conservation with 95% ethanol for 5 min. DNA was stained with propidium iodide (2,5 µg/ml in PBS) just before slide examination with a fluorescence microscope (Leica DMR) equipped with a CCD-200E video camera. At least 100 images per dose and treatment time were analysed using the Comet Assay IV software (Perceptive Instruments, Haverhill, UK). Two parameters, the Olive tail moment (OTM), defined as the product of the distance between the barycentres of the head and tail by the proportion of DNA in the tail, and the percentage of DNA in the comet tail (% DNA) were used to evaluate the extent of DNA damage in individual cells.

Statistical analysis

Each value corresponded to the mean +/- SEM of 3 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 cytotoxicity between treated and corresponding control samples and CYP3A4 activity in cultures treated with 0.5µM or 1µM compared to 0.1µM AFB1 for 24h as well as in cultures treated for 7 or 14 days compared to 1 day. For the comet assay, DNA damage was expressed as the median OTM or percent DNA in the tail of 100 cells. Comparisons between cell cultures submitted to different treatment conditions were made using ANOVA followed by pair-wise comparison using the Tukey's test. Data were considered significantly different when $p<0.05$.

RESULTS

Behavior of confluent differentiated HepaRG cells in long term culture

After differentiation from low density-seeded-cultures HepaRG cells formed typical colonies exhibiting a morphology resembling that of normal hepatocytes in primary culture with a dense cytoplasm, one or two nuclei and bile canaliculus-like structures, and were surrounded by more flattened and clearer epithelial cells corresponding to biliary cells. Examination under phase-contrast microscopy did not show any marked morphological changes during the 4-week confluence period tested (Fig. 1A).

Expression of specific transcripts

Transcripts of sixteen genes, i.e. four CYPs (CYP 1A2, 2B6, 2E1 and 3A4), three phase II enzymes (UGT1A1, GSTA1/2, and mEH), four membrane transporters (MDR1, MRP2, OCT1 and BSEP), two nuclear receptors (PXR and CAR), two enzymes involved in protection against toxic oxygen species (catalase and MnSOD) and aldolase B were analysed by RT-qPCR at different times over the 4-week period studied in confluent differentiated

HepaRG cell cultures. The data displayed in Table 2 clearly show that the levels of transcripts remained well maintained, showing no significant variation during the whole studied period whether the corresponding genes are recognized as coding for liver-specific functions (e.g. aldolase B, CYP3A4, CAR and the membrane transporters OCT1 and BSEP) or not (e.g. MDR1, MRP2, catalase and MnSOD). Moreover, when the levels of liver-specific gene transcripts were compared to those measured in freshly isolated hepatocyte samples, they were found to be frequently much lower, especially CYP1A2, CYP2E1 and BSEP, and as previously reported for some of them (Aninat et al., 2006) were in the range of the levels measured in 1-3 days primary human hepatocyte cultures.

Drug metabolizing enzyme activities

6β-hydroxylation of testosterone and deethylation of phenacetin activities catalysed by CYP3A4 and CYP1A2 respectively were also analysed at different time points and as observed for the corresponding transcripts were found to be relatively stable over the 4-week studied period. Whatever the time point considered the values were not statistically different (Fig.2). For 6β-hydroxylation of testosterone, they ranged between 600-800 pmol/min/mg proteins, being similar to those previously reported and comparable to those usually found in primary human hepatocyte cultures (Aninat et al., 2006). Similarly, during the whole confluent period studied HepaRG cells retained their responsiveness to rifampicin and 3-MC (Fig. 3). At each time point analyzed at least a 2-fold induction was observed with either inducers.

Dose-dependent and cumulative cytotoxic effects of AFB1

In a first series of experiments 24h primary human hepatocytes, differentiated HepaRG and confluent HepG2 cells were exposed to varying concentrations of AFB1 for 3, 24 and 72h.

Cytotoxic effects were estimated by measurement of intracellular ATP content. As shown in Fig. 4, AFB1 did not cause any damage to the 3 cell types after a 3h treatment while after 24h, cytotoxicity was evidenced with 5 and even 1µM in both primary human hepatocytes and HepaRG cells. The effects were much more pronounced after 72h. However the values of intracellular ATP did not drop under 60%; this was explained by the fact that, as evidenced by light microscopic examination, morphological alterations of HepaRG cells were restricted to hepatocyte-like cells and were characterized by cell rounding and cytoplasmic granular appearance (Fig. 1 B-F). An exception was with the highest mycotoxin concentration of 5µM that caused both a stronger decrease in ATP content and some damage to biliary-like cells (Fig. 1B). Furthermore, variations in the sensitivity to the mycotoxin were noticed between the 3 exposed human hepatocyte populations, probably related to differences in the levels of the enzymes involved in its metabolism. No damage was observed in HepG2 cells over the 72h exposure even with 5µM.

In another series of experiments, differentiated HepaRG cells were exposed to 0.01, 0.1 and 0.5µM AFB1 for up to 14 days. The mycotoxin was added every 2 days with each medium renewal. Morphological changes and limited decrease in both intracellular ATP content and CYP3A4 activity occurred after 7 days with 0.1 µM AFB1 (Fig. 1D and Fig. 5). No morphological changes were visualized in biliary-like cells after a 7-days exposure to AFB1 at concentrations of 0.01µM (Fig. 1F). Consistent with a cumulative cytotoxic effect of AFB1, CYP3A4 activity was significantly decreased after 14 days with 0.1µM ($p<0.001$ compared to day1).

Genotoxic effects of AFB1 identified by the Comet assay

A positive relationship was obtained between the extent of DNA damage, estimated by the OTM or the percent DNA in the tail, and AFB1 concentrations after a 3h exposure of

differentiated HepaRG cells to the mycotoxin (Fig. 6). A 9- and 4-fold increase in OTM and percent DNA in the tail respectively was recorded in 5µM AFB1-treated versus corresponding untreated cultures. DNA damage induced by the mycotoxin occurred in the absence of increased cytotoxicity estimated by intracellular ATP content (Fig. 4) and trypan blue exclusion and annexin V tests (data not shown) when compared to untreated controls. By contrast undifferentiated HepaRG cells, that did not express CYP3A4 and CYP1A2, were not sensitive to AFB1. A 1h prior and concomittant treatment of the cells with 10µM ketoconazole, a CYP3A4 inhibitor, was associated with a statistically significant reduction of DNA damage in 5µM AFB1-treated cultures. No effect was evidenced with 10µM ketoconazole alone (Table 3). As expected, MMS, a direct alkylating compound, was highly genototoxic to both undifferentiated and differentiated HepaRG cells, being slightly more DNA damaging in the latter (Fig. 6).

DISCUSSION

The use of *in vitro* liver cell models for the evaluation of hepatotoxic compounds is hampered by either their functional instability and low survival (tissue slices, primary cultures) or the loss of the major cytochromes P450 (hepatoma cell lines). Consequently, the treatment time of metabolically active liver cells from either human or animal origin is restricted to days, making them likely unsuitable for investigating mechanisms of toxicity of xenobiotics that are hepatotoxic *in vivo* only after weeks or months of reiterated exposures. In this study, we bring the first demonstration that a human hepatoma cell line expressing various xenobiotic metabolizing enzymes, including the major CYPs, membrane transporters and nuclear receptors when having reached its maximum differentiation state (Aninat et al., 2006; Le Vee et al., 2006) can be further maintained functionally stable for several weeks, and used for chronic toxicity studies using AFB1 as a reference hepatotoxic compound. No obvious

morphological changes occurred in HepaRG cell cultures during such a long-term confluence period. The cells still remained able to transdifferentiate when seeded at low density and to sustain typical cultures composed of both hepatocyte-like and biliary-like cells, each representing around 50% of the total cell population (Cerec et al., 2007) (not shown). It may be therefore assumed that the four-week functional stability of differentiated HepaRG cells at confluence can certainly be greatly extended.

Transcripts of genes involved in various pathways of xenobiotic metabolism and/or recognized as liver-specific markers were analyzed in differentiated HepaRG cells by RT-qPCR at varying time-points over a four-week period. Compared to the levels measured in freshly isolated hepatocytes those encoding liver-specific functions were lower and more comparable to the corresponding ones quantified in 1-3 days pure human hepatocyte cultures; they included aldolase B, a marker of adult hepatocytes, CYP3A4, the nuclear receptor CAR as well as the transporters OCT1 and BSEP. OCT1 is located in the basolateral membrane of the hepatocyte and transports diverse organic cations including toxins and endogenous compounds (Jonker et al., 2004) while BSEP plays a central role in bile acid secretion and bile flow (Alrefai et al., 2007). However, it must be emphasized that hepatocyte-like HepaRG cells represented only around 50% of the total cell population; thereby meaning that the levels of liver-specific transcripts should be nearly doubled to be correctly compared to those measured in pure freshly isolated hepatocyte populations. By contrast other genes were expressed at levels much closer to those found in freshly isolated hepatocytes; they include MnSOD and catalase, two enzymes involved in cellular defense against toxic oxygen species during endogenous metabolism and biotransformation of xenobiotics. In agreement with gene transcription, stability of enzyme activities and their responsiveness to prototypical inducers were also well maintained during the 4-week period as evidenced with CYP3A4 and CYP1A2 activities and their induction by rifampicin and 3-MC respectively.

As stressed for their corresponding transcripts CYP3A4 and CYP1A2 activities would be more elevated by considering only hepatocyte-like cells in HepaRG cell cultures. On this basis, when compared to the levels determined in primary human hepatocyte cultures, those expressed in HepaRG cells would even be higher for CYP3A4 (Aninat et al., 2006) and close to the lowest values for CYP1A2. Indeed, as previously reported (Guillouzo 1998), CYP1A2 activity, using phenacetin as a substrate, greatly varied in primary human hepatocytes from different donors (0.1 to 2.5 nmoles min/mg protein, n=25, after 18-24h of culture). The fold induction with prototypical inducers was also in agreement with previously reported results from primary human hepatocyte cultures (Diaz et al., 1990; Morel et al., 1990). However, it is quite important to keep in mind that a fold induction is related not only to the inducer and its concentration but also to the substrate and the basal activity; the lower the basal activity the higher the induction level. Indeed the results are not the same when ethoxyresorufin is used instead of phenacetin as a substrate for CYP1A2 determinations. Activities of CYP1A are quite low with ethoxyresorufin and consequently the fold change after induction is quite high (Diaz et al., 1990; Aninat et al., 2006). By contrast, when phenacetin is used as a substrate basal activity is higher and fold induction quite lower; the extent of CYP1A2 induction being only around 2-3 fold in both primary hepatocytes and HepaRG cells. This is supported by *in vivo* observations: after a 4 days treatment with omeprazole, the levels of CYP1A2 were increased by around 2-3 fold except in one patient having a low basal activity who showed a 15-fold induction (Diaz et al., 1990). The same conclusion could be drawn from previously reported data for CYP3A4 in HepaRG cells (Aninat et al. 2006).

AFB1 is a reference hepatotoxic compound, acting mainly by binding to DNA and proteins through its *exo* 8,9 epoxide metabolite formed mainly by CYP3A4 (Guengerich et al., 1998). We previously found that differentiated HepaRG cells were much more sensitive to AFB1

than HepG2 cells that do not express both CYP3A4 and CYP1A2 (Aninat et al., 2006). Our present results confirm this observation and show that HepaRG cells are as sensitive as primary human hepatocytes to this mycotoxin, on the basis of cytotoxicity evaluation by determination of intracellular ATP content that has been reported to be more relevant than classical viability tests. However, comparable results were observed by using the neutral red assay (not shown). Assuming that cytotoxicity induced by the mycotoxin is concentration-dependent and cumulative, differentiated HepaRG cells were exposed every two days during 14 days to varying concentrations of AFB1. As expected, in addition to a concentration-dependent toxicity following a unique exposure a cumulative toxicity was observed affecting primarily hepatocytes-like cells. A concentration as low as 0.1µM caused a decrease in CYP3A4 activity starting after one week of treatment, i.e. corresponding to three additions of the mycotoxin. The cytotoxic effects were amplified after 14 days of treatment. These observations support the idea that hepatocyte-like cells mixed with biliary-like cells that do not express the major CYPs, represent an appropriate model to identify compounds that require oxidative metabolism to be hepatotoxic. More precise characterization of phenotypic changes in the two cell populations could be conducted by their selective enzymatic detachment prior to functional analyses.

In vitro genotoxic effects of AFB1 were studied using the single cell gel electrophoresis (comet) assay which is a sensitive method for the detection of DNA damage and repair induced by genotoxic compounds in individual cells and is extensively used in genotoxicity studies. The alkaline version, used in this study, detects single- and double-strand DNA breaks as well as abasic sites (Collins 2004; Witte et al., 2007). The *exo*-8,9 metabolite of AFB1 binds to the N-7 position of guanine residues in DNA and can depurinate to give an abasic site or to be repaired by the nucleotide excision repair pathway (Bedard et al., 2006).

Using the comet assay dose-dependent DNA damage induced by AFB1 was demonstrated in differentiated HepaRG cells in the absence of any sign of cytotoxicity, indicating that DNA alterations evidenced by this test were specific of AFB1-induced genotoxic lesions. In agreement with a genotoxic effect related to the epoxide, DNA alterations were not observed in AFB1-treated undifferentiated cultures that do not express CYP3A4 and were greatly decreased in cells treated with the CYP3A inhibitor ketoconazole. Interestingly, similar dose-dependent effects of AFB1 were obtained with differentiated HepaRG cells using the micronucleus assay (Fessard et al, preliminary observations). DNA damaging effects of AFB1 were however much lower than those caused by the alkylating agent MMS. These differences might have several non-exclusive explanations: indeed it is likely that during a 3h exposition period AFB1 metabolism and formation of DNA-reactive metabolites were not complete and that a large proportion of the epoxides was inactivated by epoxide hydratase and possibly GST enzymes. Such a difference between direct and indirect genotoxic agents has already been observed in the comet assay with other human cells (Uhl et al., 1999). Moreover, while both cell types were damaged by MMS, only hepatocyte-like cells were likely to be sensitive to the mycotoxin. Further experiments will allow to define the lowest AFB1 concentration inducing detectable DNA lesions under well-defined experimental conditions that could be further applied for investigating specific DNA lesions induced by a wide variety of xenobiotics.

Taken together, our results clearly demonstrate that HepaRG cells not only can fully differentiate but in addition have the unique property to retain their differentiation state for weeks at confluence. We bring here the proof of concept that metabolically competent HepaRG cells represent a suitable model to study chronic toxicity and genotoxicity of chemicals in human liver and to identify early and late target genes of any potential hepatotoxic compound.

ACKNOWLEDGMENTS

We thank Mrs Tifen Le Charpentier, Sylvie Huet (Comet assay) and Annick Mourot for excellent technical help, the Biological Resources Centre of Rennes and Biopredic International for the supply of isolated human hepatocytes and the Ouest-Genopole imaging-cell chip platform for microscopic analyses.

REFERENCES

Alrefai W. A. and Gill R. K. Bile Acid Transporters: Structure, Function, Regulation and Pathophysiological Implications. *Pharmaceut Res* 24(10): 1802-1823. 2007.

Aninat C., Piton A., Glaise D., Le Charpentier T., Langouet S., Morel F., Guguen-Guillouzo C. and Guillouzo A. Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metab Dispos.* 34(1): 75-83. 2006.

Aninat C., Seguin P., Descheemaeker P., Morel F., Malledant Y. and Guillouzo A. Catecholamines induce an inflammatory response in human hepatocytes. *Critl Care Med.* 36(3):848-854. 2008.

Bedard L. L. and Massey T. E. Aflatoxin B1-induced DNA damage and its repair. *Cancer Lett.* 241(2): 174-83. 2006.

Cerec V., Glaise D., Garnier D., Morosan S., Turlin B., Drenou B., Gripon P., Kremsdorf D., Guguen-Guillouzo C. and Corlu A. Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. *Hepatology*. 45(4): 957-67. 2007.

Collins A. R. The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol*. 26(3): 249-61. 2004.

Diaz D., Fabre I., Daujat M., Saint Aubert B., Bories P., Michel H. and Maurel P. Omeprazole is an aryl hydrocarbon-like inducer of human hepatic cytochrome P450. *Gastroenterology*. 99(3): 737-747. 1990.

Gripon P., Rumin S., Urban S., Le Seyec J., Glaise D., Cannie I., Guyomard C., Lucas J., Trepo C. and Guguen-Guillouzo. C. Infection of a human hepatoma cell line by hepatitis B virus. *PNAS*. 99(24): 15655-15660. 2002.

Guengerich F. P., Johnson W. W., Shimada T., Ueng Y. F., Yamazaki H. and Langouet S. Activation and detoxication of aflatoxin B1. *Mutat Res.* 402(1-2): 121-8. 1998.

Guguen-Guillouzo C., Campion J. P., Brissot P., Glaise D., Launois B., Bourel M. and Guillouzo A. High yield preparation of isolated human adult hepatocytes by enzymatic perfusion of the liver. *Cell Biol Int Rep.* 6(6): 625-8. 1982.

Guillouzo A. Liver cell models in in vitro toxicology. *Environ Health Perspect.* 106 Suppl 2: 511-32. 1998.

Guillouzo A., Corlu A., Aninat C., Glaise D., Morel F. and Guguen-Guillouzo C. The human hepatoma HepaRG cells: A highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chem Biol Interact.* 168 (1): 68-73. 2007.

Hewitt N. J., Lechon M. J., Houston J. B., Hallifax D., Brown H. S., Maurel P., Kenna J. G., Gustavsson L., Lohmann C., Skonberg C., Guillouzo A., Tuschl G., Li A. P., LeCluyse E., Groothuis G. M. and Hengstler J. G. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev.* 39(1): 159-234. 2007.

Jonker J. W. and Schinkel A. H. Pharmacological and physiological functions of the polyspecific organic cation transporters: OCT1, 2, and 3 (SLC22A1-3). *J Pharmacol Exp Ther*. 308(1): 2-9. 2004.

Lasser K. E., Allen P. D., Woolhandler S. J., Himmelstein D. U., Wolfe S. M. and Bor D. H. Timing of new black box warnings and withdrawals for prescription medications. *Jama*. 287(17): 2215-20. 2002.

Le Vee M., Jigorel E., Glaise D., Gripon P., Guguen-Guillouzo C. and Fardel O. Functional expression of sinusoidal and canalicular hepatic drug transporters in the differentiated human hepatoma HepaRG cell line. *Eur J Pharm Sci.* 28(1-2): 109-17. 2006.

Lee W. M. Drug-induced hepatotoxicity. *N Engl J Med.* 349(5): 474-85. 2003.

Majer B. J., Mersch-Sundermann V., Darroudi F., Laky B., de Wit K. and Knasmuller S. Genotoxic effects of dietary and lifestyle related carcinogens in human derived hepatoma (HepG2, Hep3B) cells. *Mutat Res.* 551(1-2): 153-66. 2004.

Morel F., Beaune P. H., Ratanasavanh D., Flinois J. P., Yang C. S., Guengerich F. P. and Guillouzo A. Expression of cytochrome P-450 enzymes in cultured human hepatocytes. *Eur J Biochem.* 191(2): 437-44. 1990.

Olson H., Betton G., Robinson D., Thomas K., Monro A., Kolaja G., Lilly P., Sanders J., Sipes G., Bracken W., Dorato M., Van Deun K., Smith P., Berger B. and Heller A. Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regul Toxicol Pharmacol.* 32(1): 56-67. 2000.

Paolini M. and Cantelli-Forti G. On the metabolizing systems for short-term genotoxicity assays: a review. *Mutat Res.* 387(1): 17-34. 1997.

Sassa S., Sugita O., Galbraith R. A. and Kappas A. Drug metabolism by the human hepatoma cell, Hep G2. *Biochem Biophys Res Commun.* 143(1): 52-7. 1987.

Uhl M., Helma C. and Knasmuller S. Single-cell gel electrophoresis assays with human-derived hepatoma (Hep G2) cells. *Mutat Res.* 441(2): 215-24. 1999.

Wilkening S., Stahl F. and Bader A. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. *Drug Metab Dispos.* 31(8): 1035-42. 2003.

Witte I., Plappert U., de Wall H. and Hartmann A. Genetic toxicity assessment: employing the best science for human safety evaluation part III: the comet assay as an alternative to in vitro clastogenicity tests for early drug candidate selection. *Toxicol Sci.* 97(1): 21-6. 2007.

FOOTNOTES

Financial supports: This work was supported by EEC contracts (LIINTOP-STREP-037499 and COMICS STREP 037575), ANR contract (06SEST17) and the Ligue 35 contre le Cancer.

Reprint request should be addressed to : GUILLOUZO André, INSERM U620, Detoxication et Réparation Tissulaire, Faculté des Sciences Pharmaceutiques et Biologiques, Université de Rennes I, 35043 Rennes Cedex, France. Andre.Guillouzo@univ-rennes1.fr

LEGENDS FOR FIGURES

Figure 1. **Phase-contrast-micrographs of control and AFB1-treated differentiated HepaRG cells**.

HepaRG cells (30 days following cell seeding) were exposed to the vehicule only for 14 days (A) or to AFB1 at 5µM for 24h (B), 0.5µM for 24h (C), 0.1µM for 7 days (D), 0.1µM for 14 days (E) or 0.01µM for 14 days (F). Hepatocyte-like colonies exhibit slight morphological alterations (D and F) or major changes associated with alterations of biliary-like cells (B and E). Hepatocyte-like (H) and biliary-like (BC) cells; bile canaliculus (arrow). (Original magnification x 150)

Figure 2. **CYP3A4- and 1A2-related activities in HepaRG cells as a function of time at confluence**.

Determinations of testosterone 6β-hydroxylation (CYP3A4) and phenacetin deethylation (CYP1A2) activities were performed in differentiated HepaRG cells at several time points. Results are expressed as pmol/min/mg protein and are the mean ± SEM of three independent experiments. ns, not statistically significant.

Figure 3. **Responsiveness of drug-metabolizing enzyme activities in differentiated HepaRG cells exposed to prototypical inducers**.

Cultures were exposed for 48h to 50µM rifampicin (A), 5µM 3-MC (B) or 0.2% DMSO (control). Results are mean ± SEM of three independent experiments and are expressed as pmol/min/mg protein. Each fold induction value was indicated above corresponding diagram. * $p<0.05$ *vs* control at day 30 and ** $p<0.01$ *vs* control at day 58.

Figure 4. **Comparative acute toxicity of AFB1 to primary human hepatocytes, HepaRG and HepG2 cells.**

24h primary human hepatocytes, differentiated HepaRG cells and confluent HepG2 cells were exposed to different concentrations of AFB1 for 3h, 24h or 72h. AFB1 cytotoxicity was assayed using intracellular ATP measurements. Results are normalized to control cells and express as means ± SEM of three independent experiments. * $p<0.05$; ** $p<0.01$.

Figure 5. **Chronic toxicity of AFB1 to differentiated HepaRG cells.**

Cultures were treated every two days at each medium renewal with varying AFB1 concentrations or 2% DMSO (control). Intracellular ATP (A) and CYP3A4 activity (B) were measured. Results are expressed as percentages compared to corresponding control, set at 100%. **A.** * $p<0.05$. **B.** *** $p<0.001$ *vs* day 1.

Morphology changes in hepatocyte-like cells were graded from 0 (no changes) to + (slight alterations), + + (cells rounding; few detached cells) and +++ (many detached and dead cells)

Figure 6. **Effects of AFB1 on DNA damage in HepaRG cells.**

Undifferentiated and differentiated HepaRG cells were exposed to varying AFB1 concentrations for 3 h. Results are the mean ± SEM of 3 to 4 independent experiments. DNA damage was expressed as percentage of corresponding negative control: Olive tail moment (OTM) (A) and % DNA in the comet tail (B). MMS (5µg/ml) was used as a positive control. *$p<0.05$, statistically significant difference between undifferentiated and differentiated cells.

DMD Fast Forward. Published on March 17, 2008 as DOI: 10.1124/dmd.107.019901
This article has not been copyedited and formatted. The final version may differ from this version.

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

Genes	Forward Primer	Reverse Primer
18S	CGCCGCTAGAGGTGAAATTC	TTGGCAAATGCTTTCGCTC
Aldolase B	GCATCTGTCAGCAGAATGGA	TAGACAGCAGCCAGGACCTT
CYP3A4	CTTCATCCAATGGACTGCATAAAT	TCCCAAGTATAACACTCTACACAGACAA
CYP1A2	TGGAGACCTTCCGACACTCCT	CGTTGTGTCCCTTGTTGTGC
CYP2B6	TTCCTACTGCTTCCGTCTATCAAA	GTGCAGAATCCCACAGCTCA
CYP2E1	TTGAAGCCTCTCGTTGACCC	CGTGGTGGGATACAGCCAA
GSTA1/2	TGCAACAATAAGTGCTTTACCTAAGTG	TTAACTAAGTGGGTGAATAGGAGTTGTATT
UGT1A1	TGACGCCTCGTTGTACATCAG	CCTCCCTTTGGAATGGCAC
mEH	ACAGGCACCATCATCTCCTC	CCAGTGGGCACATAGACCTT
BSEP	TGATCCTGATGAAGGGAAGG	TGGTTCCTGGGAAACAATTC
MDR1	GCCAAAGCCAAAATATCAGC	TTCCAATGTGTTCGGCATTA
OCT1	TAATGGACCACATCGCTCAA	AGCCCCTGATAGAGCACAGA
MRP2	TGAGCAAGTTTGAAACGCACAT	AGCTCTTCTCCTGCCGTCTCT
CAR	TGATCAGCTGCAAGAGGAGA	AGGCCTAGCAACTTCGCATA
PXR	CCAGGACATACACCCTTTG	CTACCTGTGATGCCGAACAA
MnSOD	GGGTTGGCTTGGTTTCAATA	CTGATTTGGACAAGCAGCAA
Catalase	ACCAGGGCATCAAAAACCTTT	CCGGATGCCATAGTCAGGAT

DMD Fast Forward. Published on March 17, 2008 as DOI: 10.1124/dmd.107.019901
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2: **Gene expression in HepaRG cells as a function of time at confluence**

Comparative expression of various genes in differentiated HepaRG cells at several confluent time points. Results are expressed as percentages compared to day 30 after seeding, arbitrary set at 100% and are the mean ± SEM of three independent experiments. In parentheses the percentages of the values measured in a pool of freshly isolated human hepatocytes (FIH) from three donors; ns, not statistically significant.

Genes	mRNA levels (%)					
	30	37	44	51	58 (days)	p
Aldolase B	100(6)	113 ± 14	129 ± 38	155 ± 36	154 ± 28	ns
CYP3A4	100(119)	99 ± 11	108 ± 47	111 ± 31	105 ± 15	ns
CYP1A2	100(1)	111 ± 35	128 ± 25	109 ± 42	93 ± 38	ns
CYP2B6	100(32)	117 ± 12	185 ± 9	181 ± 24	208 ± 71	ns
CYP2E1	100(5)	207 ± 38	187 ± 13	169 ± 38	133 ± 16	ns
GSTA1/2	100(186)	90 ± 15	89 ± 18	106 ± 15	94 ± 10	ns
UGT1A1	100(181)	81 ± 8	96 ± 11	90 ± 3	81 ± 19	ns
mEH	100(213)	101 ± 10	105 ± 3	111 ± 13	114 ± 16	ns
BSEP	100(2)	93 ± 36	150 ± 84	181 ± 55	233 ± 37	ns
MDR1	100(303)	81 ± 10	89 ± 3	71 ± 9	72 ± 12	ns
OCT1	100(25)	124 ± 25	159 ± 20	181 ± 12	173 ± 26	ns
MRP2	100(164)	112 ± 9	133 ± 4	116 ± 11	117 ± 22	ns
CAR	100(21)	111 ± 4	143 ± 47	143 ± 26	136 ± 14	ns
PXR	100(58)	132 ± 23	172 ± 32	176 ± 28	188 ± 59	ns
MnSOD	100(317)	105 ± 17	145 ± 10	144 ± 22	121 ± 31	ns
Catalase	100(67)	84 ± 2	100 ± 20	88 ± 16	89 ± 18	ns

DMD Fast Forward. Published on March 17, 2008 as DOI: 10.1124/dmd.107.019901
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3. **Effect of ketoconazole treatment on the induction of DNA damage by AFB1 in HepaRG cells.**

Differentiated HepaRG cells (30 days following cell seeding) were exposed for 3h to varying concentrations of AFB1 in the presence or absence of 10µM ketoconazole**.**

Results are mean ± SEM of 4 independent experiments, * $p<0.05$: different from solvent control; § $p<0.05$: different from their counterparts treated only with AFB1. MMS: methyl methane sulfonate (positive control).

		DNA damage	
	Ketoconazole (10 µM)	**OTM**	**% DNA in the tail**
Control (medium)	-	0.13 ± 0.03	1.53 ± 0.29
MMS (5 µg/mL)	-	6.43 ± 1.93 (*)	22.24 ± 5.31 (*)
Control (DMSO)	-	0.10 ± 0.01	1.29 ± 0.12
	+	0.11 ± 0.01	1.36 ± 0.15
AFB1 1 µM	-	0.22 ± 0.03	2.01 ± 0.28
	+	0.18 ± 0.06	1.78 ± 0.39
AFB1 2.5 µM	-	0.50 ± 0.09 (*)	3.73 ± 0.66 (*)
	+	0.27 ± 0.02	2.45 ± 0.27
AFB1 5 µM	-	0.84 ± 0.15 (*)	5.36 ± 0.33 (*)
	+	0.35 ± 0.05 (§)	3.22 ± 0.61 (§)

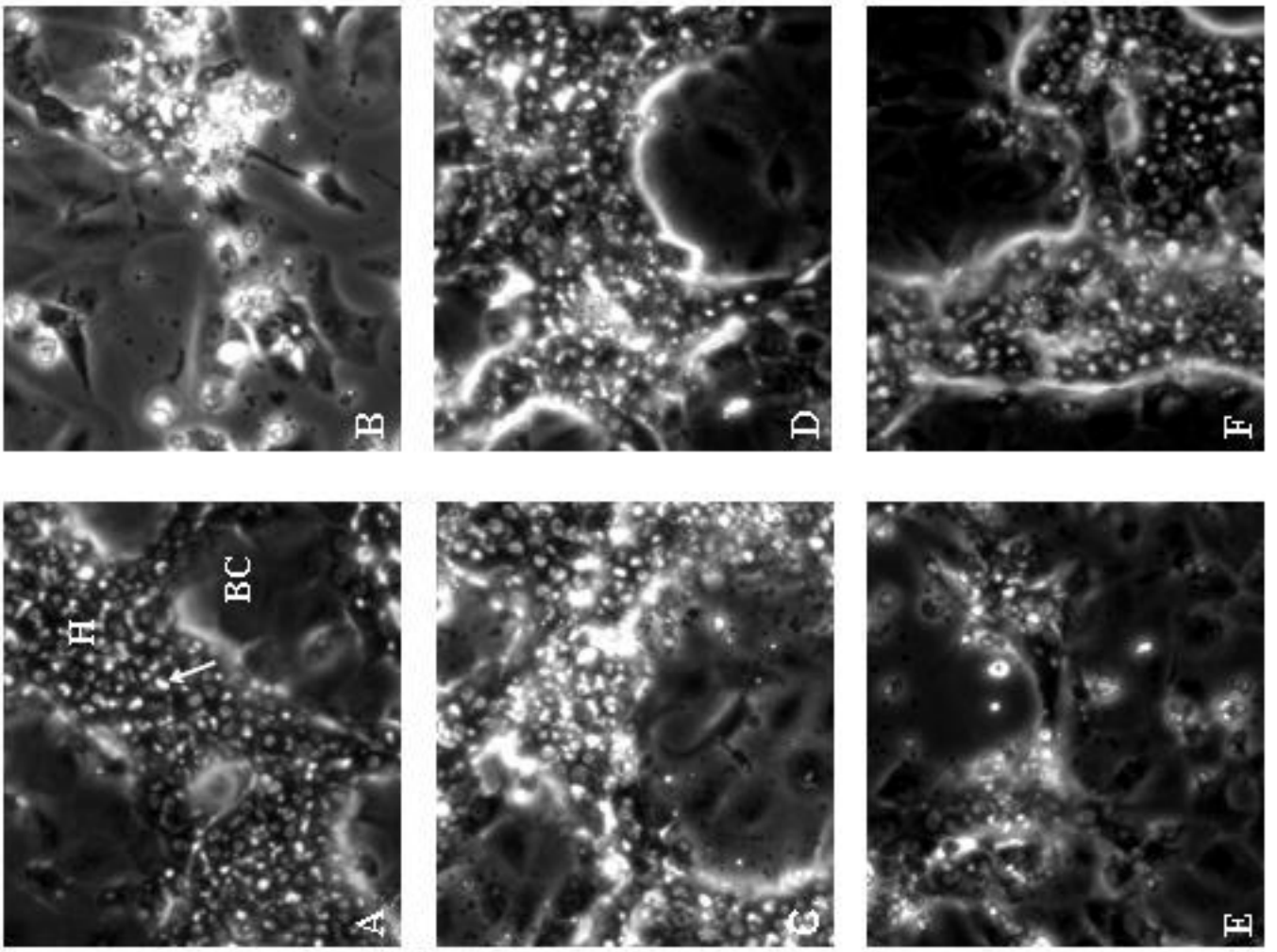
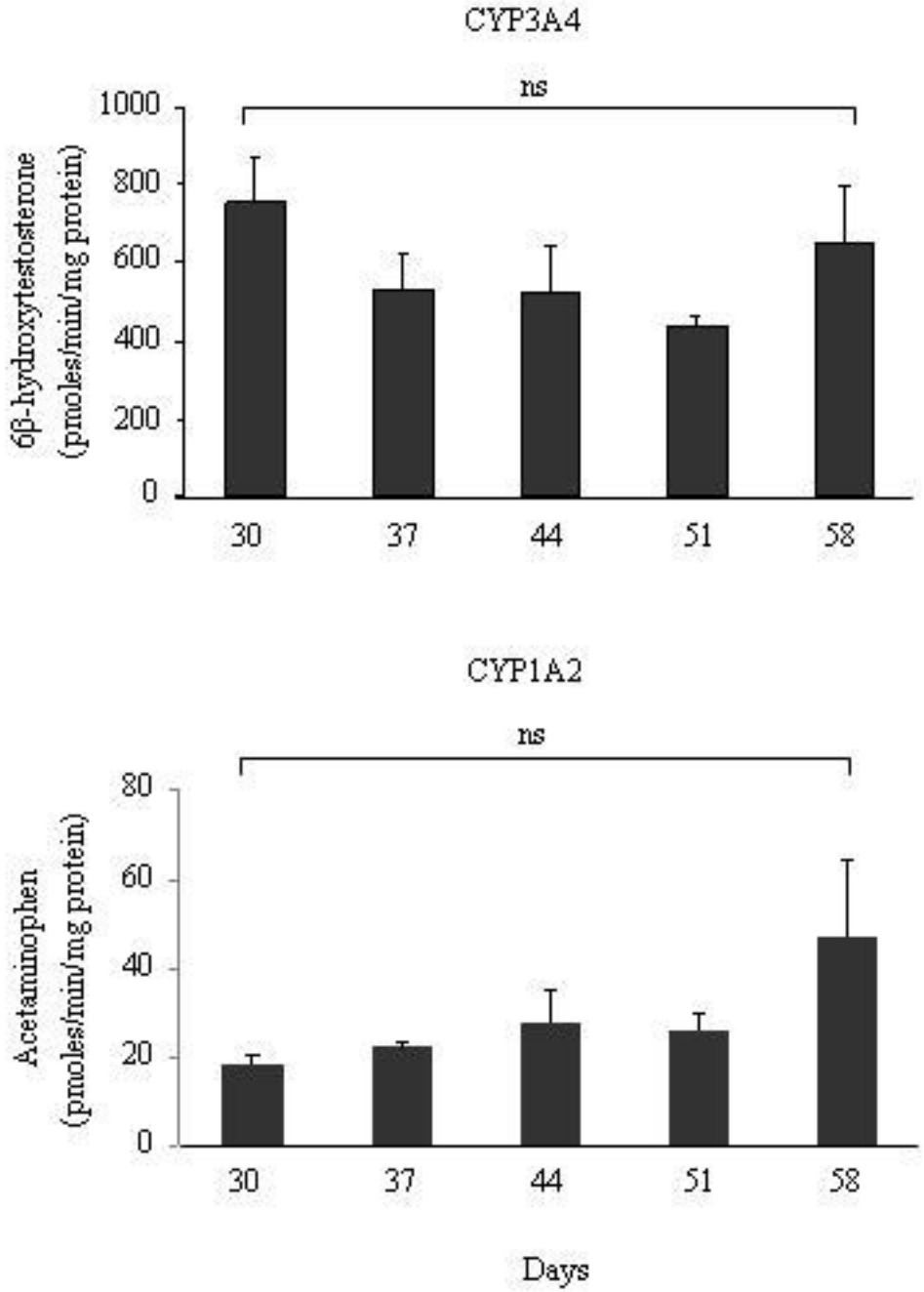


Fig. 1.

Fig. 2



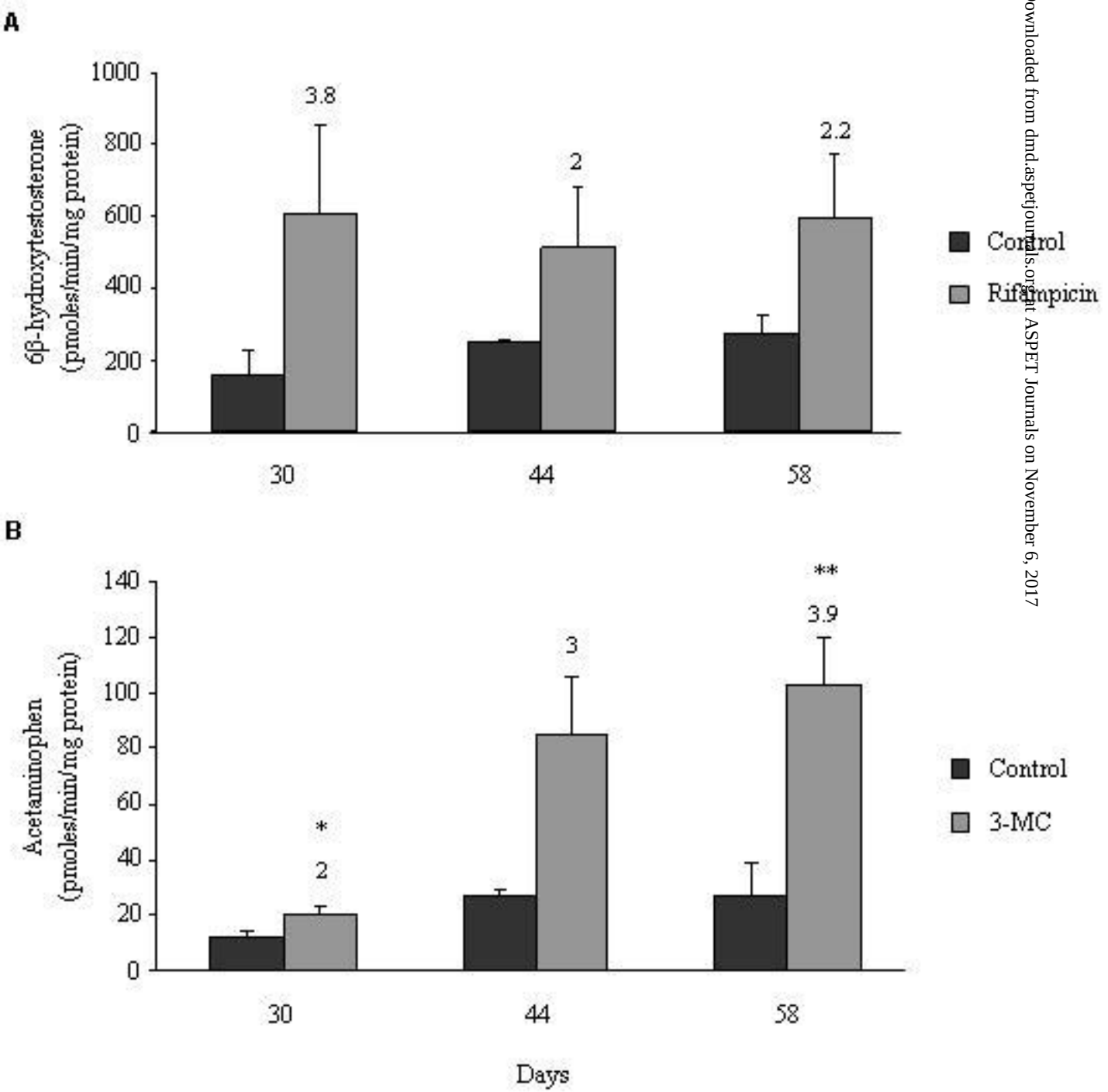


Fig. 3

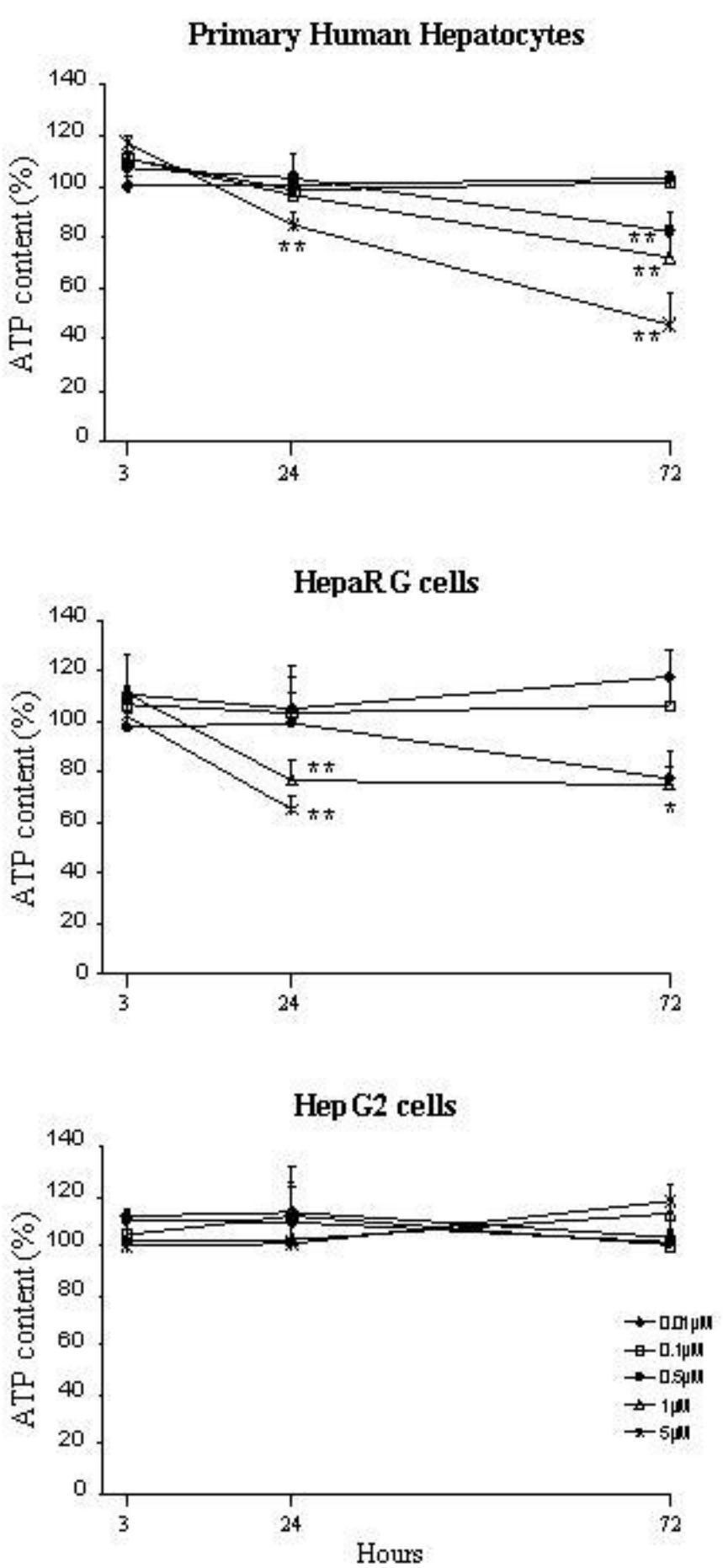

Primary Human Hepatocytes
ATP content (%)
140
120
100
80
60
40
20
0
3
24
72
**
**
**
**
HepaR G cells
ATP content (%)
140
120
100
80
60
40
20
0
3
24
72
**
**
*
Hep G2 cells
ATP content (%)
140
120
100
80
60
40
20
0
3
24
72
Hours
0.01µM
0.1µM
0.5µM
1µM
5µM

Fig.4

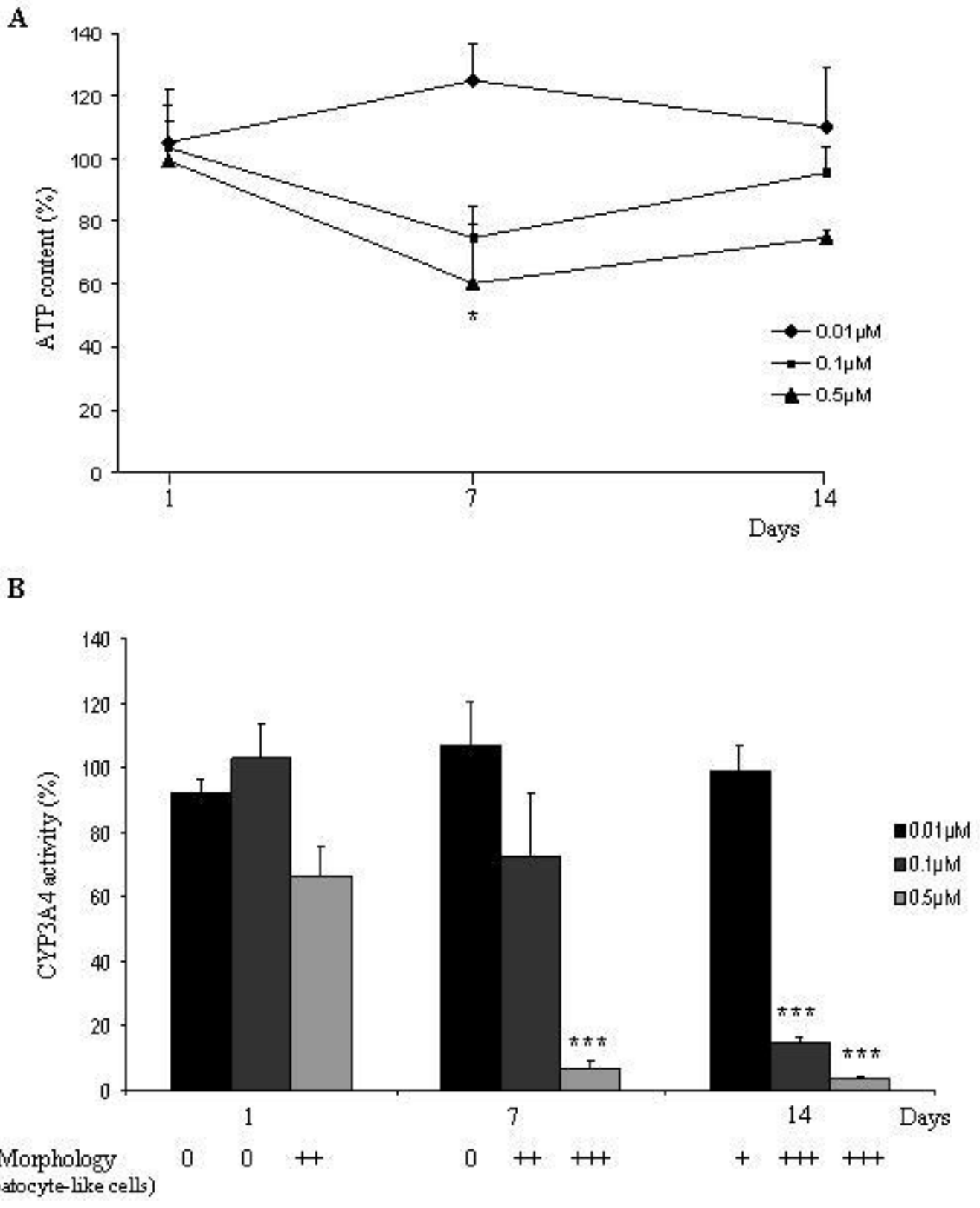

A
ATP content (%)
140
120
100
80
60
40
20
0
*
0.01μM
0.1μM
0.5μM
1
7
14
Days
B
CYP3A4 activity (%)
140
120
100
80
60
40
20
0
***
***
***
0.01μM
0.1μM
0.5μM
1
7
14
Days
Morphology
(Hepatocyte-like cells)
0 0 ++ 0 ++ +++ + +++ +++

Fig. 5

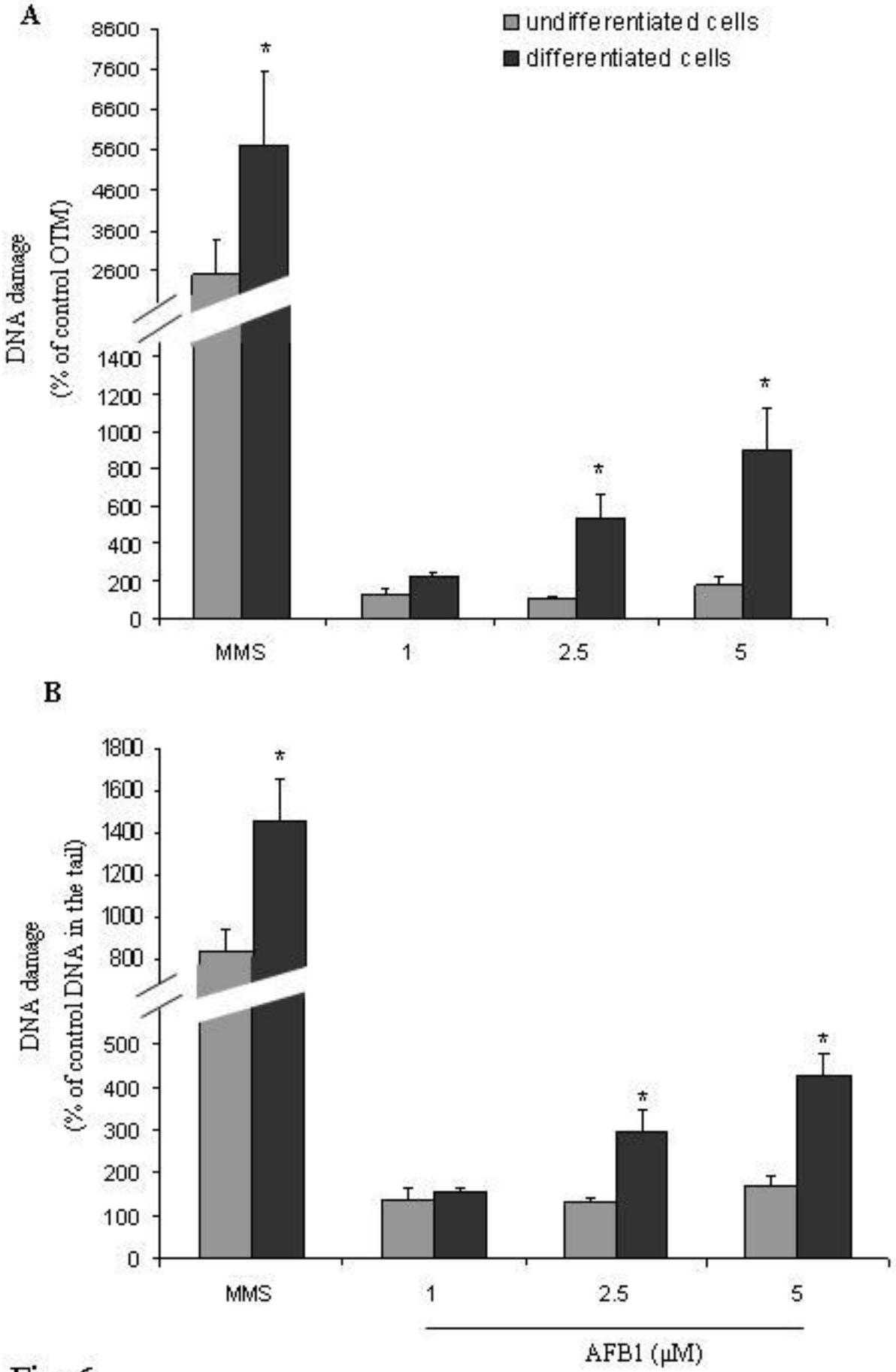

A
undifferentiated cells
differentiated cells
DNA damage
(% of control OTM)
8600
7600
6600
5600
4600
3600
2600
1400
1200
1000
800
600
400
200
0
*
MMS
1
2.5
5
B
DNA damage
(% of control DNA in the tail)
1800
1600
1400
1200
1000
800
500
400
300
200
100
0
MMS
1
2.5
5
AFB1 (μM)

Fig. 6.