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DMSO-free highly differentiated HepaRG spheroids for chronic toxicity, liver functions and genotoxicity studies

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Keywords

HepaRG cells, 3D culture model, long-term differentiation, genotoxicity, human liver injury, toxicity screening

Abbreviations

CYP, cytochrome P450; DMSO, dimethyl sulfoxide; AFB₁, aflatoxin B₁; B[a]P, Benzo[a]Pyrene; CPA, Cyclophosphamide; MMS, methylmethane sulfonate; PHH, primary human hepatocytes

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Abstract

The liver is essential in the elimination of environmental and food contaminants. Given the interspecies differences between rodents and humans, the development of relevant *in vitro* human models is crucial to investigate liver functions and toxicity in cells that better reflect pathophysiological processes. Classically, the differentiation of the hepatic HepaRG cell line requires high concentration of dimethyl sulfoxide (DMSO), which restricts its usefulness for drug-metabolism studies. Herein, we describe undifferentiated HepaRG cells embedded in a collagen matrix in DMSO-free conditions that rapidly organize into polarized hollow spheroids of differentiated hepatocyte-like cells (Hepoid-HepaRG). Our conditions allow concomitant proliferation with high levels of liver-specific functions and xenobiotic metabolism enzymes expression and activities after a few days of culture and for at least 4 weeks. By studying the toxicity of well-known injury-inducing drugs by treating cells with 1- to 100-fold of their plasmatic concentrations, we showed appropriate responses and demonstrate the sensitivity to drugs known to induce various degrees of liver injury. Our results also demonstrated that the model is well suited to estimate cholestatic and steatotic effects of drugs following chronic treatment. Additionally, DNA alterations caused by four genotoxic compounds (Aflatoxin B₁ (AFB₁), Benzo[a]Pyrene (B[a]P), Cyclophosphamide (CPA) and Methyl methanesulfonate (MMS)) were quantified in a dose-dependent manner by the comet and micronucleus assays. Their genotoxic effects were significantly increased after either an acute 24h treatment (AFB₁: 1.5-6 µM, CPA: 2.5-10 µM, B[a]P: 12.5-50 µM, MMS: 90-450 µM) or after a 14-day treatment at much lower concentrations (AFB₁: 0.05-0.2 µM, CPA: 0.125-0.5 µM, B[a]P: 0.125-0.5 µM) representative to human exposure. Altogether, the DMSO-free 3D culture of Hepoid-HepaRG provides highly differentiated and proliferating cells relevant for various toxicological *in vitro* assays, especially for drug-preclinical studies and environmental chemicals risk assessment.

Introduction

The critical interspecies variations between human and animals regarding drug metabolism capacities reinforce the need for efficient human *in vitro* models¹⁻³. In line with the 3R's strategy (reduce, replace, refine) which aims to reduce and optimize the use of animals for *in vivo* testing, many laboratories are developing alternative human cell-based models that are more predictive than commonly used standard approaches to assess toxicity. Modeling hepatic toxicity is particularly needed as drug-induced liver injuries (DILI) is the most frequent reason cited for the withdrawal of approved drugs from the market and accounts for up to 15% of the cases of acute liver failure⁴. Thus, the development of relevant *in vitro* human hepatic models is a major challenge to evaluate the toxicological effects of environmental contaminants and drugs. Among cell lines, the HepaRG line, which was derived from hepatocellular carcinoma, is composed of progenitors that have the unique ability to differentiate into a co-culture of hepatocyte-like and biliary cells⁵. HepaRG cells exhibit specific liver functions resembling those of adult Primary Human Hepatocytes (PHH)⁶⁻⁸; they retain the main characteristics of mature hepatocytes, including expression of biotransformation enzymes, nuclear receptors, transcription factors and transporters^{6,7,9-11}. This last decade, HepaRG cells have been widely used for drug screening and toxicological studies^{9,12-14} and their stable human-relevant cytochrome P450 (CYP) activities have made them largely used cells for the study of drug-induced hepatotoxicity^{6,15}. To fully differentiate into hepatocyte-like cells and reach optimal expression of metabolism enzymes, HepaRG cells are conventionally cultured for 14 days in the presence of DMSO after an initial 14-days period of proliferation to achieve high density confluence^{6,16}. Besides the long process required for their differentiation (28 days), the addition of DMSO results in reactive oxygen species (ROS) scavenging as well as the constitutive induction of several CYP, in particular the main hepatic isoform CYP3A4 which therefore does not respond to its prototype inducers^{6,17}, while DMSO withdrawal leads to a drastic drop of specific hepatic functions within one day^{6,9}.

Tests to evaluate the genotoxicity associated with food and environmental pollutants, such as the comet and the micronucleus assays, are routinely performed *in vivo* on rodents according to authorize organizations guidelines. Given the significant errors that may result from interspecies differences in xenobiotic metabolism when extrapolating for human risk assessment^{1,2}, novel *in vitro* robust models of human cells are critically needed to study the genotoxicity and mutagenesis of compounds that undergo metabolic activation. Another concern is the exposure of human hepatic cells to acute treatment with high doses of chemical compounds that are not representative of *in vivo* exposure¹⁸⁻²¹. Models in which compounds can be tested over prolonged periods are necessary to better evaluate the genotoxic effects of contaminants on health in a more realistic setting. It is now well recognized that conventional 2D cultures do not recapitulate the complex liver architecture²². In 3D culture, more physiological cell-cell and cell-matrix interactions, and maintenance of cell polarity greatly improve the expression profiles of liver genes^{23,24}. Recent studies have shown that various 3D culture models of HepaRG cells promote hepatic differentiation and metabolic functions, and increase sensitivity to DNA damages induced by pro-carcinogens^{20,25-31}. Models based on 3D structures comprising differentiated hepatic cells are thought to be particularly relevant to faithfully recapitulate key liver functions. In particular, 3D cultures are well suited to obtain phenotype-stable liver cells capable of maintaining their hepatocyte-specific polarity, viability and functions. Such 3D cultures can be obtained by the formation of HepaRG cells aggregates in ultra-low attachment plates or after embedment in a matrix such as alginate, cellulose hydrogel or gelatin³²⁻³⁴. Most HepaRG cultures use DMSO-differentiated cells before 3D seeding in order to obtain optimal hepatocyte features. To date, only two studies describe DMSO-free 3D cultures of HepaRG cells, limited data is therefore available regarding the differentiation of HepaRG spheroids in short and long-term cultures. One study refers to a strategy based on the microencapsulation of cells in alginate suitable for short-term toxicological applications³⁵, while the other focuses on the co-culturing of HepaRG cells with stellate and endothelial cells to recapitulate the key steps of fibrogenesis³⁰. Thus, a full phenotypic characterization and assessment of the suitability of DMSO-free HepaRG 3D cultures for studying chronic DILI and liver diseases are still missing.

The aim of this study was to develop a DMSO-free, easy-to-handle, rapid and robust HepaRG 3D model, named Hepoid-HepaRG, exhibiting hepatic functions and xenobiotic metabolism capacities close to mature human hepatocytes. Such aim is in adequacy with the 3R's strategy for getting relevant information on toxic and genotoxic drugs and contaminants in highly

differentiated human hepatocytes after acute and chronic treatments. We validated the robustness and performance of this novel 3D model to study DILI severity. Moreover, we demonstrated that the model is pertinent to quantify the effects of cholestatic and steatotic drugs as well as to perform genotoxicity and mutagenesis. In this work, we showed for the first time the genotoxicity of several mutagens using very low concentrations in long-term conditions, i.e. 14 days. The Hepoid-HepaRG model thus constitutes a promising *in vitro* tool for predicting chemical-induced human liver injuries both in the acute and chronic settings.

Materials and methods

Chemicals

Unless mentioned, chemicals were purchased from Sigma Aldrich. All chemicals were of the highest quality available.

Cell culture and treatment

HepaRG cells were routinely propagated as described previously⁵. The William's E medium was supplemented with 5 µg/ml insulin, 2 mM glutamine, 10 % Fetal Bovine Serum, 100 units/ml penicillin, 100 mg/ml streptomycin and 5×10^{-5} M hydrocortisone hemisuccinate. The medium was renewed every 2 to 3 days and the cells were cultured until passage 18. After 2 weeks of culture, classically differentiated HepaRG cultured in 2D monolayer were shifted to the same culture medium supplemented with 2 % DMSO for 2 more weeks.

For 3D culture, the HepaRG cells were passaged every week. Type I collagen from bovine skin was diluted into the culture medium to obtain a collagen solution at 1.5 mg/ml and the cells were added at a concentration of 5×10^5 cells/ml. This mixture of cells and collagen was poured into multiwell plates and incubated at 37 °C, 5% CO₂. After 1 h, the gels were polymerized and an equal volume of medium was added.

All short treatments (≤ 72 h) were performed in DMSO and FBS-free medium. DMSO in 2D cultures and FBS in both 2D and 3D cultures were maintained in the culture medium for long-term treatments. For mRNA and genotoxicity analysis, cells were extracted from the gels by digestion with Liberase (10 µg/ml, Roche) for 20 min at 37°C.

TPEF microscopy

Two-photon excitation fluorescence (TPEF) microscopy imaging was performed as previously described²⁴.

Quantification of DNA synthesis

The thymidine analogue 5-Ethynyl-2'-deoxyuridine EdU (10 µM, Thermo-Fisher, CAS 61135-33-9) was used to quantify DNA analysis. After 24h of incorporation, HepaRG spheroids embedded in collagen gels were fixed with 4 % paraformaldehyde (CAS 30525-89-4) for 1 h, impregnated with paraffin and embedded in blocks. Paraffin-embedded spheroids were cut 4 µm sections and EdU was detected with a fluorescent CY5-azide (10 µM).

Toxicity studies of DILI-causing compounds

Compounds with various DILI potentials were screened. Therapeutic exposure concentrations (C_{max}) were extracted from previously published data³⁶. After 5 days of culture, Hepoid-HepaRG were treated with 1x, 5x, 20x, 50x and 100x of the respective drug concentrations during a total of 14 days excepted for amiodarone (CAS 1951-25-3) and ketoconazole (CAS 65277-42-1) for which the maximal concentration was 50x because of their poor solubility. Acetaminophen (CAS 103-90-2) was diluted in the medium, whereas others compounds were diluted in DMSO with maximal concentration of DMSO never exceeding 0.4 %. Viability was assessed by measurement of cellular ATP content using the CellTiter-Glo 3D Assay (Promega). Compounds were considered hepatotoxic when the viability was reduced by at least 20 % compared to control with * $p < 0.05$ (t-test).

Albumin and urea secretion

Cell culture medium was collected every 48 h for the dosage of albumin and urea content. Albumin and urea concentrations were determined according to the manufacturer's guidelines using the Human Serum Albumin DuoSet ELISA kit (R&D Systems) and the ChromaDazzle Urea Assay (Assay Genie), respectively.

Efflux transport assay

Two fluorescent dyes, 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate (CDFDA, CAS 4091-99-0) and cholyl-L-lysyl-fluorescein (CLF, CAS 140616-46-2) were used to study the activity of the multidrug resistance-associated protein (MRP2) and the bile salt export pump (BSEP), respectively. After 10 min of washing with the uptake buffer⁷, HepaRG spheroids were incubated for 15 min at 37°C with fluorescent substrates (3 µM CDFDA or 5 µM CLF) then washed with the uptake buffer and observed under fluorescence microscopy.

Immunohistochemistry

After fixation with 4 % paraformaldehyde for 1 h, collagen gels were impregnated with paraffin and embedded in blocks. Paraffin-embedded spheroids were cut 4 µm sections, and stained for E-cadherin (#3195, 1:100, Cell Signaling), N-cadherin (#610921, 1:100, BD Biosciences) and MRP2 (#ab3373, 1:100, Abcam).

CYPs enzyme activities measurement by LC-MS

HepaRG cells were treated with vehicle (DMSO, 0.1 %) for basal activities or with 3-methylcholantrene (5 µM, 24 h, CAS 56-49-5) for CYP1A2 induction, rifampicin (50 µM, 72 h, CAS 13292-46-1) for CYP3A4 and CYP2D6 induction, or phenobarbital (3.2 mM, 72 h, CAS 50-06-6) for CYP2B6 induction. Cells were incubated with various substrates, i.e. phenacetin (CYP1A2, 200 µM, CAS 62-44-2), midazolam (CYP3A4, 50 µM, Pharmacopée Européenne, CAS 59467-70-8), bupropion (CYP2B6, 100 µM, CAS 34841-39-9), amodiaquine (CYP2C8, 100 µM, CAS 6398-98-7), warfarin (CYP2C9, 100 µM, CAS 81-81-2) and dextromethorphan (CYP2D6, 100 µM, CAS 125-71-3). Supernatants were collected after 4 h and metabolites were quantified as previously described²⁴. A CellTiter Glo-3D assay was performed to normalize activity to the number of viable cells.

Triglyceride content

Stock solutions of tested compounds were prepared in DMSO and diluted in culture medium with a maximal final concentration of 0.2 % DMSO. Steatosis studies were started at day 5 for 14 days, the culture medium was replaced every 48 or 72 h. Total triglycerides concentration was measured using the Triglyceride-Glo Assay (Promega) according to the manufacturer's protocol.

Caspase-3/7 activity

Caspase 3/7 activity was measured using the SensoLyte Homogeneous AMC Caspase-3/7 Assay (Anaspec). Briefly, Hepoid-HepaRG cells were lysed during 10 min on ice before the caspase-3/7 substrate solution (DEVD-AMC) was incubated with the cell lysates for 1.5 h at 37°C. AMC cleavage by caspase 3/7 was monitored by spectrofluorometry (V_{\max}). A CellTiter Glo-3D assay was performed in parallel to normalize the V_{\max} to the number of viable cells.

Cholestasis toxicity studies

As previously described, a stock mixture of the six more abundant bile acids (BA) was prepared according to the concentration of each BA found in normal human plasma (**Supplementary Table 1**)³⁷. Excepted for acetaminophen (CAS 103-90-2) which was directly prepared in culture medium, stock solutions of tested compounds were prepared in DMSO and diluted in culture medium with a maximal final concentration of 0.7 % of DMSO. Toxicity studies were started at day 5 for 14 days, the medium was replaced every 48 or 72 h. Viability was assessed by measurement of ATP using the CellTiter-Glo 3D Assay. To quantify the drug's ability to exert its cholestatic toxicity, the drug-induced cholestatic index (CI) was

calculated as described previously³⁷. It corresponds to the ratio of EC₅₀ with BA (+BA) divided by the EC₅₀ without BA (-BA) after drug exposure. A CI value less than 80 % was the threshold taken for determining whether the drug could have a cholestatic toxicity.

mRNA analysis

Total RNA was extracted using NucleoSpin RNA (Macherey-Nagel). RNAs were reverse-transcribed into cDNAs using the High Capacity cDNA reverse transcription kit (Applied Biosystems). RT-qPCR analysis was performed with the CFX384 Real-Time System (Biorad) using the SYBR Green PCR master mix (Applied Biosystems). GAPDH was used as the internal reference. For transcriptomic analysis, mRNAs were collected from HepaRG cultured in 2D (n = 5) or in 3D at day 4 (n = 5), day 15 (n = 5) and day 28 (n = 4). RNA samples were checked for degradation based on the RNA Integrity Number (RIN > 8). The RNA libraries were sequenced using the HiSeq 2500 (Illumina). Complementary RNA synthesis, hybridization and chip scanning were performed at the GenoBIRD platform (Nantes, FR). Data analysis was performed using R. GO terms enrichment analysis of differentially expressed genes (Fold Change > 2, p < 0.05) was performed using WebGestalt (WEB-based GENESeT) online analysis tool (Vanderbilt University, The Netherlands).

Comet Assay

Spheroids were centrifuged and incubated with Trypsin/EDTA 0.05% (Gibco) during 6 min at 37 °C to isolate cells. FBS-containing medium was added to stop the reaction. Cells were centrifuged and pellets were resuspended in 70 µl of cold PBS. 30µl of suspension was then mixed with 300 µl of LMP agarose (CAS 9012-36-6) maintained at 37 °C and 30 µl of the mixture was deposited in a well of a CometSlide 20 wells (Trevigen) which was processed as previously described³⁸. Briefly, the slides were incubated over night at 4°C in a lysis solution (Trevigen) supplemented with 10 % DMSO. DNA was then allowed to unwind during 40 min in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) prior to electrophoretic migration (30 min, 21V). The slides were bathed twice in neutralizing solution (0.4 M Tris-HCl, pH 7.5) for 5 min and in 70% ethanol for 5 min and dried at 37°C for at least 2 h. Finally, DNA was stained with SYBR Gold 1X (Invitrogen) during 30 min before being dried completely at 37 °C. Slides were observed using a fluorescence microscope (Leica DMR) equipped with a CCD-200E video camera. At least 100 comets per condition were acquired and analyzed using the Comet Assay IV software (Perceptive Instruments). The extent of DNA damage in individual cells was evaluated by the percentage of tail DNA.

Micronucleus assay

Spheroids were centrifuged and incubated with Trypsin/EDTA 0.05 % (Gibco) during 6 min at 37 °C to isolate cells. FBS-containing medium was added to stop the reaction. Cells were centrifuged and pellets were resuspended in medium supplemented with rhEGF (50 ng/ml, Peprotech) and cytochalasine B (2 mg/ml, CAS 14930-96-2), a cytostatic agent that blocks cytokinesis. After a 72 h recovery period, an osmotic shock (25 % water, 5 min) was realized prior to fixation in 4% paraformaldehyde (CAS 30525-89-4) for 20 min and staining with DAPI (0.5 µg/ml, CAS 28718-90-3). Micronuclei were scored in 1000 binucleated cells per assay. For cytotoxicity assessment, the cytokinesis-block proliferation index (CBPI) was calculated: [CBPI= (number of monocleated cells + 2 x number of binucleated cells + 3 x number of polynucleated cells)/total number of cells)].

Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Data were analyzed with one-way ANOVA. Differences were considered significant when * p < 0.05, ** p < 0.01, *** p < 0.001. All the results were from at least three independent experiments.

Results

Characterization of Hepoid-HepaRG morphology

To optimize the differentiation of HepaRG cells, we selected a collagen matrix concentration of 1.5 mg/ml that we have previously shown to be the optimum 3D culture condition allowing proliferation and differentiation of the Huh-7 hepatoma cell line and primary human hepatocytes^{24,39}. Undifferentiated HepaRG cells were embedded in the collagen matrix and cultivated for several weeks in absence of DMSO. The morphology and organization of HepaRG structures, named Hepoid-HepaRG, were first observed by TPEF microscopy. To examine the 3D organization of the cells, we performed Z-stack reconstructions at day 6, 15 and 28. TPEF scanning showed that the seeding of undifferentiated HepaRG cells into collagen gels led to the formation of cell clusters that increased in size over time in culture (**Fig. 1A**). The HepaRG cells self-organized as spheroids exhibiting an acini-like structure with a hollow lumen and an average diameter reaching $28 \pm 7 \mu\text{M}$ and $50.6 \pm 11.3 \mu\text{M}$ after 15 and 28 days of culture, respectively. Quantification studies showed a 4.2-fold increase in TPEF-autofluorescent cell signal between days 6 and 28 (**Fig. 1B**). ATP measurement, taken as a marker of viability, showed an increase for the first 20 days followed by a constant level or a slight decrease thereafter (**Fig. 1C**). Moreover, our results showed that 10 ± 2.3 to 46.9 ± 8.7 % of the HepaRG cells appeared positive for EdU incorporation depending on the culture time, with an optimal capacity at day 15 (**Fig. 1D**). The labeling slightly decreased thereafter but clearly demonstrated the potential for long-term proliferation in the 3D collagen matrix.

To assess the epithelial phenotype of 3D HepaRG cells, we looked at the mRNA expression and protein localization of E-cadherin and N-cadherin after 6, 15 and 28 days of culture (**Fig. 1E and 1F**). 2D HepaRG cultures fully differentiated by 2 % DMSO (2D-DMSO) were taken as reference. The results showed a high and constant expression of E-cadherin throughout the time in culture as compared to 2D-DMSO. By contrast, the expression of the mesenchymal marker N-cadherin remained stable throughout the culture at a level close to that of 2D-DMSO. As evidenced by the localization of E- and N-cadherins at the apico and lateral membranes (**Fig. 1E**), HepaRG cells rapidly developed in small clusters with hollow lumen and the cells appeared polarized. Overall, this morphological characterization shows that the 3D culture of HepaRG cells in a collagen matrix, in the absence of DMSO, maintains proliferation and promotes an epithelial phenotype similar to what we have described previously for PHH²⁴.

Hepoid-HepaRG exhibit characteristics of highly differentiated hepatocytes

By transcriptomic analysis, we compared the expression profiles of 3D cultured HepaRG in absence of DMSO at days 6, 15 and 28 to 2D-DMSO (at day 28), condition considered as the gold standard for HepaRG differentiation. We looked at the expression of specific liver genes, listed for most of them in the LiGEP panel defined by Kim *et al.*⁴⁰. This panel is based on RNAs that are significantly differentially expressed between liver and non-liver samples. They developed an algorithm based on RNA-sequencing analyses to assess the differentiation or maturation status of 93 liver-specific genes. The heatmap associated with the LiGEP genes revealed a strong homology of expression profiles between the 2D-DMSO and Hepoid-HepaRG (**Fig. 2A**) at days 6, 15, and 28. To confirm the role of the 3D culture on spheroids differentiation, we used RT-qPCR to measure RNA expression of *HNF4 α* , *HNF1 β* , *albumin* and *α -fetoprotein*. We confirmed that these mRNAs were maintained for at least 28 days in spheroids at levels higher or close to those detected in 2D-DMSO (**Fig. 2B**). We also analyzed the secretion of albumin and urea (**Fig. 2C**), and showed that they remained constant or increased, respectively, during the whole culture process. Notably, the secretion levels of albumin and urea were significantly higher in 3D cultures compared to 2D-DMSO. The lower expression of SOX9 in 3D HepaRG at all time points suggests that the cells preferentially differentiate into a hepatic rather than a biliary lineage when cultured in the collagen matrix, in contrast to 2D-DMSO cultures where the two lineages coexist⁶ (**Fig. 2D**). However, the biliary marker CK19 was expressed at comparable levels in both culture conditions. When examining the expression of genes previously identified as markers of ductal liver, stem cells, hepatoblasts and mesenchymal cells, we found that 2D-DMSO and 3D HepaRG at different time points in cultures were comparable (**Supplementary Fig. 1**).

3D culture of HepaRG cells promotes rapid and stable expressions and activities of phase I, II and III xenobiotic-metabolizing enzymes

We first analyzed the mRNA expression of major nuclear receptors implicated in xenobiotic metabolism regulation, i.e CAR, PXR, AhR and PPAR α (**Fig. 3A**). Their expression was comparable in Hepoid-HepaRG and 2D-DMSO. Then, we examined the expression of drug-metabolizing enzymes and transporters (DMET) which are essential to subsequent assessment of the potential hepatotoxicity of drugs and contaminants. Their expression was analyzed by transcriptomic analysis in 3D cultures at 6, 15 and 28 days in comparison with 2D-DMSO (**Supplemental Fig.2**). As confirmed by RT-qPCR analysis, the expression of these DMET in Hepoid-HepaRG at different time points were close to their expressions in 2D-DMSO (**Fig. 3**). CYPs are the major enzymes implicated in phase I xenobiotic-metabolism pathways, playing a crucial role in the bioactivation of many drugs and contaminants. In particular, CYP3A4 is the major isoform expressed in the human liver⁴¹. We validated that some isoforms were equally expressed (*CYP1A2*, *CYP2C8*, *CYP2C9*) or lower (*CYP3A4*, *CYP2B6*) in Hepoid-HepaRG compared to 2D-DMSO (**Fig. 3B**). The high expression level of CYP3A4 and to a lesser extent of CYP2B6 in 2D-DMSO compared to 3D HepaRG is explained by the addition of DMSO in the 2D culture medium. It therefore reflects an induced level rather than a basal one. Besides the mRNA expression, we estimated the functional activities of the main CYP isoforms by HPLC tandem mass spectrometry. CYP1A2, a liver specific isoform, can be regulated by a polycyclic aromatic hydrocarbon compound, 3-methylcholantrene (3-MC), well known to occur through the AhR pathway. Our result showed that the capacity of CYP1A2 to be induced by 3-MC is similar in 3D and 2D-DMSO (**Fig. 3C**). Basal activities were also comparable for four isoforms, CYP2B6, CYP2D6, CYP2C8, CYP2C9 while CYP2B6 and CYP2D6 activities were strongly or equally induced, respectively, in 3D versus 2D cultures. Indeed, the basal activity of CYP2B6 was similar between 2D-DMSO and 3D cultures but the induction rate after treatment with phenobarbital was higher in Hepoid-HepaRG (2.2 fold increase in 2D-DMSO vs 23.3, 6.7 and 5.44 fold increase in 3D at day 6, 15 and 28, respectively). Of note, CYP3A4 with has a much lower basal activity retains its induction capabilities in the 3D model after treatment with rifampicin (about 15-fold increase at the various time points), while this capability is completely lost in the 2D cultures due to the presence of DMSO.

Then, we analyzed by RT-qPCR the mRNA expression level of several xenobiotic metabolism enzymes of phase II (NAT1, NAT2, GST α , UGT1A1 and UGT1A9) and transporters (MRP2, MRP3, OCT1, NTCP and BSEP) involved in detoxifying pathways in Hepoid-HepaRG and 2D-DMSO (**Fig. 3D and E**). Our results showed that the mRNA expressions were maintained throughout the time of culture up to 28 days in Hepoid-HepaRG at level comparable to 2D-DMSO (with the exception of BSEP mRNA expressed at much higher levels in Hepoid-HepaRG compared to 2D-DMSO).

Finally, to confirm the polarization of the spheroids, we performed immunolocalization of the efflux drug-transporter MRP2. At all time points and in line with its *in vivo* distribution, MRP2 was localized at the apical/bile canalicular domain of the cells, clearly at the center of the spheroids. The functional activity of MRP2 and BSEP confirmed a clear efflux of the CDFDA and CLF, respectively, into the central lumen of the spheroids at day 6, 15 and 28 (**Fig. 3F**).

All these data demonstrate that after only few days, Hepoid-HepaRG reached comparable hepatic differentiation to the 28-days 2D cultures in the presence of the chemical adjuvant DMSO. Importantly, it should be emphasized that the advantage of Hepoid-HepaRG model in regards to expression and activities of DMET is notable whatever the time of culture considered, as soon as day 6 and up to day 28, thus paving the way to long-term treatments.

Validation of the Hepoid-HepaRG model for toxicity, liver pathologies and genotoxicity assays

Toxicity

We have demonstrated that DMSO-free HepaRG spheroids embedded in a collagen matrix exhibit high and stable levels of hepatic differentiation markers including DMET over at least 4 weeks of culture. To validate these properties in term of toxicity, we first focused on drugs recognized to cause varying degrees of DILI: severe DILI (amiodarone, amodiaquine, cyclosporine A, diclofenac, ketoconazole), high DILI (acetaminophen, chlorpromazine, coumarin, methotrexate, quinidine) or negative controls (hydrocortisone, lidocaine, progesterone, terfenadine, warfarin). These molecules have been tested at

therapeutic exposure concentrations (1x) or higher (5x, 20x, 50x, 100x). Given that the cholestatic effect of some compounds could only be detected after long-term repeated exposures⁴², the toxicity was evaluated in Hepoid-HepaRG cells after a prolonged 14-day treatment. The results are consistent with the expected degrees of DILI severity of the compounds (**Fig. 4A**): severe and high DILI drugs were detected at concentrations close to their plasmatic concentrations (1x) while drugs not considered to induce DILI did not induce any toxicity, indicating that our 3D model is appropriate to assess drug hepatotoxicity. We then focused on four drugs inducing toxicity through the formation of ROS: acetaminophen, chlorpromazine, diclofenac, and cisplatin⁴³. In line with the previously described role of DMSO as a ROS scavenger¹⁷, 3D cultured HepaRG cells showed increased sensitivity to all ROS-inducing drugs tested as compared to 2D-DMSO (**Fig. 4B**). This ROS-induced toxicity was related to a dose-response induction of caspase 3/7 activities after cisplatin and, to a lesser extent, diclofenac treatments. While, as expected, acetaminophen toxicity appeared to be disconnected from caspase 3/7-mediated apoptosis (**Supplemental Fig. 3**).

Liver pathologies: steatosis and cholestasis

To assess whether the Hepoid-HepaRG model is relevant for studying liver pathologies, we tested the activity of drugs with known steatotic and cholestatic effects using a 14-day treatment. Steatosis assessed by triglyceride quantification showed a significant increase after treatment with amiodarone (1.35 to 2.3 fold), at a level comparable to that obtained after treatment with oleic acid (1.80 to 2.46 fold), thus indicating that the HepaRG model can reproduce steatotic drug-induced pathologies. With regards to cholestasis, the accumulation of CDFDA and CLF in the hollow lumen of the spheroid is indicative of good functionality of the cells and biliary MRP2 and BSEP activities. Recently, Hendriks et al. introduced an *in vitro* cholestatic assay based on an intracellular toxic accumulation of bile acids (BA)^{37,42}. To apply this assay to Hepoid-HepaRG, we first looked at the non-toxic effect of BA at plasmatic concentrations (1x) or higher (50x, 100x). We retained a concentration of 100x for the assay since no toxicity was detected (**Supplemental Fig. 4**). We then evaluated four drugs with known cholestatic activity (cyclosporin A, chlorpromazine, bosentan, amiodarone) in the Hepoid-HepaRG model (**Fig. 4D**). Upon repeated exposure over 14 days to cholestatic drugs at different concentrations in the presence of the 100x BA mixture, a selective synergistic toxicity of all tested compounds was revealed, resulting in increased toxicity compared to drug treatments alone. Based on measurements of the cholestatic index from three independent experiments, we confirmed that the four cholestatic drugs had cholestatic activity in the Hepoid-HepaRG model. On the other hand, no synergistic toxicity was observed, as expected, after co-treatment with the non-cholestatic toxic (high DILI, see Fig. 4A) compound acetaminophen and the BA mixture.

Genotoxicity

To validate the model with respect to chemical-induced genotoxicity, we studied the effects of some well-known positive mutagens after acute (24h) and chronic treatments (14 days). We selected AFB₁, a mycotoxin considered to be the most powerful hepatocarcinogen in humans, B[a]P, a polycyclic aromatic hydrocarbure, and CPA, an alkylating agent. All three require metabolism activation to exert their toxic and genotoxic effects. Moreover, an acute treatment with the direct mutagen MMS was used as a positive control.

The comet assay was successfully adapted on the Hepoid-HepaRG model. First, we checked that the concentrations used did not induce any toxicity higher than 80 % after 24h and 14 days (**Fig. 5A and 5B**). The positive control MMS induced significant DNA fragmentation from 90 μ M. After 24h and 14 days of treatment, our results clearly demonstrated that the pro-mutagens AFB₁, CPA and B[a]P induced a significant increase of DNA damage in a dose-dependent manner. Interestingly, a 24 h treatment gave similar results as a long-term treatment with 30 to 100 times lower concentrations. We then performed mutagenesis studies by adapting the micronucleus assay to our 3D cultures. To ensure that micronucleus formation was linked to duplication of the cells, cytokinesis was blocked by cytochalasine B according to the OECD's test guideline TG 487. The percentage of binucleated cells was 30 to 60 % and the level of binucleated micronucleated (BNMN) cells varied between 2.3 and 13.8 %. The positive control MMS induced between 3.9 and 13.4 % of BNMN, depending on

the concentration. After both acute and chronic treatment, AFB₁, CPA, B[a]P statistically increased the number of BNMN at non-cytotoxic concentrations, considering the values of CBPI (**Fig. 5C**).

All together, our results demonstrate the relevance of Hepoid-HepaRG for genotoxicity and mutagenesis studies in highly differentiated human hepatocytes. In particular, the high sensitivity of the Hepoid-HepaRG model makes it particularly well suited for long-term studies at very low doses that are more representative of actual chronic human exposure.

Discussion

The current study demonstrates the establishment of a DMSO-free 3D model of highly differentiated and proliferating HepaRG cells. We showed a rapid establishment of liver functions in 3D cultured HepaRG cells within a few days after embedment of undifferentiated cells into the collagen matrix and long-term maintenance at levels close to those of 2D-DMSO. Proliferating Hepoid-HepaRG exhibit a polarized acini organization with a high level of expressions and activities of xenobiotic-metabolism enzymes, making them well suited for toxicology, physiopathology, genotoxicology and mutagenesis studies after short and long-term treatments.

The HepaRG cell line is recognized as one of the most differentiated human hepatic cell line when used with the DMSO-dependent 2D culture protocol. However, DMSO has pleiotropic effects on cell behavior, including the induction of drug-metabolizing enzymes and the suppression of hepatic homeostasis-related features such as ammonia detoxification and glycogen storage⁴⁴. This classical method to get differentiated hepatocyte-like cells is widely used and almost all 3D studies refer to the embedment of DMSO-differentiated HepaRG cells, whose differentiation is maintained by adding or not DMSO to the culture medium^{28,29,45–47}. In these cultures, withdrawal of DMSO leads to rapid dedifferentiation of the HepaRG cells, the duration of culture without DMSO is therefore often limited to a few days, making them unsuitable for long-term chronic toxicity and metabolism studies. Two DMSO-free 3D culture methods of HepaRG cells have been described to date. One described spheroids formed in spinner vessels and then microencapsulated in alginate beads that exhibit, after 14 days of culture, hepatic functions similar to those of 2D-DMSO³⁵. Our results showed that the HepaRG spheroids in collagen gels reach a high state of differentiation within one week and retain their hepatic phenotype for at least 28 days making it possible to study the toxicological effects of drugs and contaminants rapidly and after chronic treatments. In particular, we showed that the activities of xenobiotic metabolism enzymes were comparable to the 2D-DMSO and also PHH (data not shown) as early as 6 days of culture and stable thereafter. Moreover, a major advantage Hepoid-HepaRG for toxicity studies is that CYP3A4 and CYP2B6 retain their ability to be activated unlike in 2D-DMSO in which DMSO artificially induces it. This is a crucial improvement over the 2D-DMSO model for genotoxicity testing of compounds requiring bioactivation.

Classical 2D *in vitro* liver cell models for toxicity assays are generally hampered either by rapid dedifferentiation and poor survival of primary cultures or by incomplete hepatic differentiation of hepatoma cell lines. 3D culture strategies that favor cell-cell and cell-matrix interactions greatly improve the maintenance of hepatic-specific functions in primary cultures⁴⁸, hepatic cell lines^{26,30,39,49} and iPSC-derived hepatocytes⁵⁰. The size of the spheroids and their cellular organization are critical for oxygen diffusion and long-term survival. Most of the hepatic spheroids described in the literature are composed of a large number of cells with hypoxia-induced necrotic cores and do not present any particular organization at the multicellular level^{46,47}. Consistent with our observations previously made on Huh-7 cells and PHH^{24,39}, HepaRG cells cultured in collagen gels self-assemble and organize into small spheroids with a hollow lumen with a single layer of cells. HepaRG cells show high plasticity in 2D culture, being able to differentiate into biliary and hepatocyte-like cells after exposure to DMSO⁶. Our data on DMSO-free Hepoid-HepaRG showed that hepatic differentiation is similar or superior to that of cells cultured in 2D with DMSO. However, even if some biliary genes (i.e. SOX9) seem to be expressed at a lower level in 3D cultures, most biliary markers are expressed at a level close to those observed in 2D-DMSO. While some reports indicated that HepaRG cells encapsulated in 3D matrices form hepatocyte-like colonies rather than cholangiocytes²⁶, the biliary phenotype of collagen-embedded Hepoid-HepaRG cannot be excluded. The expression profile of most of the key hepatic genes in our HepaRG spheroids shows that 3D culture in the collagen matrix promotes rapid differentiation of the embedded undifferentiated cells that remain at a high level over the time of culture. The heatmap illustrating the expression

profiles of the LiGEP genes revealed a strong homology between the 3D cultures and the established model of differentiated hepatocyte culture 2D-DMSO. Moreover, in Hepoid-HepaRG, hepatic markers (i.e. Albumin, HNF1 β , HNF4 α) analyzed by RT-qPCR were significantly increased while albumin and urea expression and secretion remained at higher level for at least 28 days.

Given the interesting properties of survival and differentiation of the Hepoid-HepaRG model, we applied it to a range of applications regarding liver toxicity and genotoxicity. We demonstrated that it is particularly well suited for drug-induced toxicity studies by showing an appropriate response to different DILIs. However, drugs that do not induce DILI did not cause toxicity, showing that we did not detect false positives and that the model is sensitive but not overly so. Modeling complex liver diseases is an emerging and promising field that could provide new insights into disease mechanism and progression, and enable screening of novel drugs. Drug-induced cholestasis is associated with impaired homeostasis leading to the intracellular retention of toxic BA. The drug-transporter MRP2 in 2D cultures can also be detected in canalicular structures after treatment with DMSO^{10,51} while some previous studies showed a polarization of the transporters (i.e. PGP, MRP2, BSEP) in HepaRG spheroids formed in a spinner-bioreactor²⁸ or on ULA plates³⁷. In the present work, we demonstrated in Hepoid-HepaRG that the MRP2 protein is precisely distributed around the hollow lumen of the spheroids, forming an acini-like structure. The functional activity of two main apical BA transporters, MRP2 and BSEP, confirmed a clear efflux at the center of the spheroids. The sensitivity to cholestatic drugs was determined by comparing cell viability after drug exposure in the presence of a non-cytotoxic concentration of BA^{37,42}. Compounds with known cholestatic liability (cyclosporin A, bosentan, amiodarone, chlorpromazine) showed synergistic toxicity with BA in HepaRG spheroids compared to exposure to compounds alone while no such synergy was observed with a non-cholestatic control (acetaminophen), demonstrating that our 3D model can be useful to easily detect cholestatic compounds and quantify their activities upon prolonged exposures. Some studies reported a synergic toxicity between cholestatic compounds and BA related to an increase of oxidative stress^{37,52,53}. Comparing the CI values determined for chlorpromazine by Hendricks et al. (0.66 ± 0.03), our Hepoid-HepaRG model appears to have a greater sensibility to drug-induced cholestasis (0.40 ± 0.24) due to the DMSO-free culture conditions. With regard to lipid overload, 2D-DMSO has been reported to respond well to steatotic stimulation⁵⁴⁻⁵⁶. In this study, we showed that the 3D HepaRG model allows lipid accumulation quantification by determining triglyceride levels after induction with steatotic compounds. In accordance with the previously published data⁵⁷, it demonstrates the valuableness of human 3D hepatic models for the development of dynamic disease models and screening for NASH and NAFLD drugs using long term repeated exposure in culture. The Hepoid-HepaRG model constitutes a promising *in vitro* system to evaluate the cholestatic and steatotic liabilities of new drugs and to perform investigations of underlying disease mechanisms.

We and others have shown that matrix stiffness plays a major role in cell behavior and could also influence the proliferation of hepatic cells^{27,39,58}. It is noteworthy that the DMSO-dependent HepaRG differentiation protocol implies differential periods of proliferation and differentiation of the cells, which makes this model not optimal for mutagenesis studies^{18,19,31}. By contrast, Hepoid-HepaRG showed a proliferative capacity throughout the length of culture, while presenting highly differentiated hepatic functions. Such properties are essential for *in vitro* genetic toxicity detection of drugs and contaminants, which are currently analyzed through the comet assay and the micronucleus test⁵⁹. The fact that in our study, both acute and chronic treatments with pro-carcinogens led to DNA damage detection is highly differentiating compared to previous studies using human cell lines both in 2D and 3D cultures^{20,21,31}. Indeed, none of previous studies allowed 14-days long-term mutagenic assays in a well-differentiated and proliferating human hepatic model with low concentrations of pro-carcinogens that better reflect the actual human exposure. Therefore, the high sensitivity of Hepoid-HepaRG opens new perspectives in term of studying the genotoxicity and mutagenic potential of drugs and contaminants in human, including high-throughput screening, and may contribute substantially to reducing the use of laboratory animals for experiments.

All together, by showing the sensitivity of HepaRG cells embedded in a collagen matrix, we demonstrated that the Hepoid-HepaRG model is particularly suitable for both acute and chronic toxicity, as well as physiopathological and genotoxicity studies. In addition, since deleterious effects have been studied for only a small fraction of environmental contaminants to

date, robust and rapidly available *in vitro* human hepatic models such as Hepoid-HepaRG should be of great value for assessing the potential risk of most xenobiotic compounds whose effects on humans are still unknown. A strategy combining Hepoid-HepaRG and microfluidic devices will improve the exchange between cells and their environment, paving the way for organ-on-chip applications and new pharmaceutical developments.

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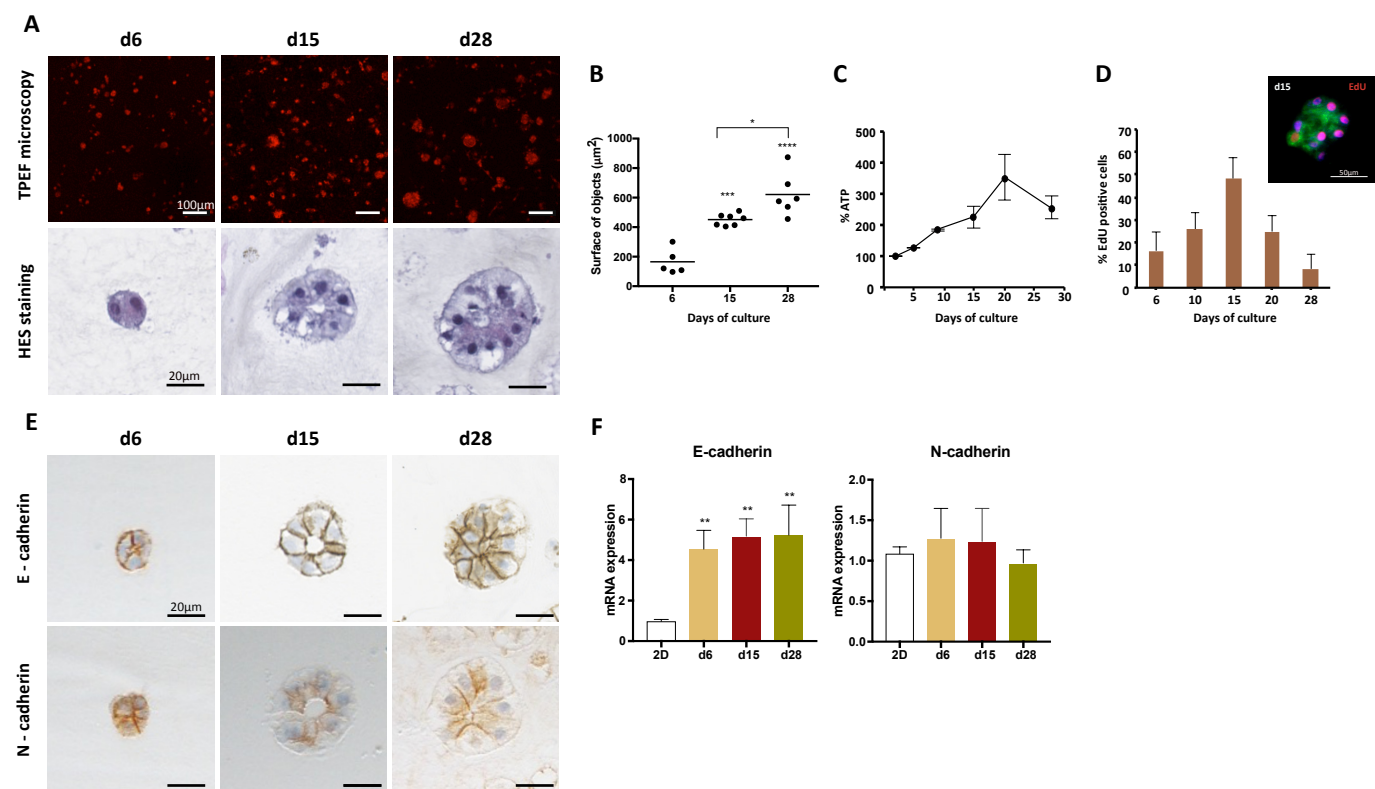


Figure 1 – 3D culture of HepaRG cells in collagen gels promotes formation of proliferating spheroids with an epithelial phenotype

A. TPEF images (10× magnification, z projection, scale bar = 100 μm), HES (hematoxylin, eosin, saffron) staining (scale bar = 20 μm) and **B.** quantification of the surface of Hepoid-HepaRG (ImageJ software) at day 6, 15 and 28. **C.** Viability assessed by cellular ATP content at different time points of culture expressed in % compared to day 2. **D.** Quantification of the rate of proliferation determined by the incorporation of EdU in 3D cultures. **E.** Immunostaining of E-cadherin and N-cadherin in Hepoid-HepaRG at days 6, 15 and 28 (scale bar = 20 μm). **F.** mRNA expression by RT-qPCR of E-cadherin and N-cadherin at day 6, 15 and 28. The results are normalized to levels in 2D-DMSO (2D).

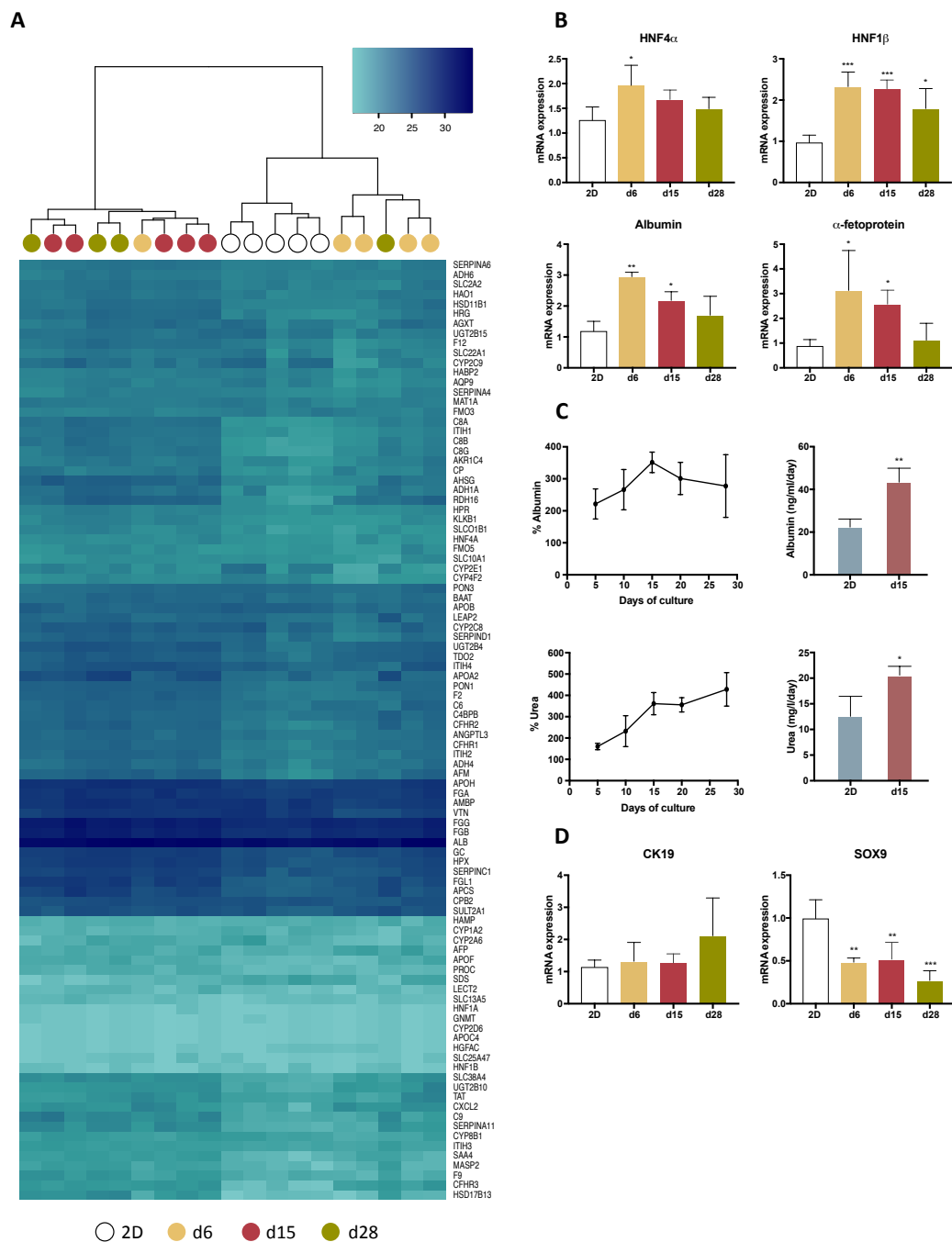


Figure 2 – Hepoid-HepaRG exhibit stable characteristics of differentiated hepatocytes

A. Heatmap representing level of major hepatic genes expression in HepaRG cells cultured in presence of DMSO (white circles, 2D) or in 3D during 6 (yellow), 15 (pink) and 28 (green) days. **B.** Quantification of the gene expression patterns by RT-qPCR of HNF4 α , HNF1 β , Albumin and α -fetoprotein at day 6, 15 and 28. The results are expressed compared to the 2D-DMSO (2D). **C.** Albumin (**upper panel**) and urea (**lower panel**) secretions in Hepoid-HepaRG (**left**) at different time points of culture and normalize to d2 and (**right**) at day 15 or in 2D-DMSO (2D) and normalize with the number of viable cells. **D.** mRNA expression by RT-qPCR of CK19 and SOX9 at day 6, 15 and 28. Results are normalized to the 2D control.

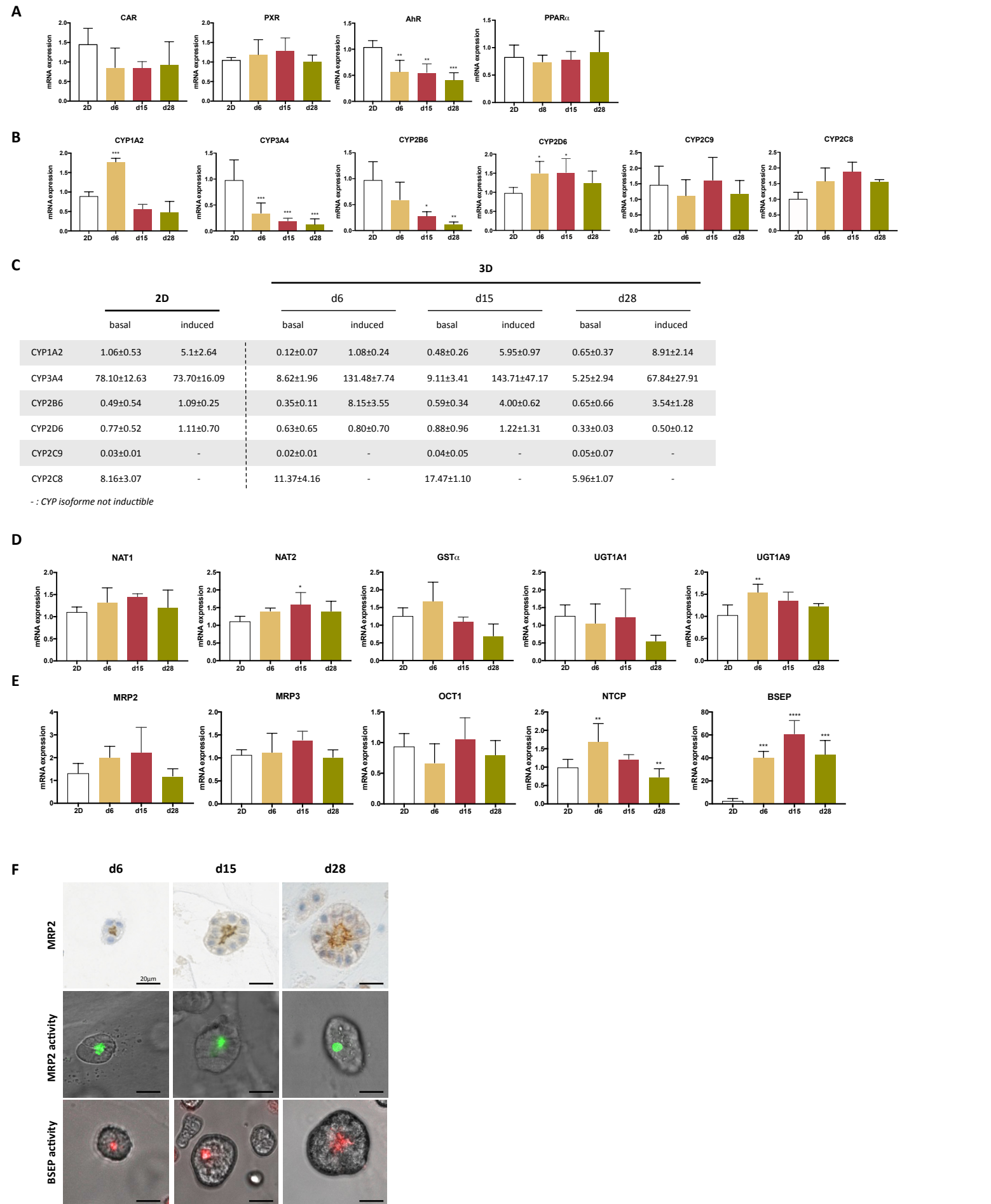
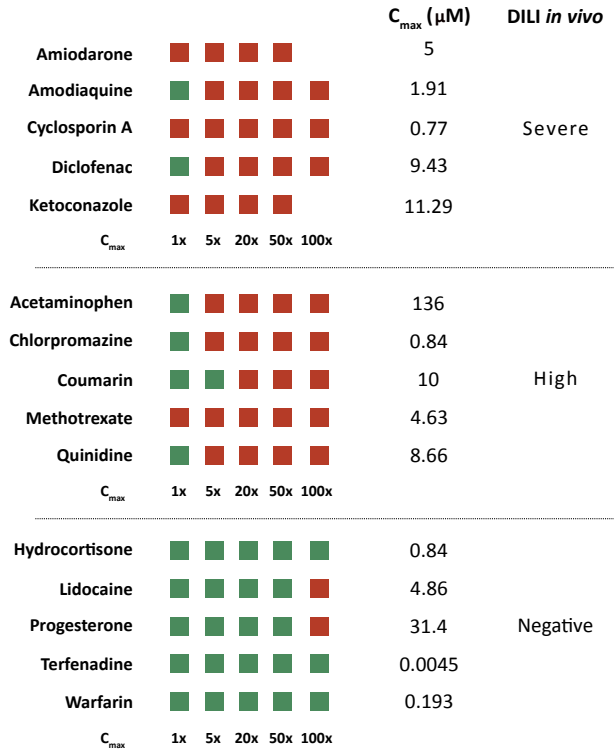


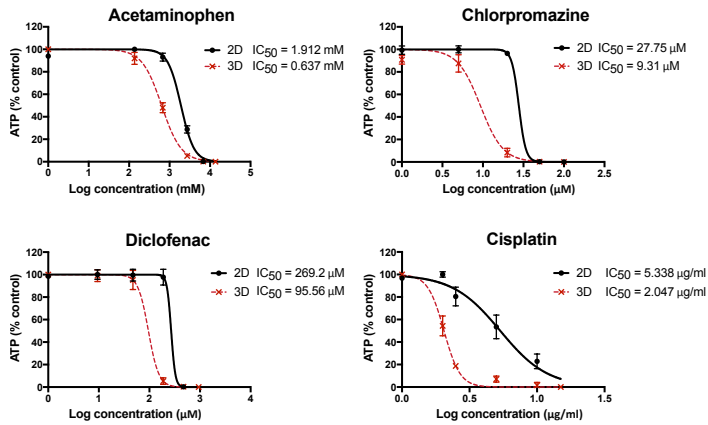
Figure 3 – Hepoid-HepaRG stably express functional xenobiotic metabolism enzymes

mRNA expression analysis by RT-qPCR of (A) transcription factors (CAR, PXR, AhR and PPAR α), (B) major CYPs isoforms involved in the liver detoxication of xenobiotics (CYP1A2, CYP3A4, CYP2B6, CYP2D6, CYP2C9 and CYP2C8), (D) phase II enzymes (NAT1, NAT2, GST α , UGT1A1 and UGT1A9) and (E) hepatic transporters (MRP2, MRP3, OCT1, NTCP and BSEP) in Hepoid-HepaRG at day 6, 15 and 28. The results are expressed compared to 2D-DMSO (2D). C. LC/MS–MS assays of the activities (pmol/min/RLU*10¹⁰) of major CYPs in Hepoid-HepaRG at day 6, 15 and 28 and in 2D-DMSO (2D) for comparison. CYP1A2 was induced by 3-methylcholantrene (5 μ m, 24 h), CYP3A4 and CYP2D6 were induced by rifampicin (50 μ M, 72 h) and CYP2B6 was induced by phenobarbital (3.2 mM, 72 h). F. Immunostaining of MRP2 in Hepoid-HepaRG (**upper panel**) and functional analysis of efflux transporters MRP2 (**middle panel**) and BSEP (**lower panel**) at days 6, 15 and 28 assessed by CLF and CDFDA secretion, respectively (All scale bars = 20 μ m).

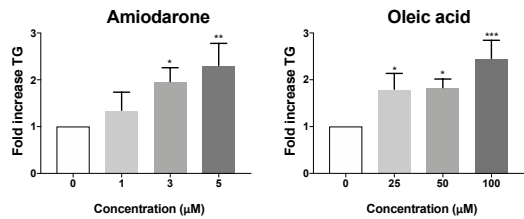
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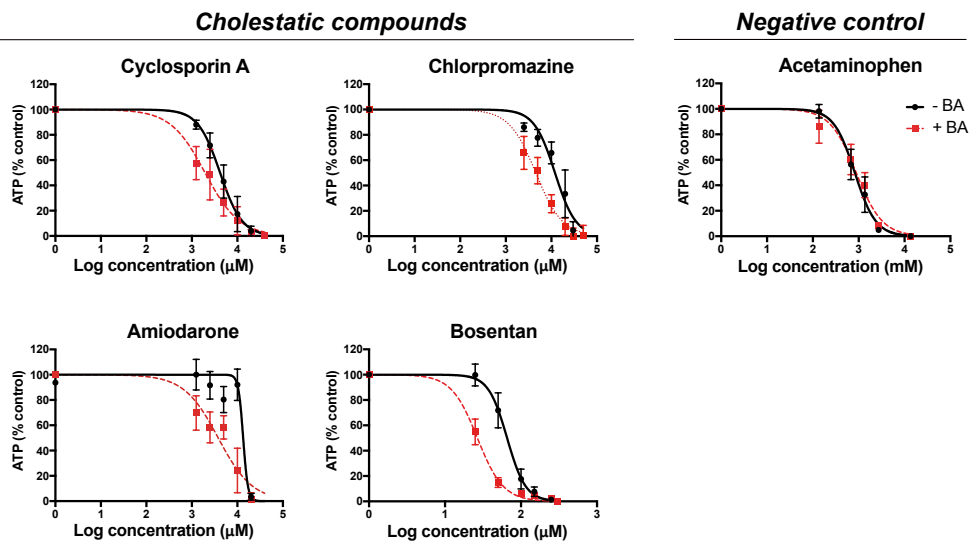
B



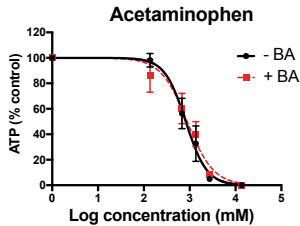
C



D



Negative control



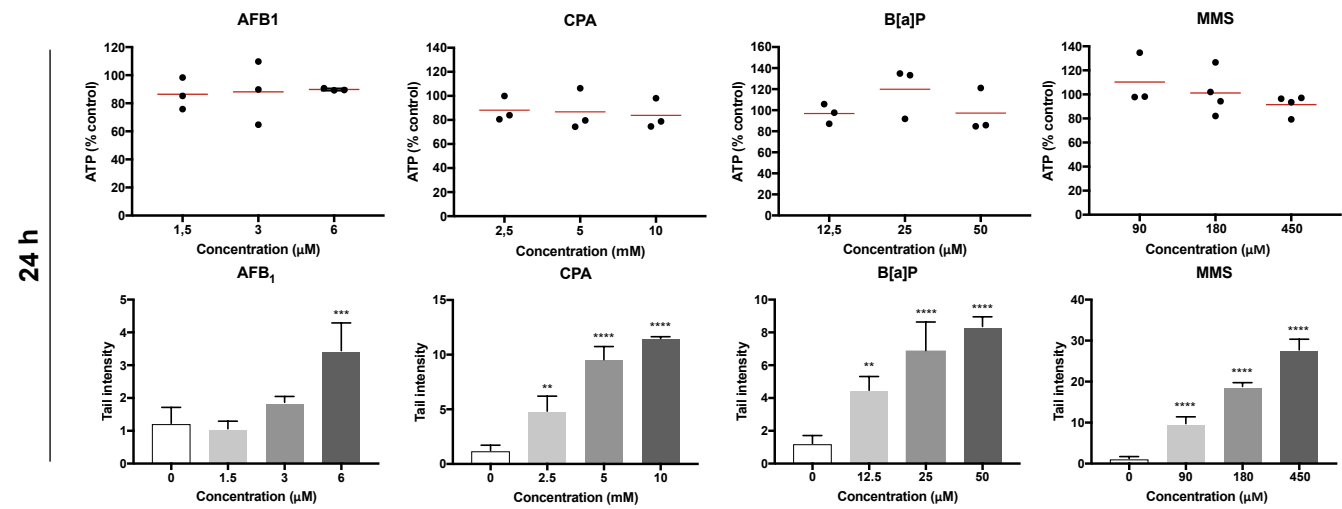
	Compound	EC ₅₀		Cholestatic Index
		- BA (μ M)	+ BA (μ M)	
Positive	Cyclosporin A	3.6	2.8	0.78
		7.9	4.5	0.57
		3.0	1.24	0.41
	Chlorpromazine	9.9	6.6	0.67
		19.8	3.5	0.18
		11	4.0	0.37
Negative	Amiodarone	12.3	0.8	0.07
		18.6	3.7	0.20
		12.0	9.1	0.76
	Bosentan	73.7	33.3	0.45
		46.3	23.9	0.52
		59.3	17.9	0.30
Negative	Acetaminophen	1128	1307	1.16
		1156	1065	0.92
		560	650	1.16

Figure 4 – 3D HepaRG culture allows the study of long-term DILI and alterations of liver functions

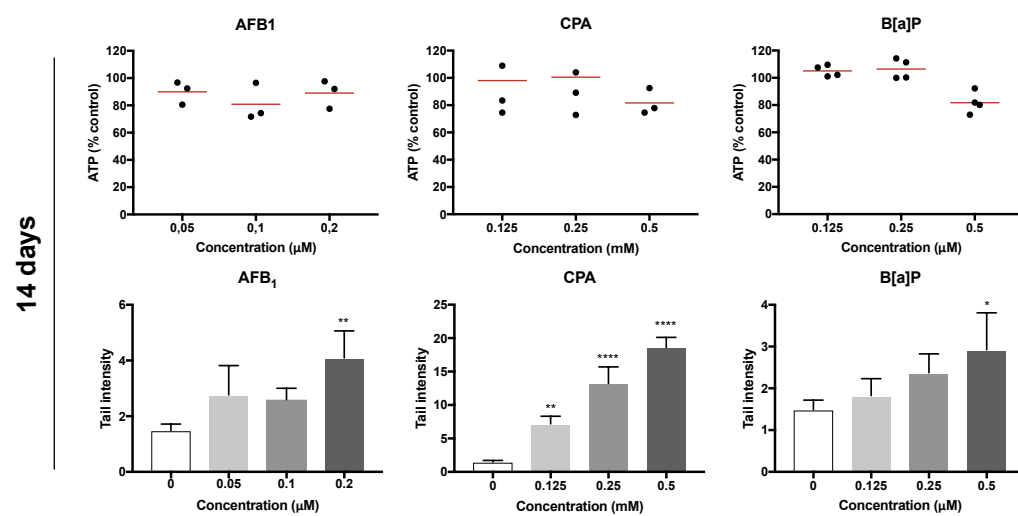
A. Hepoid-HepaRG were treated during 14 days with 1X to 100X of therapeutic exposure concentrations (C_{max}) of different compounds known to cause various degrees of hepatotoxicity. Red boxes indicated that the viability was reduced by at least 20% compared to the control with * $p < 0.05$ (t-test) whereas green boxes indicated low (< 20%) or no toxicity. Cellular ATP content was measured as marker of viability. Data of *in vivo* C_{max} and DILI classification are from ³⁶.

B. Hepatotoxicity of four compounds (acetaminophen, chlorpromazine, diclofenac, cisplatin) known to induce ROS formation in Hepoid-HepaRG compared to 2D-DMSO after 14-days of repeated exposure. **C.** Level of triglycerides accumulation after 14 days of treatment with the steatosis-inducing molecule amiodarone (Left) or oleic acid (Right). **D.** Hepoid-HepaRG were treated during 14 days with known cholestatic compounds (cyclosporine A, chlorpromazine, amiodarone, bosentan) or compound with non-cholestatic liability (acetaminophen) in the presence or absence of a non-toxic mixture of biliary acids (BA). (Left) Viability quantification. (Right) Cholestatic index values of the tested compounds determined in 3 independent experiments. Red: cholestatic index < 0.8, green: cholestatic index > 0.8. For toxicity-based experiments, viability was assessed by measuring the cellular ATP content on the last day of treatment.

A



B



C

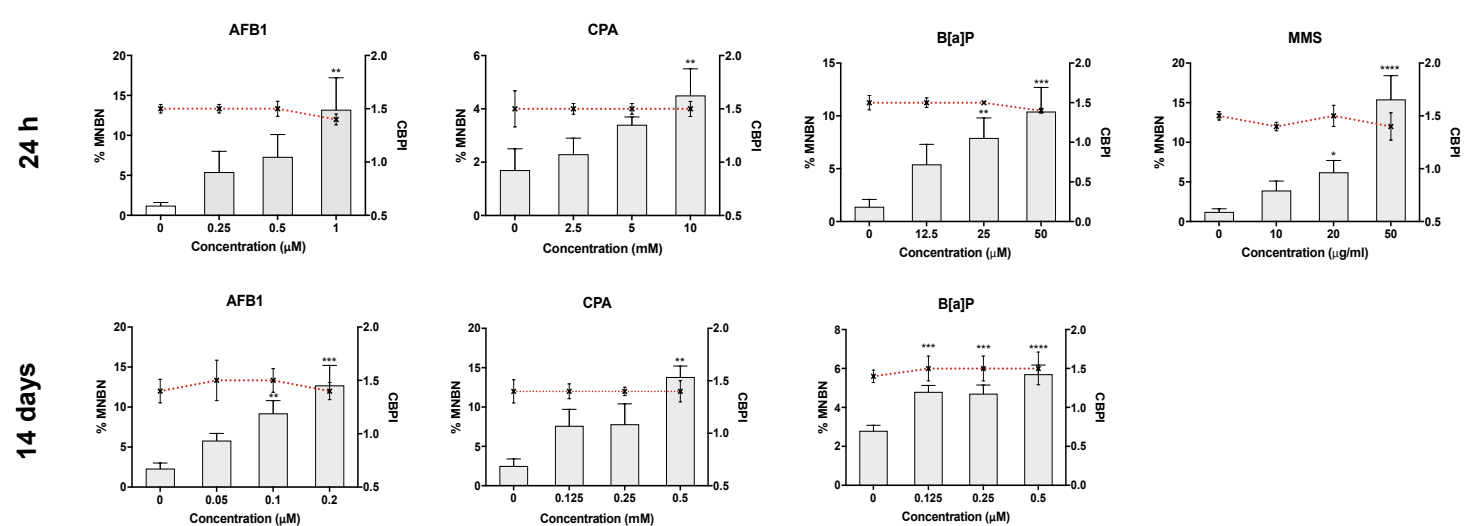
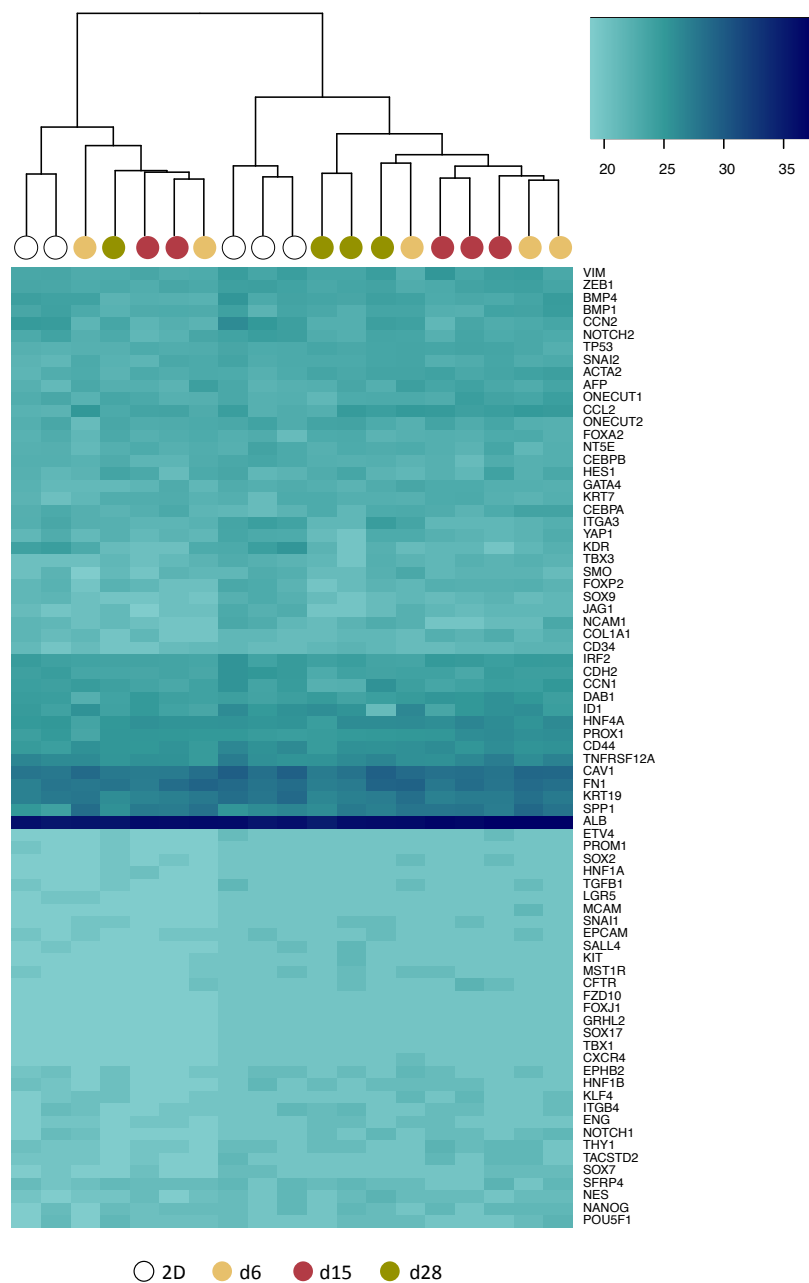


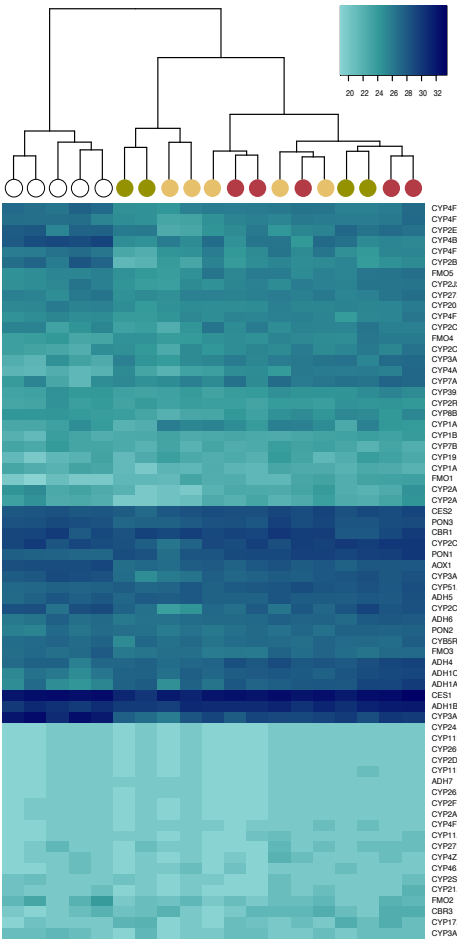
Figure 5 – Acute and chronic exposures to mutagens generate DNA damages and chromosomic aberrations in Hepoid-HepaRG

Hepoid-HepaRG were exposed to a panel of mutagens (AFB₁, CPA, B[a]P and MMS). **A. (Top panel)** Cytotoxicity assay after 24 h of treatment. The % of viability is expressed compared to treatment with vehicle (DMSO 0.1%, negative control). **(Bottom panel)** Level of DNA damage induction measured by comet assay after 24 h of treatment. DNA damage is expressed as the mean of median % tail DNA intensity **B. (Top panel)** Cytotoxicity assay after 14 days of treatment. The % of viability is expressed compared to vehicle. **(Bottom panel)** Level of DNA damage measured by comet assay after 14 days of repeated exposure. **C.** Frequency of MNBN cells (histogrammes) and cytotoxicity index CBPI (dotted line) in Hepoid-HepaRG exposed to mutagens for 24 h or 14 days.

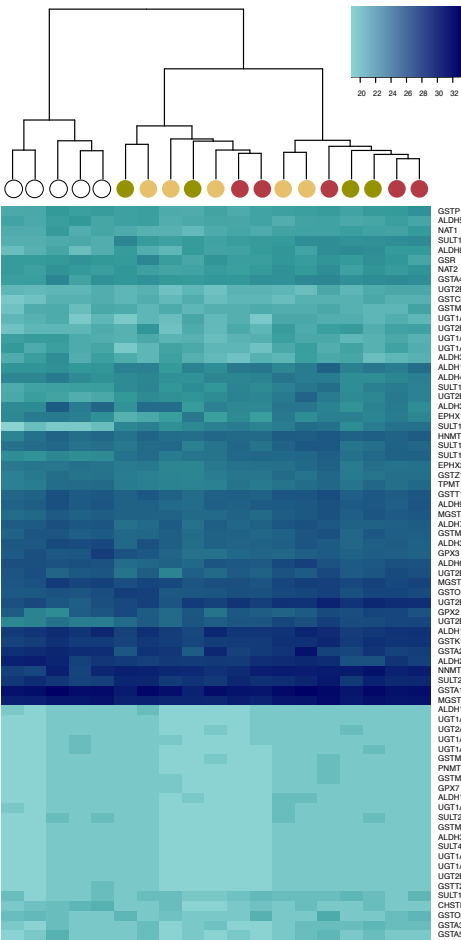


Supplemental Figure 1 : Heatmap representing levels of genes expressed in stem cells and mesenchymal cells in HepaRG cells cultured in 2D with DMSO (white circles) or in 3D during 6 (yellow), 15 (pink) and 28 (green) days. Albumin expression (ALB) : control of hepatic differentiation

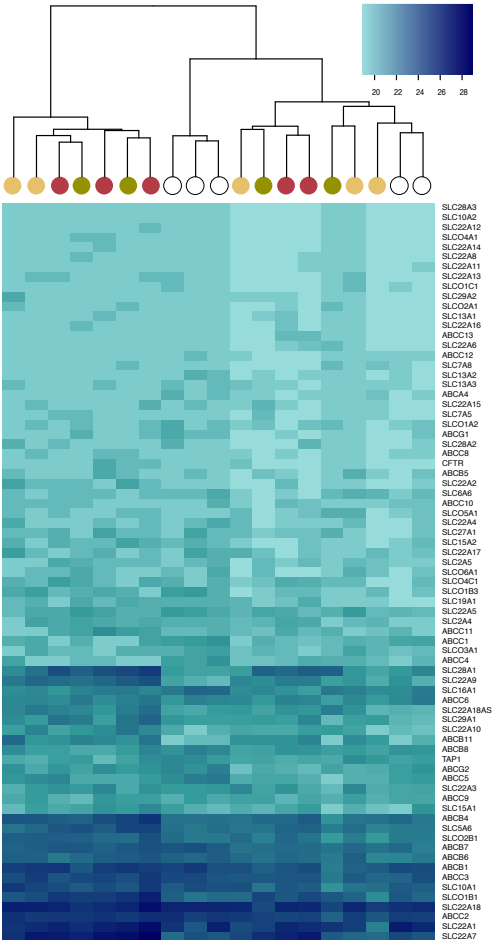
Phase I



Phase II

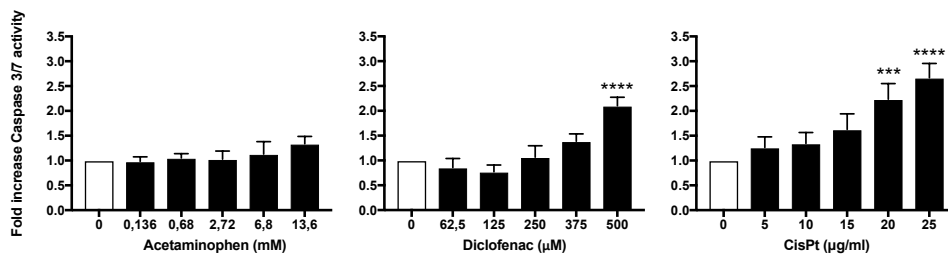


Transporters

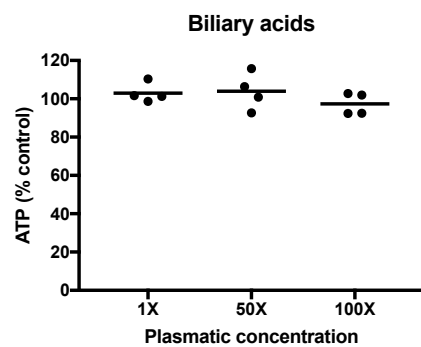


○ 2D ● d6 ● d15 ● d28

Supplemental Figure 2 : Heatmap representing levels of hepatic DMET genes expressed in HepaRG cells cultured in presence of DMSO (white circles) or in 3D during 6 (yellow), 15 (pink) and 28 (green) days.



Supplemental Figure 3 : Caspase 3/7 activity measured after a treatment of 48 h with acetaminophen or 24 h with cisplatin and diclofenac.



Supplemental Figure 4 : Hepoid-HepaRG viability measurement after a 14-day treatment with 1x, 50x or 100x the plasmatic concentration of a mixture of 6 biliary acids.