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GENOTOXICITY AND CARCINOGENICITY

Towards better prediction of xenobiotic genotoxicity: CometChip technology coupled with a 3D model of HepaRG human liver cells

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Abstract

Toxicology is facing a major change in the way toxicity testing is conducted by moving away from animal experimentation towards animal-free methods. To improve the in vitro genotoxicity assessment of chemical and physical compounds, there is an urgent need to accelerate the development of 3D cell models in high-throughput DNA damage detection platforms. Among the alternative methods, hepatic cell lines are a relevant in vitro model for studying the functions of the liver. 3D HepaRG spheroids show improved hepatocyte differentiation, longevity, and functionality compared with 2D HepaRG cultures and are therefore a relevant model for predicting in vivo responses. Recently, the comet assay was developed on 3D HepaRG cells. However, this approach is still low throughput and does not meet the challenge of evaluating the toxicity and risk to humans of tens of thousands of compounds. In this study, we evaluated the performance of the high-throughput in vitro CometChip assay on 2D and 3D HepaRG cells. HepaRG cells were exposed for 48 h to several compounds (methyl methanesulfonate, etoposide, benzo[a]pyrene, cyclophosphamide, 7,12-dimethylbenz[a]anthracene, 2-acetylaminofluorene, and acrylamide) known to have different genotoxic modes of action. The resulting dose responses were quantified using benchmark dose modelling. DNA damage was observed for all compounds except 2-AAF in 2D HepaRG cells and etoposide in 3D HepaRG cells. Results indicate that the platform is capable of reliably identifying genotoxicants in 3D HepaRG cells, and provide further insights regarding specific responses of 2D and 3D models.

Keywords Genotoxicity · Hepatocytes · HepaRG · Spheroid · CometChip

Introduction

For several years now, toxicology has been facing a major change in the way toxicity testing is conducted by moving away from animal experimentation towards animal-free methods (Krewski et al. 2020; Hartung and Tsatsakis 2021). These new approach methodologies include in vitro approaches, computational (in silico) approaches, and combinations thereof (Zaunbrecher et al. 2017). In addition to a decrease in animal testing due to scientific reasons (inaccurately predicting human health impacts), and ethical and economic concerns, there is also a need to increase efficiency in toxicity testing (Malloy et al. 2017). We face the problem of having to assess large and increasing numbers of chemicals, including agrochemicals, environmental toxicants, food additives, cosmetics, consumer care products, and nanomaterials. A recent analysis of global inventories of chemicals estimated that more than 350,000 chemicals and mixture are registered on the global market, which was a much larger number than expected (Wang et al. 2020). Therefore, non-animal high-throughput methods are now of great interest in toxicology.

Among the alternative methods, hepatic cell lines are relevant in vitro model for studying the functions of the liver. The liver is the most important organ involved in biotransformation and elimination of drugs and xenobiotics (Sevior et al. 2012). Therefore, developing in vitro assays based on human hepatic cells is one of the most critical challenges for assessing the toxicity of xenobiotics.

HepaRG cells, derived from human hepatocellular carcinoma, have been identified as a good alternative model to primary human hepatocytes (PHHs) (Guillouzo and Guguen-Guillouzo 2018). HepaRG cells express Phase I

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and Phase II enzymes, various transporters, and nuclear receptors at levels comparable to those found in PHHs, and without the disadvantages inherent to PHHs, which include limited availability, inter-individual variability, and early dedifferentiation to progenitor-like cells (Kanebratt and Andersson 2008; Hart et al. 2010; Anthérieu et al. 2010; Lübberstedt et al. 2011).

In recent years, 3D HepaRG models generated by several methods (hanging drops, ultra-low attachment plates, embedment in a matrix or bioprinting) have been reported (Gunness et al. 2013; Mueller et al. 2014; Ramaiahgari et al. 2017; Rose et al. 2021). Three-dimensional cultures are promising tools since they may reflect the in vivo environment (in particular cell–cell interactions), and are regarded as an effective model for toxicological studies. These models show improved hepatocyte differentiation, longevity, and functionality compared with 2D HepaRG cultures (Leite et al. 2012; Ramaiahgari et al. 2017; Ott et al. 2017), and are therefore a relevant model for predicting in vivo responses.

Genotoxicity testing is an important part of the safety assessment of xenobiotics since DNA damage increases the likelihood of mutations occurring and may initiate a carcinogenic process. One of the most versatile assays for genotoxicity testing is the Comet assay. The Comet assay is a sensitive method for detecting various types of DNA damage, including alkali-labile sites, abasic sites, and single and double-stranded breaks at a single-cell level. However, the Comet assay has some recognized shortcomings, for example, its labor intensity and relatively low throughput. Recently, a novel 96-well CometChip platform was developed (Wood et al. 2010; Ngo et al. 2020).

The CometChip is an array of 96 separate macrowells, each containing ~ 400 microwells, creating a pattern of non-overlapping cells to facilitate high-throughput analysis, with a high level of reproducibility, and the potential for generating a large number of data points covering a wide range of chemical concentrations.

Different studies have demonstrated that the Comet assay can be conducted using 2D HepaRG cells (Le Hégarat et al. 2010, 2014) and recently using 3D HepaRG cells (Mandon et al. 2019). However, to date, CometChip technology has only been developed on 2D HepaRG cells (Seo et al. 2019; Buick et al. 2021) and it remained to be determined whether 3D HepaRG cells could be adapted to the high-throughput 96-well format. The objective of our study was to evaluate the suitability of 3D HepaRG cells, a relevant in vitro model for predicting in vivo responses, and high-throughput CometChip technology for detecting genotoxic potential. Several compounds known to have different genotoxic modes of action, including direct and indirect genotoxic agents, were tested. The benchmark dose (BMD) approach was employed to determine the point of departure for genotoxicity data generated by the in vitro high-throughput Comet assay. The resulting quantitative genotoxicity responses were compared with similar data generated using 2D HepaRG cells.

Materials and methods

Chemicals

2-acetylaminofluorene (2-AAF), 7,12-dimethylbenz[a] anthracene (DMBA), acrylamide (AA), benzo[a]pyrene (B[a]P), cyclophosphamide (CPA), dimethyl sulfoxide (DMSO), etoposide, and methyl methanesulfonate (MMS) were purchased from Sigma (Saint-Quentin-Fallavier, France). The CometChips were obtained from Bio-techne (Rennes, France). Kits for performing the CellTiter-Glo® luminescent cell viability assays were obtained from Promega (Charbonnières-les-Bains, France).

HepaRG cell culture

The human hepatic cell line HepaRG (Biopredic International, Rennes, France) was used at passages 14–20. Cells were cultured in Williams E medium (Eurobio, Les Ulis, France), supplemented with 10% fetal calf serum (FCS) (Perbio, Brebières, France), 100 units/mL penicillin (Invitrogen Corporation, Illkirch, France), 100 µg/mL streptomycin (Invitrogen Corporation), 5 µg/mL insulin (Sigma-Aldrich, Lyon, France), 2 mM L-glutamine (Thermofisher, Waltham, MA, USA), and 25 µg/mL hydrocortisone succinate (Pharmacia & Upjohn, Guyancourt, France) at 37 °C in an atmosphere containing 5% CO_2. 1×10^6 HepaRG cells were seeded in a 75 cm^2 flask and were incubated at 37 °C with 5% CO_2, and the medium was changed every 2 days.

2D differentiated HepaRG culture

For experimentation, after 14 days of culture in a 75 cm^2 flask, cells were seeded at 2.5×10^4 cells/cm^2 in 96-well plates. For differentiation, cells were incubated for two weeks before the addition of 1.7% DMSO to the culture medium for two more weeks. In all cases, the medium was changed three times a week.

3D HepaRG spheroid formation

Cell culture and spheroid formation were performed as previously described (Mandon et al. 2019). After 14 days of culture in a 75 cm^2 flask, cells were trypsinized prior to dissociation with a syringe to obtain a suspension of isolated cells. Cells were then seeded in 96-well ultra-low attachment plates (Corning SAS, Boulogne-Billancourt, France) at a

density of 2000 cells/well. Medium was changed after 7 days and spheroids were used on day 8.

Cell treatments

All chemicals were dissolved in DMSO, except for MMS, which was dissolved in the FCS-free medium. The stock solution of each chemical was stored at − 20 °C. The solutions of MMS were prepared freshly before each experiment. Working solutions were freshly prepared by serial dilution in the FCS-free medium, with a final concentration of 0.5% DMSO in the treatment incubation. 2D and 3D HepaRG cells were exposed to various concentrations of the test chemical in a total volume of 100 µL for 48 h (24 h for MMS) at 37 °C in a humidified atmosphere with 5% CO_2. The cytotoxicity and CometChip assays were performed following the treatment. The experiments were repeated independently at least three times for each chemical.

Cytotoxicity assay

Cell viability was measured using a CellTiter-Glo® luminescent cell viability assay kit for 2D HepaRG and a CellTiter Glo® 3D kit for 3D HepaRG, according to the manufacturer's instructions. ATP luminescence was measured using a FLUOstar® Optima Microplate reader (BMG Labtech, Champigny-sur-Marne, France). The relative viability (%) was calculated by comparing the intensity levels of the treated cells to those of the vehicle controls.

CometChip assay

The CometChip assay was performed under alkaline conditions, according to the manufacturer's instructions (Trevigen). After treatment, 2D HepaRG cells were washed with phosphate-buffered saline (PBS) and then trypsinized and resuspended in a medium. After treatment, 3D HepaRG medium was removed and spheroids were washed twice with PBS. For each condition, 4 spheroids (8000 cells) were pooled in one well of a 96-well plate. After 5 min of sedimentation, PBS was removed and 100 µL of TrypLE™ without red phenol (Gibco, Courtaboeuf, France) at 37 °C was added. After 40 min of incubation at 37 °C, the dissociation of spheroids was checked under a light microscope. After dissociation, 100 µL of 2D (around 20,000 cells) and 3D (around 8000 cells) HepaRG cells were transferred into each well of a 96-well CometChip, with each well containing approximately 400 microwells. The cells were gravity loaded into the 30-micron sized microwells for 40 min at 37 °C in a humidified atmosphere with 5% CO_2. Following cell loading, the CometChip was gently rinsed with 1 × PBS and sealed with 1% low melting point agarose in PBS. The CometChip was then treated with lysis solution (NaCl 2.5 M, EDTA 0.1 M, Tris–HCl 10 mM, with extemporaneous addition of DMSO 10% and Triton X-100 1% at pH 10) for 1 h at 4 °C, and then submerged into a chilled alkaline buffer (300 mM NaOH, 1 mM EDTA, 0.1% Triton X-100) for 40 min in the dark to unwind the DNA. Electrophoresis was performed at 30 V for 50 min (1 V/cm) at 4 °C in the same solution. After neutralization with 0.4 M Tris–HCl buffer (pH 7.4) and equilibration with 0.02 M Tris–HCl buffer (pH 7.4), the CometChip was stained overnight at 4 °C with 0.2 × SYBR® Gold (Invitrogen) diluted in 0.02 M Tris–HCl buffer (pH 7.4) and then de-stained in 0.02 M Tris–HCl buffer (pH 7.4) for 1 h. Comet images were acquired using a fluorescence microscope (Leica DMR) equipped with a CCD-200E video camera. At least 100 comets per well were analyzed using Comet Assay IV software (Perceptive Instruments, Haverhill, UK). The percentage of DNA in the comet tail (% tail DNA) was used to evaluate the extent of DNA damage.

Benchmark dose analysis

The Comet dose–response data were analyzed by benchmark dose (BMD) analysis using PROAST software running in R (version 70.3, developed by The Dutch National Institute for Public Health and the Environment, RIVM), following the technical guidance (Hardy et al. 2017). The software fits data to dose–response curves using two nested models, the exponential model and the Hill model. The best choice between the two model families was made using the Akaike Index Criterion (AIC) as proposed by European Food Safety Authority (EFSA) Guidance. BMD5, BMD10, and BMD20 were calculated based on 5%, 10% and 20% increases in the response above the vehicle control, respectively. BMDL and BMDU values, the lower and upper bounds of the 95% confidence interval (CI) of the BMD, were also calculated for 5%, 10% and 20% changes in the response above the vehicle control.

Statistics

Statistical tests were conducted using R software (R, Version 4.1.1, Vienna, Austria). Normality and variance homogeneity were evaluated using Shapiro's test and Levene's test, respectively. When necessary, raw data were mathematically transformed (Log) to achieve normality and variance homogeneity before proceeding with an ANOVA. When significant, a posteriori Tukey test was performed. Significance was established as $p < 0.05$.

Results

Cytotoxicity assay

Cell viability determined by CellTiter-Glo® luminescent cell viability assay remained > 70% after 48 h of treatment for

all compounds in 2D HepaRG cells (Fig. 1). In 3D HepaRG cells, cytotoxicity was observed for B[a]P (10 and 20 µM, 63.60% ± 5.44 and 43.59% ± 4.10, respectively) and acrylamide (2000 µM, 61.95% ± 4.18) (Fig. 2).

DNA damage induced by the 7 compounds tested in 2D and 3D HepaRG cells

The level of DNA damage detected with solvent controls (DMSO) was 3.45% ± 0.42 tail DNA in 2D HepaRG cells and 2.62% ± 0.31 in 3D HepaRG cells (Figs. 1, 2). After 24 h exposure, MMS increased the percentage of tail DNA in a concentration-dependent manner in 2D and 3D HepaRG cells with a statistically significant difference at 3.125 µM (8.42% ± 2.48) and 12.5 µM (14.47% ± 1.84). Etoposide induced increases in DNA damage in 2D HepaRG cells at 5 µM; however, no increase in % tail DNA was observed in spheroids after 48 h of treatment up to 20 µM. For the five compounds requiring metabolic activation, positive responses were observed for four test compounds in 2D HepaRG cells and for all test compounds in 3D HepaRG cells. No DNA damage was observed for 2-AAF in 2D HepaRG cells, whereas an increase in % tail DNA was observed at 12.5 µM in spheroids. The pro-genotoxicants B[a]P, CPA, DMBA and AA significantly induced DNA damage in 2D and 3D HepaRG cells at 10, 250, 20, and 1000 µM, and at 5, 250, 5, and 250 µM, respectively. Overall, heterogeneity in response between individual cells was very similar between 3 and 2D HepaRG cells. However, a slightly higher heterogeneity was observed for MMS and acrylamide in 3D HepaRG cells compared to 2D HepaRG cells without bringing an explanation for now.

Benchmark dose analysis

The Hill model families provided the best fit to calculate BMDs for all chemicals in 2D and 3D HepaRG cells, except for acrylamide in 3D HepaRG cells where the exponential model 5 was used to calculate BMDs (Table 1a). B[a]P produced comparable values in 2D and 3D HepaRG cells. MMS and CPA produced higher BMD values in 3D HepaRG cells compared to 2D HepaRG cells, whereas DMBA and acrylamide produced lower BMD values in 3D HepaRG cells (Table 1a, Fig. 3).

Lowest observed effects concentrations

LOECs in 2D and 3D HepaRG cells have been reported in Table 1b and compared to previous studies where LOECs were obtained through Comet assay or CometChip technology. Overall, lower LOECs values were observed in 3D HepaRG cells compared to 2D HepaRG cells (except for MMS and CPA) and in CometChip assay compared to Comet assay.

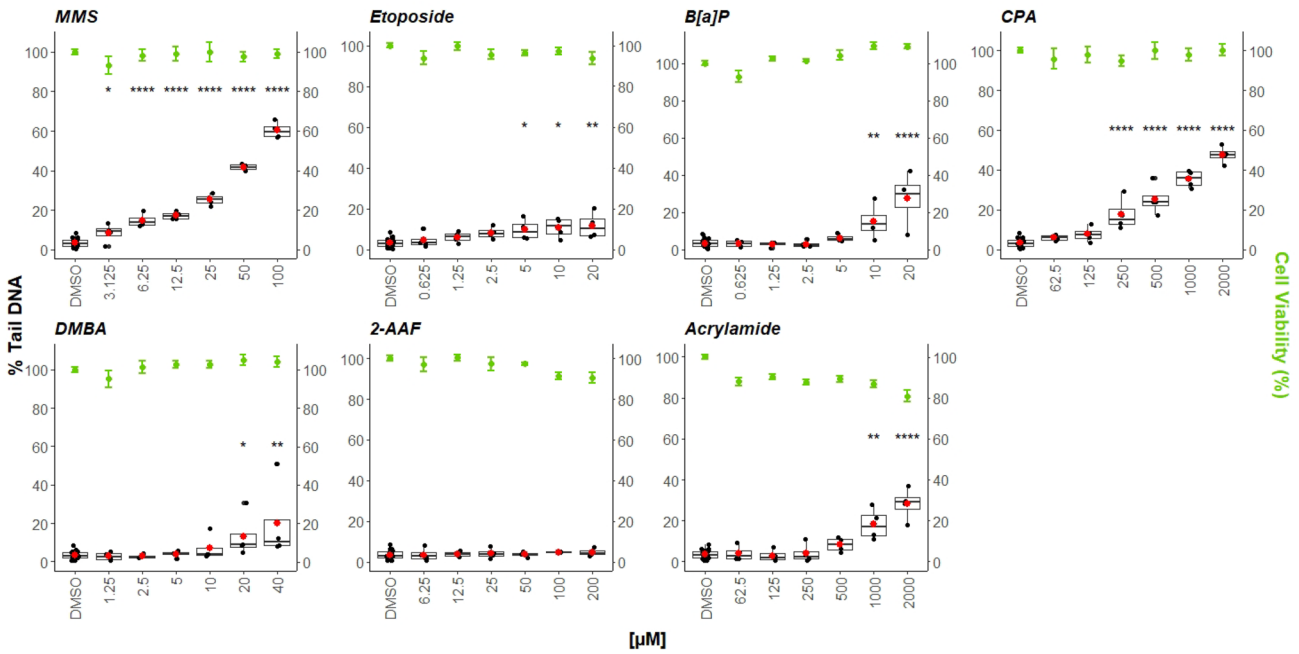

Fig. 1 DNA damage and cytotoxicity induced by genotoxicants in 2D HepaRG. Boxplot and black dots: % of tail DNA intensity (median value obtained in each experiment); red dot: mean of medians of tail intensity. Green error bar: percentage of cell viability (ratio compared to negative control). Results were calculated from at least 3 independent experiments. *p (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) was determined by one-way ANOVA (Tukey's test) (color figure online)

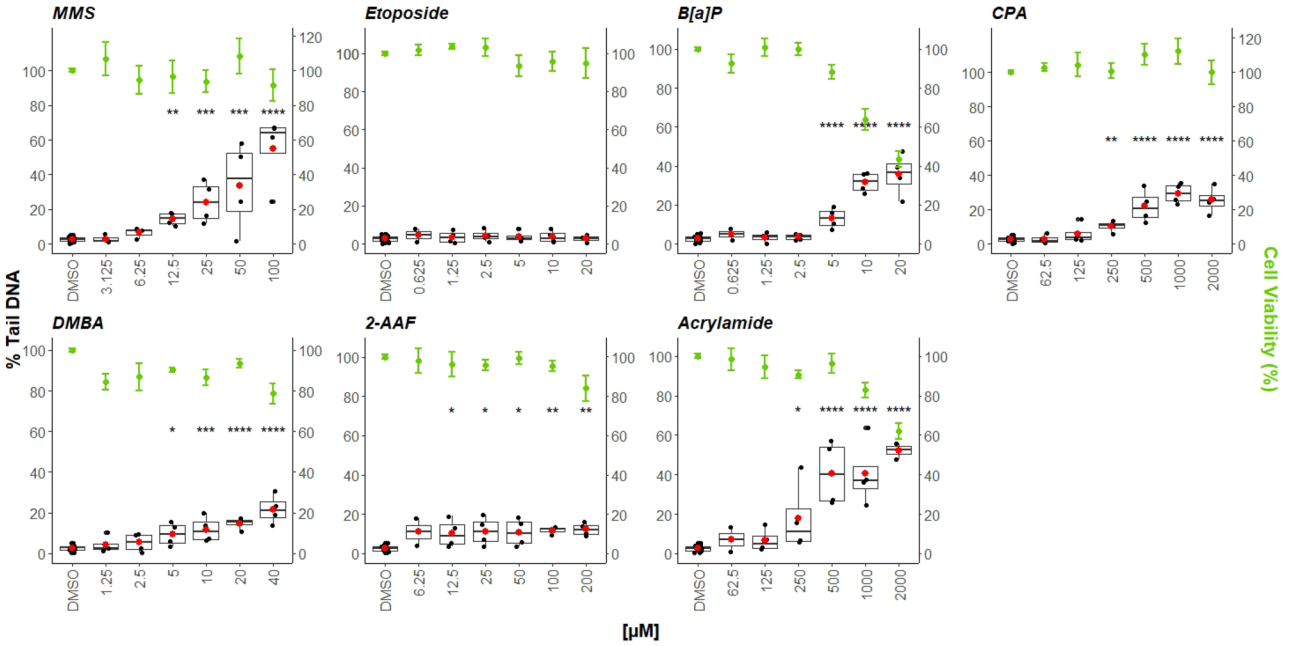

Fig. 2 DNA damage and cytotoxicity induced by genotoxicants in 3D HepaRG. Boxplot and black dots: % of tail DNA intensity (median value obtained in each experiment); red dot: mean of medians of tail intensity. Green error bar: percentage of cell viability (ratio compared to negative control). Results were calculated from at least 3 independent experiments. *p (*$p<0.05$, **$p<0.01$, and ***$p<0.001$) was determined by one-way ANOVA (Tukey's test) (color figure online)

Discussion

Currently, there is an urgent need for in vitro hepatic models that can predict genotoxic effects in humans more accurately and rapidly. The main objective of this study was to bring new insights into the development of high-throughput genotoxicity assessment in 3D liver cells.

We evaluated chemical-induced cytotoxicity in both 2D and 3D cell models, and a greater cytotoxic effect for B[a]P and acrylamide was observed in 3D HepaRG compared to 2D HepaRG cells. As 3D HepaRG cells possess higher levels of CYP activity (Gunness et al. 2013; Ott et al. 2017), it was anticipated that they would be more sensitive than 2D HepaRG cells to compounds that require metabolic activation. The cytotoxic effect of B[a]P and acrylamide may be due to their enzymatic conversion to cytotoxic metabolites. Benzo[a]pyrene is initially metabolized by cytochrome P450 (CYP) family of enzymes (CYP1A1/2, CYP1B1, and/or CYP3A4). CYP1A1 is one of the most important CYP enzymes in B[a]P bioactivation to species forming DNA adducts, leading to the ultimately reactive species, BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) (Baird et al. 2005). Acrylamide is an important monomer formed in fried and oven-cooked human foods, metabolized by CYP2E1 into a reactive genotoxic compound, glycidamide (Sumner et al. 1999; Mottram et al. 2002). These results are consistent with the improved liver-like CYP1A/CYP3A4/CYP1B1 (for B[a]P) and CYP2E1 (for acrylamide) metabolism observed with 3D HepaRG spheroids compared to 2D HepaRG cells. Ott et al. (2017) showed that CYP1A and CYP3A4 activities for 3D HepaRG cells were 142%, 872%, 54% and 38%, 649%, 1502% greater than for 2D cultures, at 24 h, 3 days, and 7 days, respectively. Gunness et al. (2013) observed that CYP2E1 enzyme activity was consistently higher (approximately sevenfold) in the 3D versus the 2D cultures and was observed during 3 weeks of cultivation.

As expected, in our study, 2D and 3D HepaRG cells were able to metabolize the different pro-genotoxicants into genotoxic metabolites, resulting in positive results in the comet assay, except for 2-AAF in 2D cells. This result was already observed in a previous study. Le Hégarat et al. (2014) also showed a negative response with the comet assay; however, they also observed a weak but significant genotoxic response with 2-AAF with the micronucleus test. This result confirmed the presence of CYP1A2 activity in HepaRG cells which bioactivates 2-AAF to N-Hydroxy-2-AAF intermediate that can bind directly to DNA (Rendic and Guengerich 2012). The positive response observed with 3D HepaRG cells could be explained by the higher level of N-acetyltransferase and CYP1A2 in 3D HepaRG implicated in the bioactivation, as also suggested (Mandon et al. 2019) and discussed previously (Ott et al. 2017).

The topoisomerase inhibitor, etoposide, failed to induce genotoxicity in 3D HepaRG cells; however, DNA damage

Table 1 Benchmark dose (BMD) (with its lower and upper confidence intervals) (a), and lowest observed effects concentrations (LOECs) (b) in 2D and 3D HepaRG cells

(a)	BMD_5 ($BMDL_5$–$BMDU_5$)		BMD_{10} ($BMDL_{10}$–$BMDU_{10}$)		BMD_{20} ($BMDL_{20}$–$BMDU_{20}$)		Model	
	2D HepaRG	3D HepaRG	2D HepaRG	3D HepaRG	2D HepaRG	3D HepaRG	2D HepaRG	3D HepaRG
MMS	7.05×10^{-5} (0–2.08×10^{-3})	0.96 (9.53×10^{-4}–3.48)	6.57×10^{-4} (6.85×10^{-6}–0.011)	1.41 (5.35×10^{-3}–4.28)	5.72×10^{-3} (1.44×10^{-4}–0.058)	2.05 (0.030–5.25)	Hill m3	Hill m5
	4.87[a]		8.491[a]					
Etoposide	7.71×10^{-5} (0–0.02)	–	8.75×10^{-4} (0–0.075)	–	9.25×10^{-3} (2.48×10^{-6}–0.28)	–	Hill m3	–
BaP	1.61 (0.22–2.99)	1.27 (0.23–1.69)	2.10 (0.45–3.68)	1.56 (0.39–2.07)	2.72 (0.90–4.52)	1.91 (0.63–2.54)	Hill m5	Hill m5
	0.005[a]		0.066[a]					
CPA	4.62 (0.029–25.70)	39.1 (9.01–80.1)	8.76 (0.15–36.20)	51.79 (15.30–98.70)	16.49 (0.75–51.1)	68.49 (26–121)	Hill m5	Hill m5
	0.25[a]		3.31[a]					
DMBA	0.41 (0.018–2.98)	2.58×10^{-3} (4.48×10^{-6}–0.076)	0.94 (0.076–4.83)	1.30×10^{-2} (7.28×10^{-5}–0.216)	2.12 (0.30–7.7)	6.27×10^{-2} (0.0010–0.59)	Hill m3	Hill m3
	1.89[a]		3.76[a]					
2-AAF	–	1.00×10^{-6} (0–6.14×10^{-4})	–	1.00×10^{-6} (0–0.0024)	–	3.96×10^{-6} (0–0.018)	–	Hill m3
Acrylamide	174.8 (46.2–256)	16.03 (0.85–77.40)	215.1 (74.6–314)	25.3 (2.22–94.5)	264.4 (119–386)	39.53 (5.59–115)	Hill m5	Expon m5

	CometChip				Comet assay			
	2D HepaRG		3D HepaRG		2D HepaRG		3D HepaRG	
MMS	+	3.125[c]	+	12.5[c]	+	–[d]	+	9[g]
		125[a]						
Etoposide	+	5[c]	–	20[c]	–	10[e]	–	2[g]
BaP	+	10[c]	+	5[c]	+	10[f]	+	20[g]
		20[a]				50[d]		
CPA	+	250[c]	+	250[c]	+	200[d]	+	1000[g]
		1600[a]						
DMBA	+	20[c]	+	5[c]	+	125[d]	+	20[g]
		25[a]						
2-AAF	–	200[c]	+	12.5[c]	–	500[d]	+	50[g]
Acrylamide	+	1000[c]	+	250[c]	+	5000[d]	+	500[g]

[a]Seo et al. (2019)

[b]In case of negative results, the LOEC is the highest concentration tested

[c]This study

[d]LeHégarat et al. (2010)

[e]LeHégarat et al. (2014)

[f]Waldherr et al. (2018)

[g]Mandon et al. (2019)

was observed in 2D HepaRG cells from 5 µM. Similar results were observed in 3D HepaRG cells (Mandon et al. 2019). Genotoxicity of etoposide on 2D HepaRG cells was also observed using different toxicogenomic approaches (Ates et al. 2018; Buick et al. 2020). Similarly, in a previous study, etoposide failed to induce DNA damage in 2D HepaRG cells at concentrations up to 10 µM (Le Hégarat et al. 2014). The difference between the two studies could be explained by the longer exposure time used in our study (48 h instead of 24 h), that could affect the cell cycle of HepaRG cells. A previous study reported that the negative response observed in 3D spheroids could be due to the inactivity of topoisomerase II in these cells, corresponding to the quiescent state of differentiated cells (Le Hégarat et al. 2014; Mandon et al. 2019). Negative response observed in 3D HepaRG cells could also be due to higher CYP3A4

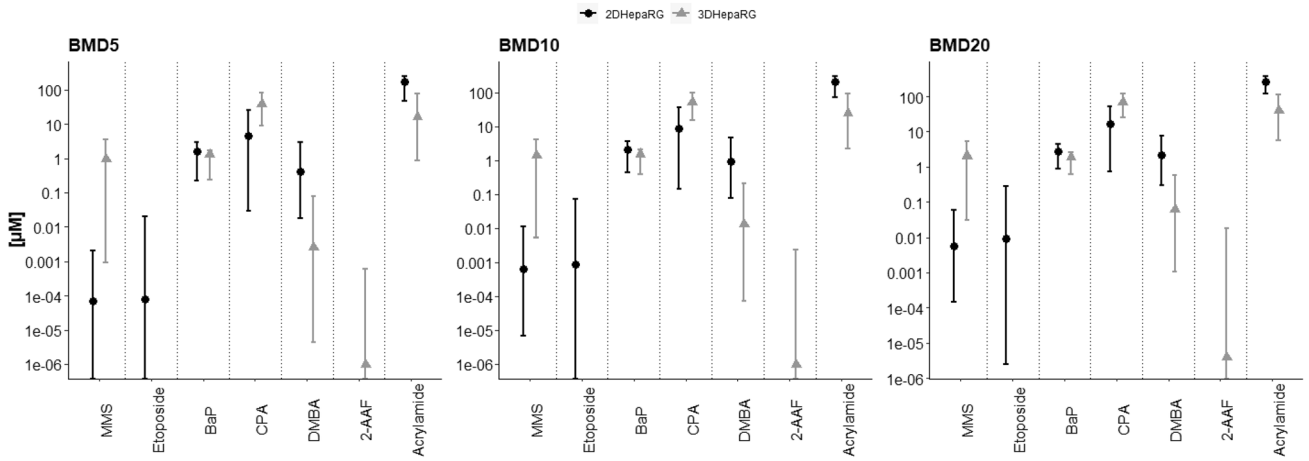

Fig. 3 Comparison of BMD5, BMD10 and BMD20 values and their confidence intervals for chemical-induced DNA damage in 2D and 3D HepaRG cells

activity in this model, in addition to high expression of drug transporters, leading to increased transport out of the cells. High drug transporter activity was shown for 3D HepaRG cells (Leite et al. 2012; Gunness et al. 2013; Ramaiahgari et al. 2017). The differences in the two cell models (2D vs. 3D) could also be due to greater synthesis of the extracellular matrix in the 3D model, which acted as a barrier to drug diffusion, particularly for large compounds (Horning et al. 2008; Mueller et al. 2014). The absence of toxicity of etoposide in 3D HepaRG could be due to its high molecular weight, making diffusion through the extracellular matrix impossible and, therefore, resulting in decreased uptake and penetration into inner layers of the spheroids. It should also be noted that through its mode of action, etoposide creates double strand breaks and the alkaline comet assay is less specific compared to the neutral comet assay to detect this kind of DNA damage (Chao and Engelward 2020).

The LOECs and BMDs for the pro-genotoxicant compounds were generally lower in 3D HepaRG compared to 2D HepaRG cells, which could be explained by the higher metabolic capacity of 3D cells, generating higher concentrations of reactive metabolites. However, conclusions based on LOECs and BMDs in this study should be interpreted with caution because the total number of cells exposed in each model was different. In the 3D HepaRG model, one spheroid of 2000 cells was exposed to 100 µL of the chemical dilutions in 96-well plates, whereas 2D HepaRG cells were exposed in 96-well plates at 70,000 cells/well to 100 µL of the chemical dilutions. The amount of chemicals per cell was 35 times higher in 3D conditions than in 2D. The LOECs and BMDs in our study were also generally lower than in other studies (Table 1a, b). The time of exposure, which was longer in our study (48 h instead of 24 h), could lead to higher toxicity of the tested chemicals. Finally, the difference in LOECs obtained between CometChip assay and Comet assay is certainly due to different protocol parameters (agarose density, electrophoresis time and conditions…).

These genotoxicity data generated from 3D HepaRG cells are valuable for human risk assessment and may serve as a model system in support of developing other human hepatocyte-based in vitro systems for genotoxicity testing. One limitation of 3D HepaRG cells is their inability to proliferate in vitro, and they may therefore not be appropriate for assays requiring cell division to produce positive responses (such as the micronucleus test). The human epidermal growth factor (hEGF) could be used to perform mitogenic stimulation in 3D spheroid as already used in 2D HepaRG cells (Josse et al. 2012). Moreover, it was recently reported that activation of Wnt signaling is sufficient to drive the proliferation of primary human hepatocytes cultured in ultra-low attachment plates (Oliva-Vilarnau et al. 2020).

In conclusion, our results indicate that the platform is capable of reliably identifying genotoxicants in 3D HepaRG cells, and provide further insights regarding specific responses of 2D and 3D models. Ultimately, this platform may become a powerful tool for compound screening concerning hepatocellular responses, while reducing the cost and time of evaluating the toxicity of xenobiotics, to develop a solid scientific basis to accelerate the risk assessment of chemicals.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethic approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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