Research Article

Evaluation of an In Vitro Three-Dimensional HepaRG Spheroid Model for Genotoxicity Testing Using the High-Throughput CometChip Platform

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Abstract

Three-dimensional (3D) culture systems are increasingly being used for genotoxicity studies due to improved cell-to-cell interactions and tissue-like structures that are limited or lacking in 2D cultures. The present study optimized a 3D culture system using metabolically competent HepaRG cells for in vitro genotoxicity testing. 3D HepaRG spheroids, formed in 96- or 384-well ultra-low attachment plates, were exposed to various concentrations of 34 test articles, including 8 direct-acting and 11 indirect-acting genotoxicants/carcinogens as well as 15 compounds that show different genotoxic responses in vitro and in vivo. DNA damage was evaluated using the high-throughput CometChip assay with concurrent cytotoxicity assessment by the ATP assay in both 2D and 3D cultures. 3D HepaRG spheroids maintained a stable phenotype for up to 30 days with higher levels of albumin secretion, cytochrome P450 gene expression, and enzyme activities compared to 2D cultures. 3D spheroids also demonstrated a higher sensitivity than 2D cultures for detecting both direct- and indirect-acting genotoxicants/carcinogens, indicating a better prediction of in vivo genotoxicity responses. When DNA damage dose-response data were quantified using PROAST software, 3D spheroids generally had lower or similar benchmark dose values compared to 2D HepaRG cells and were more comparable with primary human hepatocytes. These results demonstrate that 3D models can be adapted to the CometChip technology for high-throughput genotoxicity testing and that 3D HepaRG spheroids may be used as a reliable and pragmatic in vitro approach to better support the hazard identification and risk assessment of potential human genotoxic carcinogens.

1 Introduction

Improving current in vitro genotoxicity tests to closely mimic human responses is an ongoing task to improve hazard identification and risk assessment (Pfuhler et al., 2011). To increase the ability of in vitro mammalian cell genotoxicity tests to reliably predict genotoxicity in humans, recommendations have been made to use human-derived, tumor protein p53-proficient, and metabolically competent cells within the context of appropriately set limits of concentration and cytotoxicity (Kirkland, 2011; Kirkland et al., 2007).

Recently, the human hepatoma-derived HepaRG cell line, which expresses various levels of phase I and phase II enzymes, transporters, and nuclear receptors, has been cited as a promising cell model to be used as a surrogate for primary human hepatocytes (PHHs) for in vitro genotoxicity assessments (Guillouzo et al., 2007; Pfuhler et al., 2011). Our previous studies demonstrated that when cultured in 2D format, metabolically competent HepaRG cells expressed significantly higher levels of cytochrome P450 (CYP450) enzyme activities and showed higher sensitivity in detecting DNA damage and the micronuclei (MN) formation induced by a number of genotoxicants or carcinogens.
when compared to HepG2 cells despite both cell lines being derived from human hepatoma (Guo et al., 2020; Seo et al., 2019). 2D HepaRG cells, however, were not as sensitive as PHHs in detecting DNA damage responses induced by 10 genotoxicants or carcinogens (Seo et al., 2020).

Three-dimensional (3D) cell culture systems have been increasingly recognized as better than traditional 2D monolayer culture systems for mimicking human in vivo exposures due to their improved cell-to-cell interactions and tissue-like structures (Edmondson et al., 2014; Lauschke et al., 2016). It has been demonstrated that the environment of 3D culture is morphologically, physiologically, and functionally distinct from 2D culture (Baharvand et al., 2006). The International Workshop on Genotoxicity Testing (IWGT) agreed that 3D tissue-based models provide more in vivo-like responses in terms of cell viability, proliferation, differentiation, morphology, gene and protein expression, and function compared to standard 2D static cell culture systems (Pfüehler et al., 2020). Once validated, 3D models could be used as 2nd tier assays to follow-up endpoint-specific positives from a standard in vitro genotoxicity testing battery. The workshop also suggested that 3D liver model-based genotoxicity assays are promising but only at an early stage of development.

3D spheroids derived from human hepatic cells have gained increasing interest for genotoxicity screening due to their improved metabolic activity and hepatic function compared to 2D monolayer cultures. HepaRG spheroids have been the most reported 3D hepatic model for genotoxicity assessment. Following 24-h treatment, HepaRG spheroids showed higher sensitivity than HepG2 in 2D format in MN induction by benzo[a]pyrene (B[a]P), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), aflatoxin B1 (AFB1), and methyl methanesulfonate (MMS) using the cytokeratins-block MN assay (Conway et al., 2020; Shah et al., 2018). In addition, 3D HepaRG spheroids were shown to be more sensitive than 2D HepaRG cells to detect DNA damage induced by MMS, B[a]P, and two heterocyclic aromatic amines (HAAs) (PhIP and 2-amino-3-methyl-4H-imidazo[4,5-f]quinoline (IQ)) in the comet assay (Elje et al., 2019; Stampar et al., 2019).

A recent study successfully adapted the standard comet assay for use with 3D HepaRG spheroids developed in ultra-low attachment (ULA) plates (Mandon et al., 2019). By evaluating 11 compounds, the authors concluded that 3D HepaRG spheroids were more suitable than 2D differentiated HepaRG cells to detect DNA damage induced by human indirect-acting carcinogens (requiring metabolic activation), especially for compounds metabolized by CYP2E1 and 1A2.

Although 3D cell cultures are being successfully used for genotoxicity studies, applying 3D culture models in high-throughput genotoxicity assays is still challenging. Generally, 3D culture-based assays are considered more difficult to perform, more expensive, and their throughput is lower than that of assays performed in 2D cultures (Pfüehler et al., 2020). Several methods have been established for developing 3D culture models, such as forced-floating, hanging drop, ULA plates, agitation-based approaches, matrices, scaffolds, and microfluidic systems (Breslin and O’Driscoll, 2013; Ivanov et al., 2014). The present study used 96- or 384-well ULA plates to develop 3D spheroids since it is relatively easy to culture highly uniform spheroids in a high-throughput manner versus other methods (Ivanov et al., 2014). In addition, the 96-well CometChip platform, a recently developed high-throughput version of the comet assay, provides a rapid and sensitive method of measuring DNA damage with better reproducibility (Chao and Engelward, 2020). We envisioned that integration of the two technologies may improve the throughput of 3D assays significantly.

In the present study, HepaRG cells were used for developing 3D spheroids with the assumption that this model may generate data that is relevant to the human physiological condition. We optimized and evaluated the 3D HepaRG spheroids for genotoxicity testing by detecting DNA damage responses induced by 34 test articles: 8 direct-acting and 11 indirect-acting genotoxicants or carcinogens as well as 15 compounds that show different genotoxic responses in vitro and in vivo (Tab. 1). A wide range of concentrations was used to generate a large number of data points indicative of chemical-induced DNA damage, facilitating quantitative benchmark dose (BMD) analysis (Seo et al., 2019; Wills et al., 2016). The resulting BMDs and their upper and lower 90% confidence intervals (BMDU and BMDL) were compared with those calculated using the CometChip data generated from 2D HepaRG cells and PHHs from our previous studies (Seo et al., 2019, 2020).

2 Materials and methods

2.1 Chemicals

Eight direct-acting and 11 indirect-acting genotoxicants/carcinogens as well as 15 compounds that show different genotoxic responses in vitro and in vivo (Tab. 1) were selected for the in vitro genotoxicity test. Seven chemicals, including phenacetin, bupropion, diclofenac, omeprazole, dextromethorphan, chlorzoxazone, and midazolam, were used for CYP450 enzyme substrate cocktails. The metabolites (4-acetamidophenol (APAP), hydroxybupropion (Bup-OH), 4-hydroxy diclofenac (Dic-OH), 5-hydroxy omeprazole (OPZ-OH), dextromethorphan (Dex), 6-hydroxy chlorzoxazone (Chlorz-OH), and 1-hydroxymidazolam (Mid-OH)) and internal standard (hydroxybupropion-d6 (Bup-OH-D6)) were used for CYP450 activity measurement. All compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA), except for IQ and PhIP, which were obtained from Toronto Chemical Research (Toronto, ON, Canada).

2.2 Cell culture

The basic medium consisted of William’s E medium (Thermo Fisher, Waltham, MA, USA) supplemented with 2 mM GlutaMax™ (Thermo Fisher) and 100 μg/mL primocin (InvivoGen, San Diego, CA, USA). The growth and differentiation media were prepared by adding Growth Medium Supplement (Cat# ADD711C) and Differentiation Medium Supplement (Cat# ADD721C) (Lonza, Walkersville, MD, USA), respectively, to the basic medium. Undifferentiated HepaRG human hepatoma cell line (HPR101) was obtained from Biopredic International (Saint Grégoire, France). Cells were cultured and differentiated at 37°C in a humidified atmosphere with 5% CO₂ according to the supplier’s protocol with minor modifications.
### Tab. 1: Reported genotoxicity and carcinogenicity of test chemicals

| Group                                      | Chemical | CAS#       | Ames | Genotoxicity assay findings | Carcinogenicity | References                                                                                                                                 |
|--------------------------------------------|----------|------------|------|-----------------------------|----------------|------------------------------------------------------------------------------------------------------------------------------------------|
| Direct-acting genotoxicants/ carcinogens   | 4-NQO    | 56-57-5    | (+)  | (+) for CA, MLA, pig-a, comet | (+) for oral tumor | Le Hegarat et al., 2014; Dertinger et al., 2012; David et al., 2018; Kanojia and Vaidya, 2006                                                |
|                                            | CdCl\textsubscript{2} | 10108-64-2 | (+/-) | (+) for MN, CA, MLA, HPRT, comet | (+) for lung, prostate, etc.; Group 1 | Kirkland et al., 2016; Guo et al., 2016; Seo et al., 2019                                                                                     |
|                                            | Cisplatin | 15663-27-1 | (+)  | (+) for MN, pig-a, comet     | (+) for lung tumor, leukemia; Group 2A | Bhalli et al., 2013; Dertinger et al., 2019; Bemis et al., 2018                                                                              |
|                                            | Colchicine | 64-86-8    | (-)  | (+) for MN, CA, MLA, (-) for comet | No adequate studies | Kirkland et al., 2016; FDA/CDER, 2013; Seo et al., 2020                                                                                     |
|                                            | ENU      | 759-73-9   | (+)  | (+) for MLA, CA, MN, pig-a, HPRT, comet, UDS | (+) for kidney, mammary tumor, etc.; Group 2A | David et al., 2018; Dertinger et al., 2012; Habas et al., 2017; Kirkland et al., 2016; Le Hegarat et al., 2014; Seo et al., 2019, 2020; Bemis et al., 2018 |
|                                            | Etoposide | 33419-42-0 | (+)  | (+) for MLA, CA, MN, SCE, HPRT, comet; (-) for pig-a | Increased risk for acute myeloid leukemia in human; Group 2A | Kirkland et al., 2016; David et al., 2018; Yamamoto and Wakata, 2016; IARC, 2000                                          |
|                                            | HQ       | 123-31-9   | (-)  | (+) for MLA, MN, CA, comet   | (+) for liver and kidney adenoma; Group 3 | IARC, 1999; Kirkland et al., 2016; Peng et al., 2013                                                                                       |
|                                            | MMS      | 66-27-3    | (+)  | (+) for MLA, CA, MN, pig-a, HPRT, comet, UDS | (+) for nasal, nervous system tumor, etc.; Group 2A | Habas et al., 2017; Liu et al., 2019; Kirkland et al., 2016; Seo et al., 2019, 2020; Dertinger et al., 2012; Bemis et al., 2018 |
| Indirect-acting genotoxicants/ carcinogens | 2,4-DAT  | 95-80-7    | (+)  | (+) for MLA, CA, MN, comet, UDS, but (-) for comet in HepaRG and HPRT | (+) for liver tumor; Group 2B | Le Hegarat et al., 2014; Severin et al., 2005; Seo et al., 2019, 2020; Kirkland et al., 2016; Guo et al., 2020 |
|                                            | 2-AAF    | 53-96-3    | (+)  | (+) for MLA and UDS, but (-) for comet in HepaRG | (+) for liver, bladder, mammary gland, and skin tumors | Kirkland et al., 2016; Shigano et al., 2016; OECD, 2020                                                                                   |
| Group   | Chemical | CAS#      | Ames                  | Genotoxicity assay findings | Carcinogenicity\(^a\)                                      | References                                                                 |
|---------|----------|-----------|-----------------------|----------------------------|------------------------------------------------------------|---------------------------------------------------------------------------|
|         |          |           |                       |                            | In vitro                                                   | In vivo                                                                 |
|         |          |           |                       |                            | and HPRT                                                    | (-) for pig-a after single dose, but (+) after 28-day exposure           | IARC, 1994                                                                |
|         | Acrylamide | 79-06-1  | (-)                   |                            | (+) for MLA, SCE, CA, but (-) for UDS                     | (+) for UDS, comet, TG, SCE, MN, CA                                    |                                                                            |
|         | AFB1     | 1162-65-8 | (+)                   |                            | (+) for CA, MN, UDS, HPRT mutations                        | (+) for MN, CA, UDS, TG in liver, pig-a after 28-day exposure; (-) for comet | OECD, 2020; Kirkland et al., 2016                                           |
|         | B[a]P    | 50-32-8   | (+)                   |                            | (+) for MLA, CA, MN, pig-a, comet, HPRT, UDS               | (+) for comet, MN, pig-a, TG                                           | Le Hegarat et al., 2010; Kirkland et al., 2016; Shah et al., 2016, 2018; Graupner et al., 2014; Guo et al., 2020; Wang et al., 2018; Dertinger et al., 2019 |
|         | CPA      | 6055-19-2 | (+)                   |                            | (+) for MLA, CA, MN                                        | (+) for comet, MN, pig-a, TG                                           | Yusuf et al., 2000; Kirkland et al., 2016, 2019; Seo et al., 2019, 2020; Le Hegarat et al., 2010, 2014; Bhalli et al., 2013; Dertinger et al., 2012, 2019 |
|         | DMBA     | 57-97-6   | (+)                   |                            | (+) for MLA, CA, MN, comet, UDS                           | (+) for MN and pig-a in blood, comet in liver, CA in BM, TG             | Le Hegarat et al., 2014; Kirkland et al., 2016; Shi et al., 2011           |
|         | DMNA     | 62-75-9   | (+)                   |                            | (+) for MLA, CA, MN, HPRT, comet, UDS                     | (+) for MN and comet in liver, UDS, TG; generally (-) for MN in BM      | Kirkland et al., 2016; Seo et al., 2019                                    |
|         | IQ       | 76180-96-6| (+)                   |                            | (+) for SCE, UDS, MN; (+/-) for CA and comet;              | (+) for CA in blood, comet, TG; (-) for MN, SCE and for CA in BM        | Kirkland et al., 2016; Le Hegarat et al., 2010                              |
|         | PhiP     | 105650-23-5| (+)                   |                            | (+) for CA, MN, comet, HPRT, UDS                          | (+) for MN, UDS, comet, TG; (-) for CA                                 | Kirkland et al., 2016; Shah et al., 2018                                  |
| Group | Chemical  | CAS#     | Ames | Genotoxicity assay findings | Carcinogenicity | References |
|-------|-----------|----------|------|------------------------------|----------------|------------|
|       |           |          |      | In vitro | In vivo                   |             |            |
| In vitro (+) but in vivo (–), and Ames (+) | Styrene  | 100-42-5 | (+/-) | (+) for CA, SCE, MLA, comet | (+/-) for comet, MN, CA, SCE | (+) for lung, liver, mammary gland tumors; Group 2A | Moore et al., 2019; IARC, 2019b |
|       | 3-MCPD   | 96-24-2  | (+)  | (+) for comet | (–) for pig-a, UDS, MN, comet, gpt, Spi<sup>−</sup> mutations | (+) for renal tubule tumor, Group 2B | IARC, 2013; Onami et al., 2014; Ozcagli et al., 2016 |
|       | DFPBA    | 156545-07-2 | (+) | (+) for MLA, comet, MN | (–) for pig-a, MN, comet | No data available | Masuda-Herrera et al., 2019 |
|       | EDAC     | 25952-53-8 | (+)  | (+) for MLA, MN | (–) for pig-a, MN, comet in liver | No data available | Kirkland et al., 2019; ECHA, 2021; Custer et al., 2015 |
|       | HOPO     | 13161-30-3 | (+), E | (+) for MN | (–) for pig-a, MN, comet | No data available | Dobro et al., 2018, 2019; Custer et al., 2015 |
|       | PBA      | 98-80-6  | (+)  | No data available | (–) for pig-a, MN, comet | No data available | Masuda-Herrera et al., 2019 |
| In vitro (+) but in vivo (–), and Ames (–) | 4-Nitrophenol | 100-02-7 | (–) | (+) for CA with S9; (–) for SCE, comet, HPRT; (+/-) for MN; I for MLA | (–) for MN | (+) in mice dermal study | Fowler et al., 2012; Eichenbaum et al., 2009; Hartmann and Speit, 1997; Hu et al., 2009; NTP, 1993 |
|       | Ethyl acrylate | 140-88-5 | (–) | (+) for MLA, CA; (+/-) for MN | (+/-) for MN; (–) for SCE, CA, comet, gpt and Spi<sup>−</sup> mutations | (+) for forestomach tumor in rats and mice, Group 2B | Ellis-Hutchings et al., 2018; Fowler et al., 2012; IARC, 2019a; Przybojewska et al., 1984 |
|       | Phthalic anhydride | 85-44-9 | (–) | (+) for MLA, comet; (–) for SCE, γH2AX; (+/-) for MN, CA | No data available | (–) in rats and mice | NTP, 2022c; Fowler et al., 2012; Smart et al., 2011; Ella et al., 1994 |
|       | Sodium xylene-sulfonate | 1300-72-7 | (–) | (+) for SCE; (–) for CA; (+/-) for MN, E for MLA | No data available | (–) in rats and mice dermal studies | Fowler et al., 2012; Kirkland et al., 2016; NTP, 1998 |
|       | TBHQ     | 1948-33-0 | (–) | (+) for MLA, CA, DNA damage; (–) for HPRT and lacZ mutations; (+/-) for MN; | Borderline (+) for comet in liver; (–) for MN, CA | (–) in rats and mice | Fowler et al., 2012; Kirkland et al., 2016 |
| In vitro (–) but in vivo (+) | 1,4-Dioxane | 123-91-1 | (–) | (+) for CA, SCE, MN, MLA | (+) for MN in liver and TG; (–) for MN and pig-a in blood | (+) in rats and mice; Group 2B | Gi et al., 2018; IARC, 1999; Itoh and Hattori, 2019; Morita and Hayashi, 1998; NTP, 2022b |
10K, or 20K cells) were plated into each well of 96- or 384-well ULA round-bottom plates (Corning) in 100 μL or 50 μL medium, respectively. The culture medium was refreshed every 2-3 days by replacing half of the medium with fresh medium using VIAFLO 96/384 electronic pipettes (INTEGRA Biosciences, Hudson, NH, USA). The spheroids were incubated at 37°C in a humidified atmosphere with 5% CO₂ and maintained for up to 30 days. Images of HepaRG spheroids were acquired by a Leica DMI4000B light microscope on Days 3, 6, 10, 20, and 30 after seeding, and the diameters of spheroids were measured using the Leica Application Suite software (Leica Microsystems, Wetzlar, Germany).

### 2.4 Characterization of 3D spheroids
#### Albumin secretion
Twenty-four hours after differentiation medium was refreshed on Days 10, 20, and 30, 100 µL of culture medium was collected to a 96-well plate for the analysis of albumin secretion. The albumin secretion was quantified using a colorimetric assay (BioAssay Systems, Hayward, CA, USA).

### 2.3 Formation of 3D spheroids
Fully differentiated HepaRG cells were dissociated from tissue culture dishes using TrypLE™ Express (Thermo Fisher) and the cells were passed no more than 5 times. Absence of mycoplasma contamination in differentiated HepaRG cells was confirmed using the LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich).

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min concentration in the supernatant was determined using the Human Serum Albumin DuoSet Enzyme-linked Immunosorbent Assay (ELISA) Kit (R&D Systems, Minneapolis, MN, USA). Spheroids were lysed with RIPA buffer (Thermo Fisher), and protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher). The albumin levels were presented as ng/mg protein.

**Histology and immunohistochemistry**

HepaRG spheroids were collected and fixed in 4% paraformaldehyde in Dulbecco’s phosphate-buffered saline (DPBS) at 4°C overnight on Days 10, 20, and 30. Fixed spheroids were suspended in liquid HistoGel (Thermo Fisher) and solidified at 4°C overnight. The solidified gel was gently pushed into a biopsy cassette, and the HistoGel-containing HepaRG spheroids were embedded in Formula R®, paraffin embedding medium (Leica Biosystems, Wetzlar, Germany). Paraffin-embedded blocks were sectioned at approximately 5 µm thickness, and the sections were mounted on positively charged glass slides. Spheroid sections were deparaffinized in xylene and hydrated in a graded series of ethanol solutions. One set of sections was stained with hematoxylin and eosin (H&E), the other three sets were immunohistochemically stained for cytokeratin 19 (CK19, biliary epithelial cells marker), Ki67 (proliferation marker), and multidrug resistance-associated protein 2 (MRP2, a canalicular multispecific organic anion transporter). Spheroid sections were incubated with the following primary antibodies: mouse monoclonal anti-CK19 (MAB3238, Sigma-Aldrich), rabbit monoclonal Ki67 (RM-9106, Thermo Fisher), and mouse monoclonal anti-MRP2 (ab3373, Abcam, Waltham, MA, USA) at dilutions of 1:200 (CK19) and 1:100 (Ki67 and MRP2) for 1 h. Slides then were incubated with the appropriate secondary antibodies: rat anti-mouse IgG-Cy3 or donkey anti-rabbit IgG F(ab')2 fragment-Cy3 (415-165-166 or 711-166-152, respectively, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The sections were counterstained and mounted with Vectashield/DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). Stained sections were examined, and microphotographs were taken with a Nikon Eclipse Ni-E upright motorized microscope (Tokyo, Japan).

**CYP450 enzyme activities**

The basal activities of CYP450 enzymes in HepaRG spheroids were measured by incubating the spheroids with seven CYP substrates as described previously with minor modifications (Seo et al., 2019, 2020). Briefly, spheroids were incubated for 2 h with 100 µL differentiation medium containing 40 µM chlorozoxazone (CYP2E1) or substrate cocktails of 100 µM phenacetin (CYP1A2), 100 µM bupropion (CYP2B6), 20 µM diclofenac (CYP2C9), 20 µM omeprazole (CYP2C19), 20 µM dextromethorphan (CYP2D6), and 50 µM midazolam (CYP3A4) at 37°C in a humidified atmosphere with 5% CO2. At the end of incubation, the supernatants were collected, and the protein was removed by adding twice the volume of acetonitrile containing 100 ng/mL of Bup-OH-D6 (internal standard). The individual metabolites released into the medium were quantified by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) using a Shimadzu 20A ultra-fast liquid chromatograph (UFLC) system coupled with an AB SCIEX 3200 QTRAP mass spectrometer (SCIEX LLC, Framingham, MA, USA). Chromatographic separation was performed using an Atlantis T3 C18 column (4.6 x 150 mm, 5 µm) with an Atlantis T3 VanGuard pre-column (3.9 x 5 mm, 5 µm) (Waters Corporation, Milford, MA, USA) maintained at 40°C. Samples (injection volume of 4 µL) were separated at a flow rate of 0.5 mL/min using a gradient mobile phase containing water (Solvent A) and acetoniitrile (Solvent B), both containing 0.1% formic acid. The gradient was as follows: 0-0.2 min, 5% Solvent B; 0.2-10 min, 5-95% Solvent B; 10-10.5 min, 95-5% Solvent B; 10.5-15 min, 5% Solvent B for column re-equilibration. The mass spectrometer with electrospray ionization (ESI) source was operated in the negative ion mode for Chlorz-OH (CYP2E1) and in the positive ion mode for the other six metabolites. Multiple reaction monitoring (MRM) was used for quantitation with the following ion transitions: m/z 152.2-110.1 for APAP (CYP1A2), m/z 256.1-238.2 for Bup-OH (CYP2B6), m/z 312.1-230.0 for Omepr-OH (CYP2C9), m/z 362.2-214.2 for OPZ-OH (CYP2C19), m/z 258.2-157.2 for Dex (CYP2D6), m/z 184.0-120.0 for Chlorz-OH (CYP2E1), and m/z 342.1-203.1 for Mid-OH (CYP3A4). Following a 2-h exposure to CYP substrates, the spheroids were lysed with RIPA buffer, and the protein concentrations were measured using the Pierce BCA Protein Assay Kit. Final CYP450 activities were expressed as pmol metabolite/min/mg protein. The activities of CYP450 in PHHs (Lot# HH1085, In Vitro ADMET Laboratories, Columbia, MD, USA) were obtained from our previous study (Seo et al., 2020).

**Gene expression of phase I and phase II enzymes**

The basal gene expression levels of 14 phase I and 5 phase II enzymes were measured using quantitative real-time PCR (qPCR) at the mRNA level. Total RNA was extracted from 2D cultured cells at Day 3, 5K 3D spheroids (spheroids containing 5K HepaRG cells) at Day 10 and cryopreserved PHHs (Lot# HH1085) using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). RNA concentration and quality were measured using a NanoDrop 8000 spectrophotometer (Thermo Fisher). cDNA was synthesized from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed using the FastStart Universal Probe Master (Roche Applied Science, Indianapolis, IN, USA) with the following TaqMan™ probes (Applied Biosystems): CYP1A1 (Hs01054796_g1), CYP1A2 (Hs00167927_m1), CYP1B1 (Hs00164383_m1), CYP2A6 (Hs00885049_s1), CYP2A13 (Hs00711162_s1), CYP2B6 (Hs03044634_m1), CYP2C8 (Hs00946140_g1), CYP2C9 (Hs00426397_m1), CYP2C19 (Hs00426380_m1), CYP2D6 (Hs04931916_gH), CYP2E1 (Hs00559367_m1), CYP3A4 (Hs00604506_m1), CYP3A5 (Hs00241417_m1), CYP3A7 (Hs00426361_m1), N-acetyltransferase 1 (NAT1; Hs00265080_s1), sulfotransferase 1A1 (SULT1A1; Hs00738644_m1), SULT2A1 (Hs00234219_m1), UDP-glucuronosyltransferase 1A1 (UGT1A1; Hs02511055_s1), UGT1A6 (Hs0152477_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH;
and 2D HepaRG cells were subsequently exposed to various concentrations of cisplatin in 0.9% NaCl (Sigma-Aldrich), and the other 31 chemicals (CdCl₂) and dimethylnitrosamine (DMNA) in deionized water, were freshly prepared before each experiment. Working solutions (100 μL) were added into each well of the plates and incubated for 15 min at 37°C. Subsequently, spheroids were dissociated into single cell suspensions by gentle pipetting, followed by addition of 100 μL differentiation medium to stop the reaction. Next, the cells were transferred into each well of a 96-well CometChip (Trevenig, Gaithersburg, MD, USA), and the comet assay was conducted as described previously (Seo et al., 2020). Comet images were acquired automatically using a Cytation 5 Cell Imaging Multi-Mode Reader (BioTek) and analyzed using Trevenig Comet Analysis Software to calculate the percentage of DNA in the tail (% tail DNA). DNA damage responses also were expressed as the relative ratio of DNA damage (fold-change) compared to their respective controls (Fig. S1).  

2.8 Quantification of DNA damage dose-response data

BMD analysis was performed using PROAST software (version 70.1). DNA damage dose-response data were analyzed using both exponential and Hill models that are recommended by the European Food Safety Authority (EFSA) for the analysis of continuous data (EFSA et al., 2017). The critical effect size (CES) of 0.5, indicating a 50% increase (BMD₅₀) in % tail DNA over the vehicle control response, was chosen for the in vitro comet assay (Seo et al., 2021). The BMD₅₀ and its BMDU and BMDL were calculated simultaneously for each data set. Uncertainty of the BMD estimates, indicated by the BMDU/BMDL ratio, was used to evaluate the statistical quality of the data (Slob, 2017).

2.9 Statistical analysis

Data are presented as the mean ± SD from at least three independent experiments. The statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Holm-Sidak test for albumin secretion, CYP450 activity, and gene expression data of phase I and phase II enzymes (SigmaPlot 13.0, Systat Software, San Jose, CA, USA). ANOVA followed by Dunnett’s test was used for comparing the CometChip data between treatment groups and the vehicle control group. A value of p < 0.05 was considered statistically significant.

3 Results

3.1 HepaRG spheroid formation and size

HepaRG cells were grown to confluence in complete growth medium for 14 days and then differentiated with complete differentiation medium for another 14 days. Two morphologically distinct cell populations were observed, i.e., clustered granular hepatocyte-like cells surrounded by relatively clear biliary epithelial-like cells (Fig. 1A). After seeding in 96- or 384-well ULA plates, differentiated HepaRG cells, at three different densities (5K, 10K, and 20K/well), aggregated into spheroids without any supporting exogenous extracellular matrix after 3 days of culture (Fig. 1B). At Day 3, the diameters of 5K, 10K, and 20K spheroids averaged 369.4 ± 29.9 μm, 537.5 ± 31.0 μm, and 658.5 ± 56.2 μm, respectively. The spheroids became more compact over a period of 30 days, along with 15.8-31.6% reduction in size. At Day 30, 5K, 10K, and 20K spheroids were sized 310.9 ± 14.5 μm, 396.7 ± 10.3 μm, 450.2 ± 17.5 μm, respectively.

1 doi:10.14573/altex.2201121s
Fig 1: 2D and 3D HepaRG cultures
(A) Cell morphology of 2D HepaRG cells cultured in growth medium for 14 days and in differentiation medium for an additional 14 days (200 x magnification).
(B) HepaRG cells were seeded at 5,000, 10,000, and 20,000 (5K, 10K, and 20K) per well in ultra-low attachment (ULA) plates and cultured for up to 30 days. The morphology and size of 3D spheroids were measured at Days 3, 6, 10, 20, and 30 using a light microscope. Scale bar = 500 µm.

Fig. 2: Characterization of HepaRG spheroids
(A) H&E staining of 5K, 10K, and 20K spheroids. (B) Representative images of vacuolation, canalicular-like and ductule-like structures, necrosis, and mitotic figure visible in stained spheroids. (C) Immunofluorescent staining with the biliary epithelial cell marker CK19, proliferation marker Ki67, and canalicular marker MRP2 (5K spheroids data shown; see Fig. S11 for 10K and 20K spheroids). (D) Albumin secretion was measured by ELISA and expressed as ng/mg protein. The data are presented as the mean ± SD (n ≥ 3). Significance was determined by one-way ANOVA followed by Holm-Sidak test (*p < 0.05 for comparison of 5K, 10K, and 20K spheroids, #p < 0.05 for 2D HepaRG cells vs 5K or 10K spheroids).

H&E, hematoxylin and eosin; CK19, cytokeratin 19; MRP2, multidrug resistance-associated protein 2.
Immunofluorescent staining showed expression of the biliary marker CK19 protein in the spheroids for at least 30 days of cultivation, with expressions enhanced over time (Fig. 2C, Fig. S2). Ki67 protein was detected in the spheroids, indicating cellular proliferation potential. During 30 days of culture, 5K and 10K spheroids expressed relatively higher levels of Ki67 protein compared to 20K spheroids. The expression of MRP2 protein also was confirmed on the canalicular membrane of cells in the spheroids, indicating that bile canaliculi structures were formed in 3D HepaRG spheroids (Fig. 2C).

### 3.2 Histological and immunohistochemical analyses of HepaRG spheroids

Morphological features of 5K, 10K, and 20K spheroids at Days 10, 20, and 30 were assessed with H&E staining (Fig. 2A). The spheroids consisted of large, polygonal cells with round to oval single nuclei (rarely binucleated), 1-2 nucleoli, clear and/or granular cytoplasm with vacuolation and/or rarefaction, and low nucleus to cytoplasmic ratio. Most cells in spheroids morphologically resembled hepatocytes and occasionally appeared to form canalicular-like structures. There were also rare ductule-like structures that appeared to have a central lumen lined with cuboidal cells (Fig. 2B). Mitotic figures were seen occasionally. Viable cells were observed at the centers of the majority of 5K spheroids, whereas higher incidences of necrotic cores were observed in 10K and 20K spheroids at Days 20 and 30 (Fig. 2A).

### 3.3 Albumin secretion in 2D and 3D HepaRG cultures

Albumin secreted into culture medium within 24 h after seeding was measured in both 2D and 3D cultures (Fig. 2D). Sig-
Fig. 4: DNA damage and cytotoxicity of 19 genotoxicants/carcinogens in 2D and 3D HepaRG cultures
2D and 3D HepaRG cultures were exposed to 8 direct-acting (A) and 11 indirect-acting genotoxicants/carcinogens (B) for 24 h. Part of the DNA damage and cytotoxicity data in 2D HepaRG cells was obtained from our previous study (Seo et al., 2019). The relative cell viability (% of control, indicating cytotoxicity) was measured by ATP assay (right y-axis and top two lines). DNA damage (% tail DNA; left y-axis and bottom two lines) was detected using the CometChip assay. The red lines and black dotted lines represent the results of 3D and 2D HepaRG cultures, respectively. The data are expressed as the mean ± SD (n ≥ 3). Significance was determined by one-way ANOVA followed by Dunnett’s test (*p < 0.05 vs vehicle control). See Table 1 for abbreviations of the compounds tested.
2D and 3D HepaRG cultures were exposed to 15 compounds including five in vitro (+) but in vivo (-) and Ames (+) compounds (A); five in vitro (+) but in vivo (-) and Ames (-) compounds (B); and five in vitro (-) but in vivo (+) compounds for 24 h. The relative cell viability (% of control, indicating cytotoxicity) was measured by ATP assay (right y-axis and top two lines). DNA damage (% tail DNA; left y-axis and bottom two lines) was detected using the CometChip assay. The red lines and black dotted lines represent the results of 3D and 2D HepaRG cultures, respectively. The data are expressed as the mean ± SD (n ≥ 3). Significance was determined by one-way ANOVA followed by Dunnett’s test (*p < 0.05 vs vehicle control). See Tab. 1 for abbreviations of the compounds tested.
significantly higher albumin secretion (ng/mg protein) was shown in 3D spheroids compared to 2D HepaRG cells over an incubation period of 30 days. When compared to 2D cultures, the albumin level of 5K spheroids increased by 9.0-, 10.8-, and 23.4-fold with values of 818.2, 922.6, and 334.9 ng/mg protein (vs 91.3, 85.2, and 14.3 ng/mg protein in 2D) at Days 10, 20, and 30, respectively. Additionally, 5K spheroids produced 1.5-1.6-fold and 2.3-2.9-fold higher albumin levels than 10K and 20K spheroids at Days 10 and 20, respectively. At Day 30, the albumin secretion in 5K spheroids declined to levels equivalent to those in 10K and 20K spheroids.

3.4 CYP450 enzyme activities in PHHs, 2D, and 3D HepaRG cultures

The activities of seven major CYP450 (CYP1A2, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4) enzymes were measured by determining the formation of metabolites released into the medium via HPLC-MS/MS (Fig. 3A). The activity of CYP2E1 in PHHs was 1.6 pmol metabolite/min/mg protein, while it was below the quantification limit in both 2D HepaRG and 5K, 10K, or 20K spheroids under our experimental conditions (data not shown in Fig. 3A). The 5K and 10K spheroids maintained high activities for the other six CYPs over a culture period of 30 days, while a sharp decline was observed in 20K spheroids at Day 30. The 5K spheroids showed the highest CYP450 activities across the six CYPs, followed by 10K and 20K spheroids (5K > 10K > 20K). Specifically, the CYP activities in 5K spheroids were 1.3-2.0-fold and 1.5-3.8-fold higher than those in 10K and 20K spheroids, respectively, while the CYP activities in 10K spheroids were consistently higher (1.6-2.4-fold) than those in 20K spheroids.

When compared to 2D HepaRG cells, 5K and 10K spheroids displayed significantly higher activities for CYP1A2 (2.2-4.9-fold), 2B6 (6.7-25.0-fold), 2D6 (7.7-20.4-fold), and 3A4 (2.9-8.3-fold) for up to 30 days. Similar or slightly higher activities were observed for CYP2C9 (0.7-2.7-fold) and 2C19 (0.7-2.3-fold) between 2D and 3D cultures. CYP2D6 activity was 32.5-fold higher in PHHs than in 2D HepaRG cells, whereas the CYP activities in 10K spheroids were consistently higher (1.6-2.4-fold) than those in 20K spheroids.

3.5 Gene expression of phase I and phase II enzymes in 5K spheroids

Gene expression of phase I and phase II enzymes was determined using qPCR (Fig. 3B). In agreement with increased CYP450 enzyme activities seen in 3D cultures, 5K spheroids showed significantly higher mRNA expression than 2D HepaRG cells for 12 phase I enzymes: CYP1A1 (2.7-fold), 1A2 (11.6-fold), 1B1 (1.9-fold), 2A6 (8.2-fold), 2A13 (7.1-fold), 2B6 (11.7-fold), 2C8 (5.3-fold), 2C9 (1.3-fold), 2C19 (7.6-fold), 2D6 (2.6-fold), 2E1 (2.7-fold), and 3A4 (3.2-fold). CYP1A5 and 3A7 expression did not differ significantly between 2D HepaRG cells and 5K spheroids. The gene expression of CYP1A1, 1B1, 2A6 (undetermined), and 3A4 in PHHs was significantly lower than in 2D and 3D HepaRG cultures, whereas PHHs had considerably higher levels of CYP2D6, 2E1, 3A5, and 3A7 gene expression compared to 2D and 3D HepaRG cultures. For 5 phase II enzymes, no significant changes were found for NAT1 in 5K spheroids and PHHs compared to 2D HepaRG cells. Two sulfotransferases, SULT1A1 and 2A1, were increased in 3D spheroids (1.8-2.2-fold) and PHHs (2.1-3.3-fold), whereas two UDP-glucuronosyltransferases, UGT1A1 and 1A6, were slightly lower in 3D spheroids (0.8-fold) and PHHs (0.3-0.8-fold) compared to 2D HepaRG cells.

3.6 Cytotoxicity profiles of the 34 tested compounds in 2D and 3D HepaRG cultures

Following 24-h exposure, the cytotoxicity of 34 test articles was evaluated over a wide range of concentrations by the ATP assay. Seven out of 19 genotoxicants/carcinogens (CdCl2, colchicine, ENU, AFB1, CPA, IQ, and PhIP) and five out of 15 compounds that show different genotoxic responses in vitro and in vivo (3,5-difluorophenylboronic acid (DFPBA), PBA, 4-nitrophenol, TBHQ, and 1,4-dioxane) were more cytotoxic in 3D spheroids than in 2D cultures (Tab. 2, Fig. 4, 5). Other compounds induced similar cytotoxic effects between 2D and 3D cultures with one exception, ethyl acrylate, which showed higher cytotoxicity in 2D cultured cells than in 3D spheroids.

3.7 DNA damage profiles of the 34 tested compounds in 2D and 3D HepaRG cultures

To minimize false-positive genotoxicity responses in the CometChip assay, a cytotoxicity cutoff value of 70% was chosen for evaluating DNA damage responses (Koppen et al., 2017). When no cytotoxicity was observed, the highest test concentration used for subsequent testing was 10 mM, as recommended by the OECD guidance for genetic toxicology testing (OECD, 2017). Table 2 summarizes DNA damage responses of the 34 compounds in 2D and 3D cultures. At the maximum concentration for each compound, 14 out of 19 (73.7%) genotoxicants/carcinogens induced DNA damage in 3D spheroids, while 10 out of 19 (52.6%) genotoxicants/carcinogens were positive in 2D cultured cells after a 24-h treatment. For the 8 direct-acting genotoxicants/carcinogens, CdCl2 was the only compound that showed different DNA damage responses between 2D and 3D cultures, i.e., a 3.1-fold increase in DNA damage was observed in 3D spheroids, but not in 2D cultured cells (Fig. 4A, Tab. 2). Two compounds (4-nitroquinoline 1-oxide (4-NQO) and cisplatin) induced relatively higher % tail DNA in 3D spheroids than in 2D cultured cells (2.7-3.2-fold vs 1.6-1.9-fold increase over control, respectively). When the lowest effective concentrations (LECs) were compared, 4-NQO and MMS had lower LEC values in 3D spheroids compared to 2D cultured cells (1.88 vs 5 μM and 93.8 vs 125 μM, respectively). Cisplatin, ENU, and etoposide showed higher LEC values (25 vs 2 μM, 1600 vs 800 μM, and 50 vs 9.4 μM, respectively) in 3D spheroids than in 2D cells. HQ and aequorin colchicine produced negative responses in both culture models.

At the maximum concentrations, all 11 indirect-acting genotoxicants/carcinogens induced higher % tail DNA in 3D spheroids compared to 2D cultured cells (Fig. 4B, Fig. S1). Five compounds, acrylamide, B[a]P, cyclophosphamide (CPA), 7,12-dimethylbenzanthracene (DMBA), and DMNA, induced DNA damage in both culture models, while three compounds, 2,4-diaminotoluene (2,4-DAT), IQ, and PhIP, were positive only in
Tab. 2: Comparison of DNA damage induced in 2D and 3D HepaRG cell cultures

| Compound | Max conc. (μM) | Cytotoxicity (%) | LEC | Relative ratio | Outcome |
|----------|---------------|------------------|-----|---------------|---------|
|          | 2D | 3D | 2D | 3D | 2D | 3D | 2D | 3D | 2D | 3D | 2D | 3D |
| **Genotoxicants/carcinogens** | | | | | | | | | | | | | |
| Direct-acting genotoxicants/ carcinogens | | | | | | | | | | | | | |
| 4-NQO | 5 | 5 | 33 | 27 | 5 | 1.88 | 1.6 | 2.7 | + | ++ | |
| CdCl₂ | 8 | 4 | 13 | 35 | - | 3 | 1.1 | 3.1 | - | ++ | |
| Cisplatin | 50 | 50 | 16 | 33 | 2 | 25 | 1.9 | 3.2 | + | ++ | |
| Colchicine | 40 | 4 | 33 | 22 | - | - | 1.1 | 1.0 | - | - | |
| ENU | 3,200 | 2,400 | 26 | 29 | 800 | 1,600 | 3.0 | 3.6 | ++ | ++ | |
| Etoposide | 100 | 100 | 19 | 26 | 9.4 | 50 | 3.0 | 3.0 | ++ | ++ | |
| HQ | 200 | 200 | 37 | 37 | - | - | 1.1 | 1.3 | - | - | |
| MMS | 500 | 500 | 19 | 25 | 125 | 93.8 | 16.2 | 10.3 | +++ | +++ | |
| Indirect-acting genotoxicants/ carcinogens | | | | | | | | | | | | | |
| 2,4-DAT | 8,000 | 8,000 | 17 | 31 | - | 6,000 | 1.2 | 3.1 | - | ++ | |
| 2-AAF | 400 | 400 | 30 | 31 | - | - | 1.1 | 1.7 | - | * | |
| Acrylamide | 5,000 | 5,000 | 34 | 27 | 3,750 | 937.5 | 4.2 | 4.3 | ++ | ++ | |
| AFB1 | 5 | 3.75 | 33 | 33 | - | - | 0.9 | 2.0 | - | * | |
| B[a]P | 100 | 100 | 13 | 29 | 20 | 25 | 2.2 | 2.9 | ++ | ++ | |
| CPA | 10,000 | 5,000 | 26 | 21 | 1,600 | 1,250 | 2.0 | 3.6 | + | ++ | |
| DMBA | 1,000 | 1,000 | 35 | 23 | 25 | 750 | 2.1 | 3.2 | ++ | ++ | |
| DMNA | 10,000 | 10,000 | 15 | 22 | 4,000 | 78.1 | 5.1 | 10.6 | +++ | +++ | |
| IQ | 375 | 250 | 33 | 31 | - | - | 187.5 | 1.1 | 2.7 | - | - | |
| PhIP | 750 | 375 | 21 | 31 | - | - | 187.5 | 0.8 | 2.2 | - | - | |
| Styrene | 10,000 | 10,000 | 17 | 18 | - | - | 0.8 | 1.8 | - | - | * | |

Compounds that show different genotoxic responses in vitro and in vivo

| In vitro (+) but in vivo (-), and Ames (+) | In vitro (+) but in vivo (-), and Ames (-) | In vitro (-) but in vivo (+) |
|------------------------------------------|------------------------------------------|-----------------------------|
| 3-MCPD | 10,000 | 10,000 | 16 | 33 | 7,500 | 5,000 | 2.2 | 4.5 | ++ | ++ | |
| DFPBA | 500 | 375 | 34 | 33 | - | - | 1.5 | 1.5 | - | - | |
| EDAC | 100 | 100 | 24 | 19 | - | - | 1.3 | 1.5 | - | - | |
| HOPO | 750 | 750 | 26 | 33 | - | - | 1.3 | 1.3 | - | - | |
| PBA | 2,500 | 1,875 | 20 | 21 | - | - | 1.2 | 1.5 | - | - | |
| 4-Nitrophenol | 250 | 187.5 | 22 | 27 | - | - | 1.2 | 1.3 | - | - | |
| Ethyl acrylate | 5,000 | 10,000 | 28 | 16 | 3,750 | - | 3.1 | 1.5 | ++ | - | |
| Phthalic anhydride | 7,500 | 7,500 | 14 | 17 | - | - | 1.3 | 2.0 | - | * | |
| Sodium xylenesulfonate | 10,000 | 10,000 | 17 | 37 | - | - | 1.1 | 1.4 | - | - | |
| TBHQ | 250 | 93.8 | 21 | 33 | - | - | 1.1 | 1.4 | - | - | |
| 1,4-Dioxane | 10,000 | 7,500 | 13 | 20 | - | - | 1.1 | 1.4 | - | - | |
| Dicyclanil | 500 | 1,000 | 1 | 28 | - | - | 1.4 | 1.1 | - | - | |
| DMTP | 500 | 500 | 18 | 33 | - | - | 1.1 | 1.3 | - | - | |
| Estragole | 2,500 | 5,000 | 20 | 28 | - | - | 1.5 | 1.1 | - | - | |
| LMG | 500 | 500 | 11 | 23 | - | - | 1.7 | 1.8 | - | - | * | *

a The highest concentration tested in the CometChip assay. b Cytotoxicity was determined using the ATP assay following a 24-h treatment. c LEC, the lowest effective concentration, determined by one-way ANOVA followed by Dunnett's test, is the lowest concentration that induced a significant increase in % tail DNA. d The relative ratio compared to the % tail DNA of the vehicle control at the concentration shown in the table. e The ratio ≤ 1.5-fold (p ≥ 0.05 vs vehicle control, green color); *, 1.5 < ratio ≤ 2, but p ≥ 0.05 (green color); +, 1.5 < ratio ≤ 2; ++, 2 < ratio ≤ 5; ++++, the ratio > 5 (p < 0.05, red color). See Table 1 for abbreviations of the compounds tested.
3D HepaRG spheroids (Tab. 2). The relative DNA damage ratios of 2-acetylaminofluorene (2-AAF), AFB1, and styrene increased by 1.7-2.0-fold in 3D spheroids, but this was not statistically significant compared to the control group. Acrylamide, CPA, and DMNA had lower LEC values in 3D spheroids than in 2D cultures (937.5 vs 3750 μM, 1250 vs 1600 μM and 78.1 vs 4000 μM, respectively), while the LECs of B[a]P and DMBA were lower in 2D cultured cells than in 3D spheroids (20 vs 25 μM and 25 vs 750 μM, respectively).

For the 15 compounds that showed different genotoxic responses in vitro and in vivo, 3D and 2D cultures showed consistent positive/negative calls for all the compounds except ethyl acrylate (Fig. 5, Tab. 2). Ethyl acrylate was positive in 2D cultured cells at concentrations ≥ 3750 μM, while it did not induce significant DNA damage in 3D spheroids. 3-chloro-1,2-propanediol (3-MCPD) was the only compound that induced positive responses in both culture models, with lower LEC (5000 vs 7500 μM) and higher relative ratio observed in 3D spheroids than in 2D cultured cells (4.5-fold vs 2.2-fold). Overall, 3D cultures showed improved sensitivities for detecting DNA damage responses induced by the 34 compounds compared to 2D cultures.

3.8 Benchmark dose analysis of DNA damage responses in 2D and 3D HepaRG cultures

A covariate analysis was performed using PROAST. BMD50 values of the 15 positive DNA damage responses in 3D spheroids were divided into four groups without overlapping their 90% confidence intervals (Fig. S3A). CdCl2 and 4-NQO were the most potent, followed by DMNA, cisplatin, MMS, B[a]P, and etoposide (Tab. S1). 3-MCPD and 2,4-DAT were the least potent DNA damage inducers. For 2D cultures, the 12 positive compounds were divided into three groups without overlapping their 90% confidence intervals (Fig. S3B). 4-NQO, MMS, etoposide, B[a]P, and cisplatin were the most potent; followed by acrylamide, DMBA, ethyl acrylate, ENU, and DMNA; 3-MCPD and CPA were the least potent compounds to induce DNA strand breaks.

The exponential model was used to compare BMD values between 2D and 3D cultures (Fig. 6). For the 11 compounds that were positive in both culture models, the calculated BMD50 values with 90% confidence intervals overlapped for ENU, etoposide, MMS, acrylamide, B[a]P, and 3-MCPD in 2D and 3D cultures. The other five compounds had non-overlapping BMD50 values, with lower values in 3D than in 2D cultures. Specifically, the BMD50 values with 90% confidence intervals for 4-NQO and DMBA were next to each other, while the BMD50 values for cisplatin, CPA, and DMNA in 3D cultures were significantly lower than those in 2D cultures. We further compared BMD values generated from 3D HepaRG and PHH comet data of three individual donors from our previous study (donor information was presented in Tab. S2) (Seo et al., 2020, 2021). Six out of the nine compounds (67%) had overlapping BMD50 and upper and lower confidence intervals. While the other three compounds, ENU, MMS, and DMBA, had significantly lower BMD50 values in PHHs than in 3D HepaRG spheroids.

4 Discussion

Growing efforts are being invested in developing new approach methodologies with the goal of reducing animal use for safety assessment of drugs and other chemicals (Parish et al., 2020). 3D spheroids have been recognized as promising in vitro alternative models due to their greater physiological relevance compared to 2D cultures. However, low throughput remains a concern due to technical challenges and cost. To improve the 3D assay throughput, the present study used ULA plates as a spheroid culture system and adapted the CometChip technology to 3D culture conditions for high-throughput assessment of DNA damage. It is worth noting that 384-well ULA plates provide a more cost-efficient and less time-consuming option than 96-well plates for generating large numbers of uniform spheroids reproducibly, enabling high-throughput toxicity assays. HepaRG cells were used for developing 3D cultures given that HepaRG spheroids provide a hepatocyte-like model system with physiologically relevant levels of drug metabolism, functionality, and improved sensitivity for detecting genotoxic responses compared to their 2D counterparts (Ramaiahgari et al., 2017; Mandon et al., 2019).

We first optimized the cell number and culture time of 3D spheroids by monitoring the morphology and metabolic capacity of spheroids plated at different densities (5K, 10K, and 20K cells) and cultured for up to 30 days. HepaRG spheroids retained a stable phenotype but became tighter and more compact over time (Fig. 1B). However, necrotic cores were observed in 10K and 20K spheroids at Days 10, 20, and 30 (Fig. 2A,B). The formation of a necrotic core is attributed to hypoxia due to insufficient diffusion of oxygen and nutrients in large-sized (10K and 20K) spheroids compared to small-sized (5K) spheroids (Edmondson et al., 2014). Consequently, 5K spheroids showed the highest level of albumin secretion and baseline activities for the six CYPs over a 30-day culture period, while these activities in 20K spheroids had decreased notably at Day 30 (Fig. 2D, 3A), likely due to the size- or time-dependent necrotic core formation and the loss of hepatocyte functions (Cox et al., 2020). In agreement with previous findings (Gunness et al., 2013; Leite et al., 2012; Ott et al., 2017; Ramaiahgari et al., 2017), the present study demonstrated a significantly higher liver functionality and metabolic capacity in 3D HepaRG spheroids compared to 2D cultures.

In addition to their metabolic competence, another advantage of HepaRG cells is their ability to differentiate into hepatocyte- and cholangiocyte-like cells (Le Hegarat et al., 2010). Polarized HepaRG cells in 3D spheroids form tissue-like architectures, facilitating cell-cell interactions and junctional signaling (Ramaiahgari et al., 2017). The immunofluorescent detection of CK19 (biliary epithelial cell marker) and MRP2 (canalicular marker, Fig. 2C) in 3D spheroids indicates biliary epithelial cell differentiation (Malinen et al., 2014) and the formation of bile canaliculi-like structures that are involved in the detoxification of xenobiotics (Gunness et al., 2013; Ramaiahgari et al., 2017). In addition, the expression of Ki67 demonstrates that differentiated HepaRG spheroids retain a proliferative capability, suggesting...
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that the spheroids may potentially be used in genotoxicity assays that require cell division, i.e., the micronucleus assay and mutation assays.

We evaluated the performance of 3D HepaRG spheroids for genotoxicity testing using two groups of compounds, i.e., 8 direct-acting and 11 indirect-acting genotoxicants/carcinogens and 15 compounds that show differing genotoxic responses in vitro and in vivo (Tab. 1). All 8 direct-acting genotoxicants/carcinogens showed similar or slight increases in cytotoxicity and DNA damage in 3D spheroids versus 2D cultures with one exception, CdCl₂ (Fig. 4). CdCl₂ was consistently negative in the 2D HepaRG comet assay (Le Hegarat et al., 2014; Seo et al., 2019), but it induced significantly increased cytotoxicity and DNA strand breaks in 3D cultures. CdCl₂ has been classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC), and several mechanisms, i.e., mainly oxidative stress and inhibition of DNA repair, are involved in its carcinogenicity (IARC, 2012; Nair et al., 2013). 3D HepaRG spheroids exhibited a similar behavior to that of PHHs in detecting the cytotoxic and genotoxic potential of CdCl₂ (Seo et al., 2020). CYP450 has been reported to be involved in Cd metabolism and Cd can cause increases in CYP1A1, 2B22, 7A1, and 8B1 in chicken, fish or pig (Wang et al., 2021). We speculate that increased CYP activities may contribute to the induced DNA damage in 3D spheroids.

Compared to 2D HepaRG cells, 3D spheroids showed a higher sensitivity in detecting DNA damage of the 11 indirect-acting genotoxicants/carcinogens, with three compounds (2,4-DAT, IQ, and PhIP) positive solely in 3D cultures (Fig. 4B, Tab. 2). 2,4-DAT, a hepatocarcinogenic aromatic amine, is hydroxylated by CYP1A2 and then activated via O-acetylation to the mutagenic metabolite 4-acetylamino-2-aminotoluene by N-OAT (OEHHA, 2015). CYP1A2 also is involved in the oxidation of two HAAs (IQ and PhIP), forming DNA-reactive metabolites, including N-hydroxy-HAAs and N-acetoxy-HAAs via O-esterification (IARC, 1993; Kirkland et al., 2016; Stampar et al., 2019). Significantly increased levels of CYP1A2 expression and activity in 3D spheroids (Fig. 3) may produce high levels of genotoxic metabolites of the three aromatic amines and subsequently induced DNA damage. In contrast, 2-AAF, another aromatic amine also hydroxylated by CYP1A2, caused slightly increased DNA damage in 3D spheroids compared to 2D cultures (1.7- vs 1.1-fold) in the present study, but this was not statistically significant (Tab. 2). However, a recent study reported a positive response of PhIP and a negative response of IQ in both 2D and 3D HepaRG cultures using the comet assay, while 2-AAF was negative in 2D but positive in 3D cultures (Mandon et al., 2019). The discrepancy between the two studies may be due to different exposure times (24 h vs 48 h) and/or weak positive responses (~10% of tail DNA) in the comet assay (Fig. 4B).

The mycotoxin AFB1 requires metabolic activation by mainly CYP3A4 and 1A2 to form a very reactive 8,9-exo-epoxide metabolite (Kirkland et al., 2016; Le Hegarat et al., 2010). Although
3D spheroids expressed significantly higher mRNA levels and activities of CYP3A4 and 1A2, AFB1 induced greater cytotoxicity but not significant DNA damage in 3D spheroids compared to 2D cultured cells. AFB1 at 2.5 and 5 μM induced statistically significant increases in % tail DNA in 2D HepaRG cells after a 3-h exposure, while extended exposures of 24-h and 52-h showed no significant increases in DNA damage (Buick et al., 2021; Le Hegarat et al., 2010). AFB1 is known to induce mainly bulky DNA damage (Weng et al., 2017), but the alkaline comet assay is not sensitive to detect potentially carcinogenic bulky adducts. A recent study reported that application of DNA synthesis inhibitors (hydroxyurea (HU) and 1β-D-arabinofuranosyl cytosine (AraC)) significantly increased AFB1-induced DNA damage in HepaRG cells, demonstrating improved sensitivity of the CometChip assay by using HU/AraC to trap nucleotide excision repair (NER) intermediates for detecting bulky DNA adducts (Ngo et al., 2020). It requires further investigation as to whether the HU/AraC approach can be applied to other chemical classes, i.e., aromatic amines 2,4-DAT, 2-AAF, IQ, and PhIP for increasing the sensitivity of detecting bulky DNA adducts.

All five indirect-acting compounds that induced positive responses in both cultures showed similar or higher % tail DNA in 3D spheroids than in 2D HepaRG cells. Two polycyclic aromatic hydrocarbons, B[a]P and DMBA, are metabolically activated by CYP1A1 and 1B1 with the aid of epoxide hydrolase to form the ultimate carcinogenic metabolites, B[a]P-7,8-diol-9,10-epoxide and DMBA-3,4-diol-1,2-epoxide (Shimada and Fujii-Kuriyama, 2004). The two potent alkylating agents CPA and DMNA require metabolic activation by CYP2B6 and 2E1, respectively, to form highly reactive intermediate compounds that bind to the O~- and/or N7-position of guanine (Kirkland et al., 2016). Acrylamide is metabolized by CYP2E1 to a more DNA-reactive compound, glycidamide (Besaratinia and Pfeiffer, 2007; Manjanatha et al., 2006). Increased DNA damage responses in 3D HepaRG spheroids might be explained to some extent by 2-25-fold elevated gene expression levels and/or activities of CYPs that were involved in the metabolic activation of these compounds, i.e., CYP1A1, 1B1, 2B6, and 2E1 (Fig. 3B).

We further expanded our study and tested the DNA damage potential of 15 compounds that show different genotoxic responses in vitro and in vivo and found that only one compound, 3-MCPD, was positive in both 2D and 3D cultures. 3-MCPD was mutagenic in the Ames bacterial revertant mutation assay (IARC, 2013). The compound, along with its metabolites β-chlorolactic acid and glycidol, induced DNA damage under in vitro conditions (Ozcagli et al., 2016). However, 3-MCPD did not increase the frequencies of MN and pig-a mutant in red blood cells as well as gpt and Spi- mutations in the kidney and testis of F344 gpt-delta rats dosed with 3-MCPD 40 mg/kg bodyweight for four weeks (Onami et al., 2014). No DNA strand breaks were induced in the leukocytes, liver, kidney, testis, and bone marrow of rats dosed with 3-MCPD 60 mg/kg body weight for two days (El Ramy et al., 2007). Ethyl acrylate induced concentration-dependent increases in mutant frequency and chromosome aberrations in mouse lymphoma cells when tested without exogenous activation (Moore et al., 1988). However, it failed to induce sister chromatid exchange, DNA damage, chromosomal aberration, gpt or Spi- mutations in rodents in vivo (Ellis-Hutchings et al., 2018; IARC, 1999). In the present study, ethyl acrylate was more cytotoxic and increased % tail DNA in 2D cultured cells, but the increase was not significant in 3D spheroids (Fig. 5). Both 3-MCPD and ethyl acrylate induced tumors in rodents and were classified as Group 2B possible carcinogens to humans (IARC, 1999, 2013). However, ethyl acrylate is mainly metabolized by carboxylesterase-mediated hydrolysis and GSH conjugation (Ellis-Hutchings et al., 2018). Forestomach tumors observed in rodents were considered not relevant for human carcinogenicity and NTP delisted ethyl acrylate from the 9th edition of the Report on Carcinogens (Suh et al., 2018). The other eight in vitro (+) but in vivo (-) compounds were all negative in the CometChip assay conducted with 2D and 3D HepaRG models in the present study. These compounds did not induce tumors in rodents or had no in vivo data available (Tab. 1) (Kirkland et al., 2016). Based on these observations, we suggest that 3D HepaRG spheroids may significantly reduce the false-positive rate in current genotoxicity testing and serve as a reliable in vitro model in identifying compounds that indeed cause genotoxicity/carcinogenicity in humans.

Five compounds that were in vitro (-) but in vivo (+) all showed negative responses in the 3D HepaRG CometChip assay (Tab. 2, Fig. 5C). All five compounds were negative in the Ames test, but induced mutations and tumors in rodents, and 1,4-dioxane was the only compound in this group that was classified as a Group 2B possible human carcinogen by IARC (Tab. 1) (IARC, 1999). Although further investigation is required to determine whether the mutagenic and carcinogenetic effects induced by the other four compounds (dicyclanil, dimethyl terephthalate (DMTP), estragole, and leucomalachite green (LMG)) in rodents could reflect human responses, this observation demonstrates that the comet assay may not be as reliable as mutagenicity assays, i.e., pig-a assay, transgenic rodent (TGR) gene mutation assays for identifying mutagens/mutagenic carcinogens. Thus, the results provide evidence supporting that the comet assay may not be an appropriate follow-up test for in vitro mutagenicity assays to screen for in vivo mutagenicity (Robison et al., 2021).

Quantitative dose-response analysis of CometChip data using BMD modeling can promote derivation of a more precise point of departure than an LEC because the CometChip’s high-throughput feature enables the generation of a large number of data points covering low concentrations (Seo et al., 2019; Wills et al., 2016). It is worth noting that two promutagens, CPA and DMNA, showed significantly lower BMD50 values in 3D than in 2D HepaRG cultures likely due to the increased CYP450-mediated bioactivation of the two compounds in 3D HepaRG spheroids (Fig. 6). There was also a trend that PHHs have relatively lower or similar BMD50 values when compared with those generated from HepaRG comet data. In addition, we found that compared to 2D cultured cells, 3D spheroids had BMD50 values that were much closer to those of PHHs for almost all the compounds tested. These results suggest that 3D HepaRG spheroids more closely resemble PHHs than 2D HepaRG cells for generating DNA...
damage dose-response data. Therefore, for quantitative purposes, 3D HepaRG models might be preferred over 2D cultures to generate data for estimating health-based guidance values using quantitative in vitro to in vivo extrapolation approaches for human health risk assessment.

In conclusion, 3D HepaRG spheroids provide a useful hepatocyte-like in vitro model system with increased metabolic capacity and functionality compared to 2D HepaRG cells. The CometChip technology facilitates a high-throughput application of 3D HepaRG models. The improved sensitivity for detecting DNA damage responses of known genotoxicants/carcinogens in 3D spheroids suggests that 3D HepaRG spheroids predict in vivo genotoxicity responses better. Quantitative analysis using high-throughput dose-response data from 3D HepaRG spheroids generated BMD values close to those in PHHs. Overall, the developed in vitro system using metabolically competent 3D HepaRG spheroids can enhance the performance of in vitro genotoxicity testing by generating data that are more relevant to the human metabolic condition. Since chemicals may cause genotoxicity via various mechanisms (i.e., DNA damage, chromosomal aberration, and mutation), and the comet assay detects only DNA strand breaks, an integrated test battery that measures different genotoxicity endpoints using metabolically competent human hepatocyte spheroids is warranted for providing information for appropriate follow-up in vivo testing, thus reducing unnecessary animal studies.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

**Data availability**

Supplementary materials are available upon request from the corresponding author.

**Acknowledgements**

This study was supported by the U.S. Food and Drug Administration (FDA), National Center for Toxicological Research (NCTR, project number E0773701). J.E.S. was supported by appointments to the Postgraduate Research Program at the National Center for Toxicological Research (NCTR) administered by the Oak Ridge Institute for Science Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration (FDA). We greatly appreciate Drs Javier Revollo and Qiangen Wu for their critical review of this article.