Evaluation of genotoxicity using automated detection of γH2AX in metabolically competent HepaRG cells

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Abstract

The in situ detection of γH2AX was recently reported to be a promising biomarker of genotoxicity. In addition, the human HepaRG hepatoma cells appear to be relevant for investigating hepatic genotoxicity since they express most of drug metabolizing enzymes and a wild type p53. The aim of this study was to determine whether the automated in situ detection of γH2AX positive HepaRG cells could be relevant for evaluation of genotoxicity after single or long-term repeated in vitro exposure compared to micronucleus assay. Metabolically competent HepaRG cells were treated daily with environmental contaminants and genotoxicity was evaluated after 1, 7 and 14 days. Using these cells, we confirmed the genotoxicity of aflatoxin B1 and benzo(a)pyrene and demonstrated that dimethylbenzanthracene, fipronil and endosulfan previously found genotoxic with comet or micronucleus assays also induced γH2AX phosphorylation. Furthermore, we showed that fluoranthene and bisphenol A induced γH2AX while no effect had been previously reported in HepG2 cells. In addition, induction of γH2AX was observed with some compounds only after 7 days, highlighting the importance of studying long-term effects of low doses of contaminants. Together, our data demonstrate that automated γH2AX detection in metabolically competent HepaRG cells is a suitable high-throughput genotoxicity screening assay.

Introduction

Human exposure to a broad range of toxic compounds is virtually unavoidable because of the increasing contamination of air, water and food. Numerous environmental pollutants and food contaminants are known to induce mutations and cancers in animals but they are, with a few exceptions such as the aflatoxin B1 and benzo(a)pyrene, only suspected to exert mutagenic effects in human. However, chronic exposure to low doses of thousands of synthetic compounds generated by industries is strongly suspected to cause health issues, especially cancers. Therefore, the evaluation of the genetic toxicity of these chemicals remains a challenge.

The initial genotoxicity testing relies on a large battery of in vitro assays (1) that show a high sensitivity but suffer from a lack of specificity with an abnormally high rate of false positives compared with carcinogenicity studies in rodents (2). This led authors to propose follow-up actions to further discriminate the chemicals that trigger DNA damage by combining for each compound data on toxicity, genotoxicity, physicochemical properties and metabolism (3,4). However, there is still an obvious need to develop innovative methods for genotoxicity risk assessment to improve its prediction after single or long-term repeated in vitro exposure (1).

The liver is the major organ involved in the biotransformation of xenobiotics since it receives the blood stream coming from the digestive tract and since the adult hepatocytes express the most abundant
panel of drug metabolizing enzymes (5). The liver is the prime target of toxic xenobiotics and their down-stream reactive metabolites as demonstrated by the fact that drug-induced liver toxicity is the most frequent cause for the withdrawal from the market of approved medications (6). Because of the poor correlation between human and animal toxicity of chemicals, cultures of human primary hepatocytes are recognized as the most pertinent models for in vitro xenobiologic metabolism and toxicity studies (5). Their use is, however, limited because of their reduced life-span and the overall decrease in the expression of liver specific functions including xenobiologic-metabolizing enzymes (XMEs) in standard culture conditions (5). In addition, large variations in functional activities of XME have been reported in human hepatocytes isolated from different donors and the scarce and unpredictable availability of human biopsies limit considerably their use.

Hepatoma cell lines are an alternative to normal hepatocytes for genotoxicity studies (7–9). However, most cell lines have reduced expression of XMEs compared with primary cells (10), which may limit the assessment of genotoxicity mediated by downstream metabolites. The human HepaRG hepatoma cells are bipotent hepatic progenitors with high proliferation potential that exhibit the remarkable ability to differentiate into bile and hepatocyte-like cells (11). This cell model represents a unique in vitro system to investigate the molecular pathways of hepatocyte differentiation (11). HepaRG hepatocyte-like cells express most of the major Phase I, II and III enzymes (12,13) and are now used worldwide as an alternative in vitro model to primary hepatocytes for studying metabolism and toxicity of xenobiotics (14–16).

Differentiated HepaRG hepatocyte-like cells seem particularly suitable for the prediction of genotoxicity (16–21). These metabolically competent cells can be kept confluent and quiescent over several weeks while retaining a long-term expression of XMEs, which is a useful feature for chronic toxicity and genotoxicity studies. In addition, they can re-enter the cell cycle and proliferate when plated at low density, which allows comet and micronucleus assays to be performed (20). Furthermore, they express a wild type tumour suppressor p53 gene (11) and retinoblastoma protein (H. Dubois-Pot-Schneider and A. Corlu, personal communication) two major regulators of DNA repair and cell cycle DNA damage check-point.

A novel in vitro genotoxicity assay has been recently developed based on the in situ detection of the phosphorylation of the histone H2AX in human cells (7–9,22–24). The phosphorylation of the histone H2AX also called γH2AX occurs after a DNA double strand break in cell genome and reflects a global genotoxic insult that may originate from different types of DNA damage (4,24). The aim of this study was to determine whether the γH2AX assay in HepaRG hepatocyte-like cells using an automated in situ detection of positive cells could be relevant for toxicity and genotoxicity studies after single or long-term repeated in vitro exposure compared with micronucleus assay. For this purpose, 10 environmental contaminants were selected (supplementary Data 1, available at Mutagenesis Online) including the mycotoxin aflatoxin B1 (AFB1), the alkyllating agent methyl methane sulfonate (MMS), the organochlorine insecticide and acaricide endosulfan (Endo), the phenylpyrazole class insecticide fipronil (Fipro), the brominated flame retardant tetrabromobisphenol A (TBBPA), three polycyclic aromatic hydrocarbons [benzo[a]pyrene (BaP), dibenzoylmethane (DMBA), fluoranthene (Fluo)] and two plasticizers [bisphenol A (BPA) and diethylhexylphthalate (DEHP)]. While some of these compounds, such as AFB1 and MMS are known to induce genotoxicity and are often used as positive controls for in vitro testing, controversial results have been reported regarding the genotoxicity of the other selected compounds.

The AFB1 and the MMS trigger genotoxicity through different mechanisms. MMS is a direct genotoxic chemical while AFB1 induces DNA damage following CYP3A4-dependent biotransformation in the reactive exo-8,9 epoxide metabolite. B[a]P induces genotoxicity via its metabolism by the CYP1A1 and/1B1 into various metabolites including the highly reactive benzo[a]pyrene -7,8-dihyd- drodiol-9,10-epoxide. DMBA is metabolized via the activities of epoxide hydrolases and the CYP1A1 and 1B1 to produce several epoxides generating DNA adducts. The data are more controversial about the genotoxicity of Fluo, which produces mutagenic metabolites following activation by hepatic microsomes (25), however, its mutagenicity in vivo remains controversial. The Endo and Fipro and/or their metabolites formed through the activity of the CYP2B6 and CYP3A4 are hepatotoxic compounds (26) but are not considered as carcinogens although previous articles reported genotoxic effects in vitro (27,28). Similarly, the BPA, DEHP and TBBPA mainly metabolized by UDP-glucuronosyltransferases and/or sulfotransferases are not powerful genotoxic agents but previous studies have reported that these compounds were able to induce DNA damage (8,29–32). We thus investigated whether these compounds could induce phosphorylation of H2AX in HepaRG cells that express most Phase II enzymes.

**Materials and methods**

**Reagents**

The environmental contaminants (supplementary Data 1, available at Mutagenesis Online): Aflatoxin B1, (AFB1), benzo[a]pyrene (B[a]P), bisphenol A (BPA), diethylhexyl phthalate (DEHP), 7,12-dimethylbenz[α]anthracene (DMBA), endosulfan (Endo), fipronil (Fipro), fluoranthene (Fluo), methyl methanesulfonate (MMS), tetrabromobisphenol A (TBBPA) and insulin were purchased from Sigma (Saint Quentin Fallavier, France). William’s E medium was purchased from Eurobio (Les Ulis, France). Foetal calf serum (FCS) was obtained from Lonza (Levallois-Perret, France). Penicillin, streptomycin and 1-glutamine were purchased by Life Technologies (Saint Aubin, France).

**HepaRG cell culture and treatment conditions**

HepaRG cells were seeded at a density of 5 x 10⁴ cells/cm² and cultured in William’s E medium supplemented with 10% of FCS, 100 units/ml penicillin and 100 μg/ml streptomycin, 5 μg/ml insulin, 2 mM 1-glutamine and 50 μM hydrocortisone hemisuccinate. After 2 weeks, cells were cultured in the same medium supplemented with 2% dimethysulfoxide (DMSO) for two more weeks prior to selective seeding of hepatocytes-like cells at a density of 1.6 x 10⁴ cells/cm². For toxicity assays, cells were exposed daily to contaminants according to the protocol (supplementary Data 2, available at Mutagenesis Online). William’s E medium was supplemented with 2% FCS and 1% DMSO for acute and chronic treatments.

**MTT assay**

The cytotoxicity was assessed using the Thiazolyl Blue Tetrazolium Bromide (MTT). Briefly, cells were incubated with MTT (0.5 mg/ml) in William’s medium without FCS for 1 h at 37°C. The formed crystals were dissolved in DMSO at room temperature for 10 min and the absorbance was read at 490 nm with a microplate reader (Polarstar Omega, BMG Labtech). The MTT values reflecting the number of viable cells were expressed in percentage relatively
to the absorbance determined in control cultures. IC₉₀ were calculated using linear regression and expressed as mean ± SEM (the standard error of the mean) of at least three independent experiments.

**γH2AX and micronucleus assays**

The γ-H2AX test was performed using Cellomics™ Phospho-H2AX Activation kit (Thermo scientific) following the manufacturer's instruction. Briefly, after treatment of 1, 7 or 14 days, cells were rinsed with phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde in PBS for 15 min at room temperature before permeabilisation using 0.1% saponin. Nonspecific binding was blocked by incubating the cells with 2% of foetal bovine serum diluted with the manufacturer buffer for 15 min. Then, the cells were incubated with anti-phospho-H2AX antibody solution for 1 h at room temperature. Following washes with the buffer provided, the Dylight 549-labeled secondary antibody solution containing Hoechst 33342 dye (1 µg/ml) was incubated for 45 min. After washing, the plate was read on Cellomics™ Arrayscan VTI HCS Reader (Thermo Scientific) and at least ten pictures per well in triplicate were analyzed corresponding to a minimum of 6000 cells scored. The green fluorescence corresponding to γ-H2AX foci and co-localizing with Hoechst was integrated, weighted with nuclear number and expressed as a fold change. The treated culture was compared with control condition in order to obtain an induction factor. Relative cell count (RCC or final cell count (treated)/final cell count (control) × 100) was also assessed by the automated counting of Hoechst-stained nuclei in order to compare with the MTT data.

The micronucleus assay was performed as previously described (18). Briefly, after selective seeding in 48 well plates, cells were daily treated for 1 or 7 days prior to 3 days treatment with human epidermal growth factor (EGF). Cells were fixed for 10 min in 4% paraformaldehyde before permeabilisation with PBS containing 10% donkey serum and 0.1% saponin. F-actin was labeled with 1/400 phalloidin conjugated to sulforhodamine for 20 min and nuclei stained with Hoechst 33342 (1 µg/ml) for 5 min. Ten images per wells were taken with Cellomics™ HCS Reader. The number of nuclei was automatically assessed by image analysis using Image J software while micronuclei were manually and blindly scored. At least five pictures were scored until to reach a total of 2500 cells. All experiments were performed in triplicate. Criteria used for identifying micronuclei were: area less than one-third the nucleus, no overlap with the nucleus (distinct borders) and same aspect as the chromatin. The induction factor calculated corresponds to the ratio of the number of micronucleated (MN) cells in treated cultures compared with the number of MN cells in vehicle-treated control cultures.

**Data analysis**

Genotoxicity was considered positive when treatments produced a level of toxicity ≤50% [decrease in MTT or final cell count (RCC)] and induced at least a statistically significant 1.5-fold increase in γH2AX staining or micronucleus count (24). For choosing the appropriate concentrations in the micronucleus assay, the cytotoxicity was based on RCC despite the fact that RCC and RPD might be slightly different for some compounds and that the HepaRG cells were not in the same stage for H2AX assay and micronucleus scoring. Error bars represent the standard error of the mean (SEM). Statistical analyses were performed using Student's t-test. Statistically significant increases in H2AX phosphorylation after treatment were compared with controls using Student's test with Excel software; *P < 0.05; **P < 0.01.

**Results**

We first measured the acute and chronic cytotoxicity in order to determine the IC₉₀ of the 10 environmental contaminants in HepaRG hepatocyte-like cells (supplementary Data 3, available at Mutagenesis Online). In our experimental conditions, the differentiation status of HepaRG cells was maintained over the 14 days of treatment as demonstrated by the constant CYP3A4 expression level (supplementary Data 2, available at Mutagenesis Online). The cells were exposed to high concentrations of each compound with a single exposure for 24 h or to lower concentrations of each contaminant for daily treatments during 7 and 14 days (supplementary Data 3, available at Mutagenesis Online). The cytotoxicity was evaluated using the MTT assay at 1, 7 or 14 days and IC₉₀ were calculated (Table 1).

With the exception of the Fluo, DEHP and MMS, the cumulative treatments resulted in enhanced cytotoxic effects over time with decreasing IC₉₀ between 24 h and 7 days. For instance, a 35-fold IC₉₀ decrease was observed for the B(a)P. Prolonging the treatment up to 14 days kept on lowering significantly IC₉₀ of AFB1 (0.4 to 0.1 µM after 7 and 14 days, respectively), B(a)P (10 to 7 µM), DMBA (12 to 6 µM), Endo (57 to 12 µM) and TBBPA (38 to 25 µM).

It is widely admitted that *in vitro* genotoxicity assays must be performed at concentrations of compounds that maintained the viability of treated cultures above the IC₉₀ value (24). In this study, we selected three to four doses of each compound (supplementary Data 1 and 3, available at Mutagenesis Online) inducing moderate levels of cytotoxicity to perform the γH2AX assay (supplementary Data 4 and 5A, available at Mutagenesis Online) with automated detection using the Cellomics™ device (Figure 1). On separate 96-well plates, we confirmed with the MTT assay (Figure 1) that the viability remained for all compounds above 60% of the value obtained for control cultures demonstrating that these concentrations were compatible with genotoxicity studies on HepaRG cells.

Polycyclic aromatic hydrocarbons [B(a)P, DMBA and Fluo], AFB1, MMS, Endo and BPA showed a significant induction of H2AX positive cell index after 24 h whereas DEHP, TBBPA and Fipro did not (Figure 1). Interestingly, we showed for the first time in HepaRG cells the genotoxicity of Fluo (10 µM) and BPA (40 µM)

<table>
<thead>
<tr>
<th>IC₉₀ ± SEM</th>
<th>AFB1</th>
<th>B(a)P</th>
<th>BPA</th>
<th>DEHP</th>
<th>DMBA</th>
<th>Endo</th>
<th>Fluo</th>
<th>Fipro</th>
<th>MMS</th>
<th>TBBPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>1.8 ± 0.2</td>
<td>355 ± 15</td>
<td>108 ± 5</td>
<td>313 ± 25</td>
<td>292 ± 27</td>
<td>109 ± 4</td>
<td>217 ± 40</td>
<td>118 ± 22</td>
<td>240 ± 15</td>
<td>86 ± 6</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.4 ± 0.1</td>
<td>10 ± 2</td>
<td>87 ± 2</td>
<td>292 ± 25</td>
<td>12 ± 3.5</td>
<td>57 ± 4</td>
<td>257 ± 31</td>
<td>32 ± 2</td>
<td>177 ± 5</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.1 ± 0.05</td>
<td>7 ± 2</td>
<td>89 ± 2</td>
<td>349 ± 16</td>
<td>6 ± 0.5</td>
<td>12 ± 4</td>
<td>263 ± 32</td>
<td>33 ± 3</td>
<td>197 ± 33</td>
<td>25 ± 5</td>
</tr>
</tbody>
</table>

HepaRG cells were treated with a wide range of concentrations for the 10 selected compounds (supplementary Data 3, available at Mutagenesis Online) during 1, 7 or 14 days. The toxicity was evaluated by MTT assay and IC₉₀ (µM) ± SEM was determined using the data from three independent experiments.
at 24 h using γH2AX assay. Fluo did not show any effect on HepG2 cells in previously reported data performed, however, at lower doses (8,9) while BPA was also found positive (8). We next investigated the γH2AX staining following 1 or 2 weeks of drug exposure at low doses. Fipro that did not affect H2AX staining after 24 h induced genotoxicity after 7 or 14 days. For BPA, Fluo, and B(a)P that increased the γH2AX index at 24 h, the response became negative after chronic exposure over 7 and 14 days of treatment. Interestingly, a single exposure to 100 µM DMBA induced a 1.7-fold increase in the γH2AX positive cell number at 24 h and a 3.7- to 12-fold increase after 7 days at concentrations of 2–4 µM. The continuous exposure up to 14 days did not further enhance the γH2AX induction compared to the 7 day-exposure. Finally, the TBBPA and DEHP showed no effect on γH2AX staining.

Since the γH2AX assay is based on an automated approach, we evaluated whether an all-in-one assay double staining combining the γH2AX assay and the counting of Hoechst 33342-stained nuclei could provide a cytotoxicity endpoint in order to avoid performing a separate MTT assay. The Relative Cells Count (RCC) was established from the count of Hoechst-stained nuclei and was compared to MTT assay (supplementary Data 6, available at Mutagenesis Online). The two methods provided relatively similar results although for high concentrations of DMBA and Endo, viability levels appeared higher when using MTT. This result is in accordance with a previous study (23). However, regardless of the test used, the levels of cytotoxicity induced by the selected doses remained below 50% of the control which was the maximal cytotoxicity level accepted for genotoxicity observation. These data support the use of the γH2AX and RCC

Figure 1. MTT assay and detection of phospho-histone H2AX in HepaRG cells treated with low doses of environmental contaminants. HepaRG cells were treated with the indicated concentrations of contaminants for 1, 7 or 14 days. The toxicity was evaluated by MTT assay and the genotoxicity was measured using automated in situ detection of γH2AX positive cells. The control value was arbitrarily set at 1 and results were expressed as relative to control. Bars represent the average of at least three independent experiments (SEM). Statistics were realized using Student’s *t*-test: *$P<0.05$, **$P<0.01$, ***$P<0.001$. 

1 day of treatment

7 days of treatment

14 days of treatment
In order to further validate the sensitivity of the γH2AX assay, we quantified the micronuclei in HepaRG cells exposed to AFB1, B(a)P, BPA, Fipro and DMBA for 1 or 7 days before mitogenic stimulation by EGF for 3 days and visualization of Hoechst-stained micronuclei (supplementary Data 5B, available at Mutagenesis Online and Table 2). AFB1, B(a)P, Fipro and DMBA significantly increased the percentage of micronuclei confirming that the γH2AX assay provided relevant genotoxicity data correlating with the micronucleus assay in the HepaRG hepatocyte-like cells (Table 2). The correlation was particularly evidenced for DMBA and AFB1 which gave a concentration and time-dependent response after 1 and 7 days of treatment. In contrast, BPA transiently induced γH2AX at 24h but not at 7 days and did not significantly increase micronuclei whereas the genotoxicity of Fipro appeared detectable only after 7 days of treatment in the 2 tests. These data reinforce the conclusion that γH2AX is a promising biomarker of genotoxicity complementing the data obtained with the micronucleus assay as previously reported in HepG2 cells (24).

Discussion

In the present study, we investigated the feasibility and relevance of the γH2AX assay in HepaRG cells for genotoxicity studies of environmental contaminants. The activation of γH2AX was analyzed in cells exposed to low doses of the direct inducer of DNA damage MMS and the known genotoxic compounds AFB1, B(a)P and DMBA requiring metabolic activation. We also tested compounds (Endo, Fipro, Fluo, BPA, DEHP and TBBPA) that are not considered as powerful genotoxins but previously reported positive in one or several in vitro genotoxicity assays. Since differentiated HepaRG cells are metabolically active and stable for several days, the aim of the study was also to compare H2AX induction after single or long-term repeated in vitro exposures.

Our results confirmed and extended previously published in vitro genotoxicity data (Table 3) with the same compounds and using the comet, micronucleus and/or γH2AX assays. All compounds [AFB1, MMS, Endo, Fipro, DMBA and B(a)P] known to induce genotoxicity in various cell models also increased γH2AX index in HepaRG cells. However, the genotoxicity of Fipro and Endo previously reported using comet or micronuclear assays in human hepatic cells (Table 3) was here evidenced with the γH2AX staining. Moreover, for all these compounds, the concentrations inducing the γH2AX activation in HepaRG cells were compatible with data available in the literature (Table 3) with the exception of the Fipro inducing genotoxicity at 20 μM in HepaRG cells while lower concentrations of 0.2 and 1.6 μM were found positive in peripheral blood cells using the micronucleus and comet assays, respectively (27). DEHP and TBBPA are mainly metabolized by UDP-glucuronosyltransferases and/or sulforotransferases, which are well expressed in HepaRG and at much lower levels in HepG2 cells. We therefore investigated whether these compounds induced γH2AX in HepaRG cells that catalyse Phase II reactions. Both TBBPA and DEHP were negative in the γH2AX performed in HepaRG cells. There are no data showing genotoxicity of TBBPA in human cells but DNA damage was documented in liver cells of the fish fathead minnows (30). Although DEHP was found negative in most non-mammalian in vitro mutation assays, it was reported positive in comet assay in HepG2 cells (29).

The comparison between the γH2AX and micronucleus assays showed a good correlation for AFB1, B(a)P, Fipro, and DMBA but not with BPA. For BPA, the γH2AX assay was positive only at Day 1.
Table 3. In vitro genotoxicity data obtained for the environmental contaminants in various cell models

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell line</th>
<th>Treatment time (h/day)</th>
<th>Assays</th>
<th>Effective concentration (μM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B(a)P</td>
<td>HepG2</td>
<td>24 h</td>
<td>H2AX</td>
<td>0.1</td>
<td>(9,33)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Comet</td>
<td>50</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Micronucleus</td>
<td>0.25</td>
<td>This study</td>
</tr>
<tr>
<td>DMBA</td>
<td>HepaRG</td>
<td>1–14 days</td>
<td>H2AX</td>
<td>0.01</td>
<td>(9,33)</td>
</tr>
<tr>
<td></td>
<td>HepG2</td>
<td>24 h</td>
<td>H2AX</td>
<td>0.5–4</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Micronucleus</td>
<td>0.3</td>
<td>(33)</td>
</tr>
<tr>
<td>Fluo</td>
<td>HepaRG</td>
<td>1–14 days</td>
<td>H2AX</td>
<td>No effect</td>
<td>(9)</td>
</tr>
<tr>
<td></td>
<td>HepG2</td>
<td>24 h</td>
<td>H2AX</td>
<td>100</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Micronucleus</td>
<td>1.6</td>
<td>(27)</td>
</tr>
<tr>
<td>Fiprol</td>
<td>PBMC</td>
<td>72 h</td>
<td>Comet</td>
<td>0.2</td>
<td>(27)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Micronucleus</td>
<td>50–1000</td>
<td></td>
</tr>
<tr>
<td>Endo</td>
<td>HepaRG</td>
<td>7–14 days</td>
<td>H2AX</td>
<td>20</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>HepG2</td>
<td>48 h</td>
<td>Comet</td>
<td>200</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Micronucleus</td>
<td>30</td>
<td></td>
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<tr>
<td>TBBPA</td>
<td>No data available in vitro on human cells</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>HepaRG</td>
<td>1–14 days</td>
<td>H2AX</td>
<td>No effect</td>
<td>This study</td>
</tr>
<tr>
<td>BPA</td>
<td>MCF-7</td>
<td>24 h</td>
<td>Comet</td>
<td>1</td>
<td>(31)</td>
</tr>
<tr>
<td></td>
<td>HepG2</td>
<td>24 h</td>
<td>H2AX</td>
<td>No effect</td>
<td>(8)</td>
</tr>
<tr>
<td>DEHP</td>
<td>Rat hepatocytes</td>
<td>24 h</td>
<td>Micronucleus</td>
<td>No effect</td>
<td>(2,34)</td>
</tr>
<tr>
<td></td>
<td>HepaRG</td>
<td>1–14 days</td>
<td>H2AX</td>
<td>No effect</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>HepG2</td>
<td>24–48 h</td>
<td>Comet</td>
<td>2.5</td>
<td>(29)</td>
</tr>
<tr>
<td></td>
<td>LNCaP</td>
<td>24 h</td>
<td>3000</td>
<td></td>
<td>(32)</td>
</tr>
<tr>
<td>AFB1</td>
<td>HepG2</td>
<td>12–24 h</td>
<td>H2AX</td>
<td>1</td>
<td>(24,35)</td>
</tr>
<tr>
<td></td>
<td>HepaRG</td>
<td>24 h</td>
<td>Comet</td>
<td>2.5</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Micronucleus</td>
<td>0.1–0.25</td>
<td>(17,18)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>H2AX</td>
<td>0.025</td>
<td>This study</td>
</tr>
<tr>
<td>MMS</td>
<td>TK6</td>
<td>24 h</td>
<td>Comet</td>
<td>36</td>
<td>(36)</td>
</tr>
<tr>
<td></td>
<td>FL</td>
<td>24 h</td>
<td>H2AX</td>
<td>10</td>
<td>(37)</td>
</tr>
<tr>
<td></td>
<td>HepaRG</td>
<td>1–14 days</td>
<td>Micronucleus</td>
<td>90</td>
<td>(18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H2AX</td>
<td>2</td>
<td>This study</td>
</tr>
</tbody>
</table>

When available, the results in human hepatic cells are presented. The concentration is the lowest concentration inducing a significant change in the genotoxicity assay.

in our study but did not increase the micronucleus index in HepaRG cells and had no effect on HepG2 cells using the γH2AX assay (8). In contrast, the comet assay performed using MCF-7 cells was positive (31). Similarly, Fluo induced γH2AX assay in our study but had no effect in HepG2 cells using the same assay (9). For these two compounds, the data are not sufficient to conclude that these chemicals are genotoxic and the hypothesis of false positives remains a possible explanation.

The relevance of the γH2AX positive results in HepaRG cells following exposure to Fiprol and Endo is also questionable. In our study, phosphorylation of H2AX was not induced by Fiprol at Day 1 but became positive at Days 7 and 14. In addition, the micronucleus assay was also positive. Similarly, it was reported that Fiprol induced genotoxicity in PBMC using comet and micronucleus assays (27). Together, these data suggest that prolonged exposure to this chemical could trigger genotoxic effects. For Endo, a similar conclusion could be drawn based the observation that γH2AX was induced at the three time points in our study and the previous data showing positive comet and micronucleus assays in HepG2 cells (28).

The discrepancies in the effective concentrations of compounds inducing γH2AX and/or micronuclei between HepG2 (Table 3) and HepaRG are most likely related to the larger panel of Phase I and II metabolising enzymes expressed in HepaRG cells (12–15). For instance, AFB1 is converted into the highly reactive 8,9-exo-epoxide metabolite by the CYP3A4, which is expressed at a much higher level in HepaRG cells. This could explain why low doses of 0.025–0.1 μM were genotoxic in HepaRG compared to doses of 0.1 (24) to 1 μM (35) in HepG2 cells. We also showed that the effective concentration of Endo in HepaRG is lower than the effective doses found in HepG2 cells (28). Similarly, HepG2 transformants expressing high levels of CYP3A4 are more sensitive to Endo than the parental cells (33). Conversely, the compounds that do not require metabolic activation to form genotoxic metabolite(s) are likely to appear less genotoxic with higher effective concentrations in HepaRG cells since they will be metabolized by Phase I and II enzymes and secreted by phase III transporters also expressed at high levels in HepaRG cells (38).

The discrepancies on the magnitude and the timing of the genotoxicity between the two different assays may rely on the fact that γH2AX and micronucleus tests are based on different parameters and evidenced different stages/steps of genotoxicity. The detection of phosphorylated histone H2AX reflects an early response to genotoxic phenomena resulting from chromosomal breakages and/or mitotic catastrophe. Furthermore, the two tests are performed using quiescent and proliferating cells for γH2AX and micronucleus assays, respectively. These two tests performed in HepaRG cells should not be considered mutually exclusive and both assays provide important information on the mechanisms of genotoxicity.
In conclusion, this work is the first study applying metabolically competent HepaRG cells for high-throughput screening of genotoxicity using automated detection of γH2AX and demonstrates that this assay is suitable for genotoxicity and cytotoxicity screening in liver cells. In addition, the induction of γH2AX observed with some of the molecules only after 7-day exposures highlight the importance of studying the long term effects of low doses of contaminants. The HepaRG hepatocyte-like cells are suitable for chronic genotoxicity studies because they express a large set of drug metabolising enzymes (12) and show long-term functional stability during chronic treatments (16). Besides the metabolic capacity, an important aspect for genotoxicity studies is the fact that HepaRG cells express wild type tumour suppressors p53 and retinoblastoma protein, two major pathways regulating DNA repair and damage check-point during the cell cycle. It was previously reported that the use of human cells bearing functional p53 reduces the rate of false positive results in genotoxicity assays (39).

Together, our data suggest that the detection of γH2AX in HepaRG cells can be useful in acute and chronic genetic toxicology. Other carcinogenic and in vitro genotoxic compounds with different modes of action but also negative compounds need to be tested on HepaRG cells to confirm these conclusions.

Supplementary data
Supplementary Data 1–6 are available at Mutagenesis Online.

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