Performance of Comet and Micronucleus Assays in Metabolic Competent HepaRG Cells to Predict In Vivo Genotoxicity

Ludovic Le Hégarat,*,† Annick Mourot,* Sylvie Huet,* Lucie Vasseur,* Sandrine Camus,† Christophe Chesné,† and Valérie Fessard*

*ANSES, French Agency for Food, Environmental and Occupational Health & Safety, Fougeres Laboratory, Toxicology of contaminants Unit, Fougeres, France; and |Biopredic International, 8-18, rue Jean-Pecker, 35000 Rennes, France

†To whom correspondence should be addressed at Anses Laboratoire de Fougeres, Unité de Toxicologie des contaminant, 10 B rue Claude Bourgelat – Javené CS 40608, 35306 Fougères Cedex, France. Fax: +33-0-2.99.94.78.80. E-mail: ludovic.lehegarat@anses.fr.

Received September 25, 2013; accepted December 15, 2013

Genetic toxicity information is critical for the safety assessment of all xenobiotics. In the absence of carcinogenicity data, genetic toxicity studies may be used to draw conclusions about the carcinogenicity potential of chemicals. However, current in vitro assays have many limitations as they produce a high rate of irrelevant positive data and possible false negative data due to the weakness of the in vitro models used. Based on the knowledge that the majority of human genotoxic carcinogens require metabolic activation to become genotoxic, it is necessary to develop in vitro cell models that mimic human liver metabolism to replace the use of liver S9 fraction, which, though helpful for predicting the potential carcinogenicity of chemicals in rodents, is questionable in humans. We therefore investigate whether the recently described human hepatoma HepaRG cells, which express the major characteristics of liver functions similarly to primary human hepatocytes, could be a suitable model for human genotoxicity assessment. We determine the performance of comet and micronucleus assays in HepaRG cells to predict in vivo genotoxins based on the list of compounds published by European Centre for the Validation of Alternative Methods (ECVAM). Twenty compounds were tested in HepaRG cells with comet and micronucleus assays over a 24-h period. The specificity, the sensitivity, and the accuracy of the two tests were determined. We found that the comet assay had higher specificity (100%) than the micronucleus (MN) test (80%), whereas the latter was far more sensitive (73%) than the former (44%), resulting nonetheless in an accuracy of 72% for the comet assay and 75% for the MN test. Taken together, our data suggest that the HepaRG cell line can be of use in genetic toxicology and that efforts to develop competent human liver models should be increased.

Key words: hepatocytes; bioactivation; sensitivity; specificity; predictivity; genotoxicity.

All these factors could reduce the performance of in vitro genotoxicity assays for accurately predicting in vivo genotoxicity in humans. Efforts have consequently been made to develop more predictive human cell models to achieve these ends. Considering that many human carcinogens required one or several steps of phase I and/or II metabolism to become genotoxic, special attention is given to developing in vitro genetic toxicity testing on human liver cell lines. However, human primary hepatocytes cannot easily be used to detect induction of stable mutations due to their inability to divide, and the HepG2 cell line is only recommended when adding rat liver S9 due to its low liver metabolism capacities. HepaRG cells, which have been shown to express various phase I and phase II enzymes, transporters, and nuclear factors, including the constitutively activated receptor (CAR), at levels close to those found in primary human hepatocyte cultures (Aninat et al., 2006; Anthérieu et al., 2010, 2012; Kanebratt and Andersson, 2008a), probably provide the most appropriate human liver in vitro model for genotoxicity testing. Moreover, this model can proliferate after seeding at low density, which is a key issue for performing the micronucleus test. Although previous studies confirmed that comet and micronucleus assays can be set up on the HepaRG cell line (Bazin et al., 2010; Jossé et al., 2012; Le Hegarat et al., 2010), they did not evaluate the performance of predicting in vivo genotoxicity using this cell model. Indeed, performance determination, including both the sensitivity (proportion of genotoxic carcinogens that generated positive results) and the specificity (proportion of nongenotoxic compounds that generated negative results) (Cooper, 1979; Walmsley and Billington, 2011), requires the testing of more compounds.

In the present study, we first investigated the stability of the activities of five major phase I P450 enzymes (1A2, 2B6, 2C9, 2D6, and 3A4) in differentiated HepaRG cells under the cell culture conditions of our experiment. Second, we determined the performance of the in vitro comet assay and micronucleus tests using differentiated HepaRG cells. The variability of the level of DNA damage in the comet assay and the spontaneous
rate of micronucleus formation in negative controls, as well as the reproducibility for detecting the promutagen cyclophosphamide, were calculated from 10 independent experiments. In addition, the results from 20 chemical compounds selected from the list recommended by the ECVAM workgroup (Kirkland et al., 2008) enabled us to estimate the sensitivity, the specificity, and the accuracy of the two tests for predicting in vivo genotoxicity, alone or in combination.

MATERIALS AND METHODS

Chemicals. 1-ethyl-1-nitrosourea (ENU), 2-acetylaminofluorene (2-AAF), 2,4-diaminotoluene (2,4-DAT), 4-nitroquinoline (4-NQO), 7,12-dimethylbenzanthracene (DMBA), aflatoxin B1 (AFB1), cyclophosphamide (CPA), cytochalasin-B (cyt-B), dimethylsulfoxide (DMSO), etoposide, cadmium chloride (CdCl₂), D-limonene, D-mannitol, EDTA, Di-(2-ethylhexyl)-phtalate (DHEP), ethanol, estragole, eugenol, insulin, methylcarbamate, styrene, taxol, tetrachloroethyleno (TCE), phenacetin, bupropion, tolbutamide, and dextromethorphan were purchased from Sigma (St. Quentin-Fallavier, France), and midazolam from Pharmacopée Européenne. Methylmethanesulfonate (MMS) was purchased from Acros Organics (Geel, Belgium). Williams E medium was supplied by Eurobio (Les Ulis, France), foetal calf serum (FCS) from Perbio (Brebières, France), and penicillin and streptomycin from Invitrogen Corporation (Illkirch, France). Hydrocortisone hemisuccinate was purchased from Pharmacia & Upjohn (Guyancourt, France). All other chemicals were of the highest quality available.

Cryopreserved differentiated HepaRG cell culture. Cryopreserved HepaRG cells were plated and seeded at high density in 96-, 48-, and 24-well plates coated with collagen I at 216,000 cells/cm² for the cytotoxicity, comet and micronucleus assays in Williams E medium supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 2mM glutamine, and 5 × 10⁻⁵ hydrocortisone hemisuccinate. Twenty four hours after seeding, the medium was replaced with medium containing 2% of DMSO and renewed every two days. At day 7, the HepaRG cells were highly differentiated and exposed to chemicals.

Evaluation of P450 activities. For the determination of CYP450 activities, cells were incubated for 2 h with a cocktail of five substrates: 200µM phenacetin (CYP1A2), 100µM bupropion (CYP2B6), 100µM tolbutamide (CYP2C9), 50µM midazolam (CYP3A4), and 100µM dextromethorphan (CYP2D6) as described previously (Anthérieu et al., 2010; Lahoz et al., 2008). At the end of this period, aliquots of medium, 150 µl, were mixed with 150 µl acetonitrile and the samples were stored at −80°C until analysis. Metabolites formed and released into the culture medium were quantified by high performance liquid chromatography (HPLC) tandem mass spectrometry as previously described (Anthérieu et al., 2010).

Selection of the tested compounds. Twenty chemicals were selected from the list published by Kirkland et al. (2005) to determine the performance of the in vitro genotoxic assays on HepaRG cells:

- eight Ames-positive in vivo genotoxins including two direct mutagens (ENU, 4-NQO), five compounds that required metabolic activation (DMBA, 2-AAF, AFB1, 2,4-DAT, CPA), and one inorganic carcinogen, CdCl₂;
- three in vivo genotoxins negative or equivocal in the Ames test: etoposide, taxol, and styrene, the latter requiring CYP2E1 activation to become genotoxic;
- three nongenotoxic carcinogens: D-limonene, DEHP, and methyl carbamate;
- two noncarcinogens with negative in vivo genotoxic data: D-mannitol and EDTA;
- and a supplementary list of four equivocal genotoxic compounds: ethanol, TCE, eugenol, and estragole.

Preparation of compound solutions. All the compounds were dissolved in DMSO. For all assays, the final concentration of DMSO never exceeded 1%.

Comet assay. The comet assay was performed as described previously (Jossé et al., 2008; Le Hegarat et al., 2010; Fig. 1). DNA was stained with propidium iodide (2.5 µg/ml in PBS) just before blind-slide scoring with a fluorescence microscope (Leica DMR) equipped with a CCD-200E video camera. At least 100 cells per concentration and treatment time were analyzed using the Comet Assay IV software (Perceptive Instruments, Haverhill, UK). The extent of DNA damage in individual cells was evaluated by the percentage of tail DNA. Three independent experiments were conducted.
Cytokinesis-block micronucleus assay. The micronucleus assay was performed as described previously with minor modifications (Le Hégarat et al., 2010). Once differentiation was reached, cells were exposed to chemicals in FCS-free medium for 24 h. At the end of treatment, cells were washed twice with PBS, trypsinized and seeded at a density of 2.6 × 10⁴ cells/cm² in a two-chamber Lab-Tek Slide system. Twenty four hours after seeding, the medium was replaced with one containing 10% FCS and 3 µg/ml of Cyt-B for a further one cell cycle time division of 24 h leading to more than 75% binucleated cells in negative controls. The cells were washed twice with PBS followed by a 1.5 h recovery step in fresh 10% FCS supplemented medium. Cells were fixed in methanol/acetic acid, v/v, 9/1 for 10 min prior to staining with orange acridine for 10 min. Slides were observed under a Leica fluorescence microscope.

All slides were scored blindly. The criteria used for identifying micronuclei were those recommended by the HUMN project (Fenech et al., 2003). Micronuclei were scored in 2000 binucleated cells in duplicate cultures and cytotoxicity was determined in 500 cells per culture where binucleated, mononucleated, and polynucleated cells were scored.

For cytotoxicity assessment, the cytostasis was expressed as 100 – RI (Kirsch-Volders et al., 2003), where:

\[
RI = \frac{(\text{Number of binucleated cells} + 2 \times \text{polynucleated cells})}{\text{total number of cells treated}} / \frac{(\text{number of binucleated cells} + 2 \times \text{polynucleated cells})}{\text{total number of cells control}}
\]

At least one MN experiment on duplicate cultures was conducted for all compounds, and when the results obtained in the MN assay were inconsistent with comet assay, the experiment was repeated.

Statistical analysis. Results (medians) of the comet assay from the three independent experiments were analyzed with one-way ANOVA followed by Dunnert’s test. The percentages of micronucleated cells from treated and solvent control cultures were compared using the one-way Pearson’s chi-square test. In all tests, data were considered significantly different when \( p < 0.05 \).

Prediction of in vivo genotoxicity from in vitro assays performed on HepaRG cells. Sensitivity, specificity, and accuracy are determined as follows (Kirkland et al., 2005):

Sensitivity = \( \frac{A}{B} \)

Specificity = \( \frac{C}{D} \)

Accuracy = \( \frac{A + C}{B + D} \)

where A is the number of in vivo genotoxic (GTX) compounds tested as positive in the assay, B is the total number of in vivo positive GTX compounds, C is the number of in vivo nongenotoxic (NGTX) compounds tested as negative in the assay, and D is the total number of in vivo positive NGTX compounds.

RESULTS

Differentiated HepaRG Cells Maintained a High Level of CYP450 Activity

The stability of certain basal P450 activities during HepaRG cell culture was verified in three different batches. We showed that the five major liver P450 cytochromes (1A2, 2B6, 2D6, 2C9, and 3A4) were active in HepaRG cells and that their activities were similar from thawing to seven days of culture (Table 1).

The Comet Assay on HepaRG is More Specific Than Sensitive for Detecting In Vivo Genotoxins

Prior to the comet assay, a range-finding study (neutral red uptake) was performed for each compound. The maximal concentration was set according to solubility, at a 30% cytotoxicity level in the NRU assay or 10mM (data not shown).

The background level of DNA damage detected in the comet assay with solvent control HepaRG cells was always very low, between 0.7 and 2.3% of tail DNA. The promutagen CPA (500µM) increased the percentage of tail DNA (between 4.5 and 42.8%) in all experiments from 10 different batches, confirming the capacity of HepaRG cells to bioactivate CPA (Fig. 1).

Positive results were found for ENU, 4-NQO, and DMBA in the comet assay in the three independent experiments, whereas no DNA damage was detected for 2-AAF, 2,4-DAT, CdCl2, etoposide, and styrene (Table 1 and Supplementary figs. 1 and 2). As expected, D-limonene, DEHP, methyl carbamate, D-mannitol, and EDTA failed to increase DNA migration in HepaRG cells. None of the equivocal compounds, ethanol, TCE, eugenol, or estragole, induced DNA damage detected by the comet assay. The sensitivity of the in vitro comet assay was then compared to the micronucleus in HepaRG cells.

The Cytokinesis-Block Micronucleus Assay on HepaRG Cells Is Sensitive and Specific

The reproductibility of the micronucleus assay is shown in Figure 3. The percentage of binucleated cells in the control ranged between 75 and 84%, suggesting that both the cell division rate and the schedule of the MN test were adequate. The background frequency of micronuclei in the HepaRG cells was between 2.6 and 3.6% with a mean of 3.12 ± 0.3% and a 95% confidence interval of 2.8–3.43% calculated from eight independent experiments with duplicate cultures.

The positive control CPA (500µM) induced MN frequencies in HepaRG cells of between 7.12 and 16.43% (mean = 11.9 ± 3.7%), confirming that CPA is a suitable positive control and that HepaRG cells can effectively bio-activate this compound.
PERFORMANCE OF COMET AND MICRONUCLEUS ASSAYS

### TABLE 1

<table>
<thead>
<tr>
<th>CYP</th>
<th>Probe substrates</th>
<th>Metabolites</th>
<th>CYP activities (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>Acetaminophen</td>
<td>10.00 ± 1.5</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion</td>
<td>OH-bupropion</td>
<td>6.45 ± 0.97</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Tolbutamide</td>
<td>OH-tolbutamide</td>
<td>4.57 ± 2.93</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>Dextormphan</td>
<td>3.95 ± 0.64</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>1’OH-midazolam</td>
<td>48.54 ± 13.95</td>
</tr>
</tbody>
</table>

**FIG. 3.** Proportion of BN and BNMM measured in HepaRG cells in the micronucleus assay for negative solvent control and positive CPA control. Re-producibility of the genotoxic effect of CPA in micronucleus production in eight independent experiments from eight batches of cryopreserved HepaRG cells.

Table 2 shows the MN results obtained with the 20 compounds tested (see also supplementary data). The majority of genotoxic and progenotoxic chemicals induced the expected results in the MN test. ENU, 4-NQO, etoposide, taxol, CPA, DMBA, 2-AAF, 2,4-DAT, and AFB1 increased the frequency of MN concentration-dependently in Hepa-RG cells after 24 h of treatment. However, cadmium chloride gave inconsistent results, probably due to the steep slope of cytotoxicity. The aromatic amine 2,4-DAT and styrene were not clastogenic in HepaRG cells up to 10 and 5mM, respectively.

As expected, no formation of MN was observed with the nongenotoxic compounds, DEHP, methyl carbamate, D-mannitol, and EDTA. Only D-limonene gave positive results in the MN test at concentrations up to 2.5mM without significant cytotoxicity measured using the RI index at the end of the cytoto-B exposure.

Ethanol and TCE were not genotoxic in HepaRG cells up to 500 and 2.5mM, respectively. However, eugenol and estragole induced micronuclei in HepaRG cells above 1.5 and 1mM, respectively.

**Comet Assay and Micronucleus Tests in HepaRG Cells Are Robust for Genetic Toxicology Prediction**

Overall, compared to the micronucleus test, the comet assay showed higher specificity (100% compared to 80%), but lower sensitivity, 44% versus 73%. The MN test offers slightly greater accuracy than the comet assay, 75% versus 72% (Table 3).

**DISCUSSION**

The existing in vitro genotoxicity strategy needs improvement to decrease the number of false positive results and to improve the prediction of genotoxicity in humans by taking into account the specificity of human liver metabolism. In order to reach both objectives, we compared the performance of the comet assay and the micronucleus test using the metabolic competent human hepatoma cell model, HepaRG. Our results indicated that HepaRG is a suitable model for detecting direct mutagens and procarcinogens with comet assay and MN testing. The main advantages of this cell line are its metabolic capacity close to primary human hepatocytes and the growth potential of hepatoma cells, despite a lack of donor variability compared to primary hepatocytes (Andersson et al., 2012; Aninat et al., 2006; Anthérieu et al., 2010, 2012; Guguen-Guillouzo and Guillouzo, 2010; Jossé et al., 2008; Kanebratt and Andersson, 2008b). This removes the need for an exogenous system such as rat liver S9 fraction for bioactivation which could present disadvantages. In fact, rat S9 is not always an appropriate surrogate for mimicking the metabolic activities occurring in humans. For example, rat and human CYPs may differ in their substrates specificity, rat S9 contains higher levels of CYP 1A1 and CYP2B family, phase II activities are poorly responding with the cofactors used in S9 mix, and metabolites are formed in the extracellular medium (Hashizume and Oda, 2012; Ku et al., 2007). Therefore, the relevance of rat S9 may be questionable for genetic toxicology assessment and other alternatives including human hepatocyte models such as HepaRG cells should be developed.

We confirmed that a high level of CYP450 activity can be detected in HepaRG seven days after thawing at high density, CYP4503A4 being the most active followed by 1A2, 2B6, 2C9,
### TABLE 2
Results Obtained in Comet and Micronucleus Assays in HepaRG Cells

<table>
<thead>
<tr>
<th>Chemical</th>
<th>CAS No.</th>
<th>Further information</th>
<th>COMET</th>
<th>LEC</th>
<th>MN</th>
<th>LEC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ames-positive in vivo genotoxins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide (CPA)</td>
<td>6055–19–2</td>
<td>Requires metabolic activation (CYP2B6)</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>1-Ethyl-1-nitrosourea (ENU)</td>
<td>759–73–9</td>
<td>Strong gene mutagen (O(^6) alkylation)</td>
<td>+</td>
<td>2.5</td>
<td>+</td>
<td>0.625</td>
</tr>
<tr>
<td>4-Nitroquinoline (4-NQO)</td>
<td>56–57–5</td>
<td>Strong gene mutagen (N(^2) adducts)</td>
<td>+</td>
<td>0.025</td>
<td>+</td>
<td>0.003</td>
</tr>
<tr>
<td>7,12-Dimethylbenzanthracene (DMBA)</td>
<td>57–97–6</td>
<td>Requires metabolic activation (CYP1B1); forms bulky adducts</td>
<td>+</td>
<td>0.125</td>
<td>+</td>
<td>0.00781</td>
</tr>
<tr>
<td>2-Acetylaminofluorene (2-AAF)</td>
<td>53–96–3</td>
<td>Aromatic amine, requires metabolic activation (1A2)</td>
<td>−</td>
<td>0.5</td>
<td>+</td>
<td>0.250</td>
</tr>
<tr>
<td>2,4-Diaminotoluene (2,4-DAT)</td>
<td>95–80–7</td>
<td>Aromatic amine, requires metabolic activation</td>
<td>−</td>
<td>10</td>
<td>−</td>
<td>10(^a)</td>
</tr>
<tr>
<td>Aflatoxin B1 (AFB1)</td>
<td>1162–65–8</td>
<td>Requires metabolic activation (CYP2A4, 1A2)</td>
<td>4(^b)</td>
<td>0.0025</td>
<td>+</td>
<td>0.00025</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>10108–64–2</td>
<td>Inorganic carcinogen</td>
<td>−</td>
<td>0.005</td>
<td>−</td>
<td>0.01(^a)</td>
</tr>
<tr>
<td><strong>In vivo genotoxins negative or equivocal in Ames</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etoposide</td>
<td>33419–42–0</td>
<td>Topoisomerase inhibitor</td>
<td>−</td>
<td>0.01</td>
<td>+</td>
<td>0.0025</td>
</tr>
<tr>
<td>Taxol</td>
<td>33069–62–4</td>
<td>Aneugen</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>0.0025</td>
</tr>
<tr>
<td>Steryl</td>
<td>100–42–5</td>
<td>Requires metabolic activation to form styrene-oxide (CYP2E1)</td>
<td>−</td>
<td>5</td>
<td>−</td>
<td>5(^a)</td>
</tr>
<tr>
<td><strong>Nongenotoxic carcinogens</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-limonene</td>
<td>5989–27–5</td>
<td>Male rat kidney tumours</td>
<td>−</td>
<td>10</td>
<td>+</td>
<td>2.5</td>
</tr>
<tr>
<td>Di-(2-ethylhexyl)-phthalate (DEHP)</td>
<td>117–81–7</td>
<td>Liver carcinoitn in rats and mice</td>
<td>−</td>
<td>10</td>
<td>−</td>
<td>10(^a)</td>
</tr>
<tr>
<td>Methyl carbamate</td>
<td>598–55–8</td>
<td>Liver tumours in rats</td>
<td>−</td>
<td>10</td>
<td>−</td>
<td>10(^a)</td>
</tr>
<tr>
<td><strong>Noncarcinogens with negative in vivo genotoxicity data</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-mannitol</td>
<td>69–68–8</td>
<td></td>
<td>−</td>
<td>10</td>
<td>−</td>
<td>10(^a)</td>
</tr>
<tr>
<td>EDTA</td>
<td>10378–22–0</td>
<td></td>
<td>−</td>
<td>2.5</td>
<td>−</td>
<td>2.5(^a)</td>
</tr>
<tr>
<td>Supplementary list (prediction of in vitro genotoxicity results less clear)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>64–17–5</td>
<td>Human gastric and liver tumours</td>
<td>−</td>
<td>500</td>
<td>−</td>
<td>500(^a)</td>
</tr>
<tr>
<td>Tetrachloroethylene (TCE)</td>
<td>127–18–4</td>
<td>Mice liver tumours</td>
<td>−</td>
<td>2.5</td>
<td>−</td>
<td>1.25(^a)</td>
</tr>
<tr>
<td>Eugenol</td>
<td>97–53–0</td>
<td>In vitro genotoxic at high concentrations</td>
<td>−</td>
<td>1.5</td>
<td>+</td>
<td>1.5</td>
</tr>
<tr>
<td>Estragole</td>
<td>140–67–0</td>
<td>DNA adducts</td>
<td>−</td>
<td>2</td>
<td>+</td>
<td>1</td>
</tr>
</tbody>
</table>

Note. N.D., not determined; −, negative result; +, positive result; LEC, lowest effect concentration in mmol/l of the test chemical that gave positive result in the assay.

\(^a\)Highest concentration used.

\(^b\)Data previously published (Le Hegarat et al., 2010).

\(^c\)In case of negative results the LEC is the highest concentration tested.

### TABLE 3
Performance of In Vitro Comet and Micronucleus Assays in HepaRG Cells for Predicting In Vivo Genotoxins from Our Data

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Accuracy</th>
<th>Number of compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comet</td>
<td>5/11 (44%)</td>
<td>5/5 (100%)</td>
<td>9/14 (72)</td>
<td>16</td>
</tr>
<tr>
<td>Micronucleus</td>
<td>8/11 (73%)</td>
<td>4/5 (80%)</td>
<td>12/16 (75)</td>
<td>16</td>
</tr>
</tbody>
</table>

and 2D6, as previously published (Aninat et al., 2006; Anthéron et al., 2010; Gerets et al., 2012).

The reproducibility of the comet assay on HepaRG was shown by the results obtained with positive (CPA 0.5mM) controls over 10 independent experiments from 10 batches of freshly thawed cells. Whereas the baseline level of DNA breaks was low for the solvent control in all experiments, with a percentage of tail DNA below 2%, CPA clearly induced comet formation in each experiment as previously published with classic culture of differentiated HepaRG cells (Le Hegarat et al., 2010). Nevertheless, the 10 different HepaRG cultures showed a large variation of the comet assay response to CPA, with a percentage of tail DNA ranging between 4.5 and 42.8% (Fig. 2). This high variability in the genotoxic response of positive controls...
has been often reported with the in vitro comet assay. However, higher reproducibility was observed with the cytokinesis-block micronucleus assay on HepaRG cells. First, the frequency of binucleated cells after cytochalasine-B exposure was always close to 80% for the solvent control, indicating healthy dividing cells, with a binucleated micronucleated (BNMN) cell frequency of 3.12% (95% confidence intervals between 2.8 and 3.43%) indicating considerable homogeneity between the eight independent experiments and genomic stability of the HepaRG cells. For the positive control CPA (0.5mM), a significant induction of MN formation was observed in all experiments with a BNMN frequency between 7.12 and 16.43%. These results suggested that HepaRG cells are a suitable model for in vitro genotoxicity testing.

Our results showed that combining comet and MN assays on differentiated HepaRG cells can distinguish between genotoxic and nongenotoxic compounds with high specificity and sensitivity. Of 16 compounds from the ECVAM list, the MN test detected most of the genotoxic compounds, except the 2,4-DAT, the cadmium chloride, and the styrene. The comet assay was less efficient, as only five out of 11 genotoxic compounds showed a positive response after 24 h of treatment: CPA, ENU, 4-NQO, DMBA and AFB1, whereas 2-AAF, 2,4-DAT, cadmium chloride, etoposide, and styrene failed to induce DNA fragmentation in HepaRG cells, even at cytotoxic concentrations. Note that some of the same genotoxic compounds were undetected in both tests.

2,4-DAT failed to induce DNA damage and cytotoxic effects in HepaRG up to 10mM. However, a previous paper showed that 2,4-DAT induced comet formation, DNA repair, and MN induction in HepG2 cells after 20 h of treatment at concentrations of 1.46, 0.01, and 2.14mM, respectively, without cytotoxicity (Séverin et al., 2005). This absence of response in HepaRG could be due to a lack of metabolic enzymes that bioactivate 2,4-DAT to genotoxic N-hydroxylamine. Indeed, 2,4-DAT is converted to N-hydroxylamine by CYP1A2, which, after metabolization by sulfotransferases or N-acetyltransferase, are thought to react with DNA (Cheung et al., 1996). However, as we found that HepaRG cells metabolize phenacetin efficiently to acetaminophen, confirming the presence of active CYP4501A2, SUTL and/or NAT phase II enzymes could be lacking in HepaRG cells. Further studies are needed to determine the phase II enzyme activities of HepaRG cells.

Styrene did not show genotoxicity in HepaRG cells due to the absence of CYP4502E1 activity in these cells, which bioactivates styrene to the mutagenic metabolite styrene-oxide (Anthérier et al., 2010). In the same way, ethanol did not show genotoxicity in HepaRG cells up to 500mM due to the absence of CYP2E1 for metabolizing ethanol into acetaldehyde. However, it has been reported that ethanol is genotoxic on its own and induced chromosome loss, whereas its metabolite acetaldehyde was preferentially clastogenic (Kayani and Parry, 2010).

Cadmium chloride was highly cytotoxic but did not induce DNA fragmentation or MN formation in HepaRG cells. Cadmium chloride is an inorganic compound which is classified as carcinogenic in humans by the International Agency for Research on Cancer. Although defined as Ames-positive in the ECVAM list, some studies failed to demonstrate its genotoxicity in the Ames test (see review Hartwig, 2010). MN formation was reported in human lymphocytes, TK6 cells and CHO-K1 cells but not in HepG2 cells or in HepaRG cells (Brans et al., 1987; Fowler et al., 2010; Migliore et al., 1999; Mizota et al., 2011). Nevertheless, HepG2 and HepaRG might be less sensitive to genotoxicity involving oxidative stress due to a higher level in antioxidant detoxification systems compared to TK6 and CHO cells. The modified comet assay using specific enzymes such as Fpg would be a worth tool for detecting more easily the CdCl₂ induction of oxidized DNA bases in HepaRG cells. CdCl₂ genotoxicity could also be due to DNA repair inhibition and cell-proliferation induction, in agreement with its carcinogenic potential (Hartwig, 2010).

Positive results were obtained with promutagenic compounds requiring bioactivation steps to become genotoxic: CPA, AFB1, DMBA, and 2-AAF. CPA, AFB1, and DMBA were positive in both comet and micronucleus tests, whereas 2-AAF was only positive in the micronucleus test. This discrepancy could be due to the greater sensitivity of the MN assay for detecting some specific genotoxic insults such as bulky adducts while the comet assay was shown to hardly detect this damage (Valentin-Severin et al., 2003). Similar results were obtained in HepG2 cells where 2-AAF induced a positive MN response but was negative in the comet assay (Valentin-Severin et al., 2003). However, 2-AAF was highly positive in the UDS assay in HepG2 cells and in rodent liver, but gave equivocal results in the in vivo comet assay (Gaudin et al., 2009; Smith et al., 2008; Valentin-Severin et al., 2004). Considering both that 2-AAF was positive in the UDS assays in vitro and in vivo and that comet assay detects DNA strand breaks induced by DNA repair processes, a positive response is expected with 2-AAF in the comet assay. However, the amount of DNA repair strand breaks might be too low to increase significantly the DNA migration; indeed, more than 100 DNA
breaks must be induced to get a sufficient level detected with the comet assay (Collins, personal communication). 2-AAF is converted to N-Hydroxy-2-AAF by CYP1A2 in the liver. The resulting N-Hydroxy-2-AAF intermediate can bind directly to DNA, inducing genotoxicity (Yueh et al., 2001). However, N-Hydroxy-2-AAF could also be glucuronidated by UGT1A7 and 1A9, preventing genotoxic effects. On the other hand, there are three major pathways by which electrophilic metabolites can be formed: O-acetylation of N-Hydroxy-2-AAF, intramolecular transfer of the acetyl group of N-Hydroxy-2-AAF, and sulfation of N-Hydroxy-2-AAF by SULT1A1 and 1A2 (Glatt, 2000). The sulfation of N-Hydroxy-2-AAF by SULT1A1 formed highly genotoxic metabolites (Oda et al., 2012). Our results showed that 2-AAF is weakly genotoxic with a slight increase of MN above 250μM. This confirms the presence of CYP1A2 activity in HepaRG cells which bioactivates 2-AAF (Shimada et al., 1989). However, the N-Hydroxy-2-AAF metabolite is probably detoxified rather than bioactivated in HepaRG cells, as suggested by the weak genotoxic response. Our results highlight that detoxifying pathways should be considered in genetic toxicity testing. Further research on phase II expression and activities in HepaRG, especially SULT1A1, UGT1A7, and 1A9, would help outline how arylamines are metabolized and which metabolites are involved in the genotoxicity.

Etoposide induced positive results in the MN test but failed to induce comet formation, suggesting a low level of DNA topoisomerase II in quiescent HepaRG cells. Etoposide inhibits the ligation of TopII to cleaved DNA, generating DNA double-strand breaks. Two isoenzymes, TopIα and TopIIβ, are expressed in mammalian cells. TopIα is particularly important for DNA replication and is usually present at high levels in fast-growing cancer cells, whereas the β isoform is mainly involved in transcription-related processes, although the physiological functions of this isoform have not yet been defined (Dewese and Osheroff, 2009; Grue et al., 1998; Ju et al., 2006; Niimi et al., 2001; Nittis, 2009). Both human isoforms are etoposide targets although only an etoposide-inhibited form of the human TopIIβ DNA cleavage core has been described by crystallography (Wendorff et al., 2012; Wu et al., 2011). In addition, the lack of positive response in the HepaRG comet assay with etoposide suggests that TopIIα is not active, as expected in quiescent cells, and that the level of TopIIβ activity is rather low and undetectable by the comet assay.

In general, negative results were obtained in the comet and MN assays in HepaRG cells with nongenotoxic compounds: DHEP, methyl carbamate, D-mannitol, and EDTA. Only D-limonene induced a concentration-dependent increase of MN formation in HepaRG cells above 2.5mM, whereas the comet assay remained negative up to 10mM. However, genotoxicity studies on D-limonene found no mutagenicity of limonene and its metabolites (Kim et al., 2013). Nonetheless, a recent study seems to indicate that D-limonene could affect chromosome segregation and cytokinesis, resulting in aneuploidy (Mauro et al., 2013). These results could explain the absence of DNA damage in the comet assay and induction of micronucleus formation in our study on HepaRG cells. Further experiments with fluorescence in situ hybridization (FISH) centromeric staining should be performed to explain the mechanism of action of D-limonene.

Among the compounds from the supplementary list, for which in vitro genotoxicity results are ambiguous, ethanol and TCE gave negative results. TCE, used as a dry-cleaning solvent and as a chemical intermediate, also gave negative results in the Ames test, but was positive in in vitro MN tests on CHO-K1 and HepG2 via oxidative stress production (Hu et al., 2008; Wang et al., 2001). Our results indicate that TCE failed to exert genotoxic results in human hepatocytes and HepaRG cells.

Eugenol and estragole showed positive results only in the micronucleus test after 24 h of exposure. Eugenol, present in a variety of essential oils, is a flavoring substance authorized by the European Commission for use in foodstuffs and is also widely used as an analgesic and antiseptic in clinical dentistry. Although results were negative in the Ames test, either with or without metabolic activation, some genotoxic effects were reported in various tests on mammalian cells. Chromosome aberrations induced by eugenol in CHO cells and V79 were enhanced by the presence of S9 (Rompelberg et al., 1996), whereas no increase in Unscheduled DNA Synthesis (UDS) was observed in primary rat and mouse hepatocytes. Recently, micronuclei were found with eugenol in V79, CHL, CHO, and HepG2 cells after 3 h of treatment with S9, but the eugenol was negative in human lymphocytes and TK6 cells (Fowler et al., 2012). The authors indicated that MN formation with eugenol was always concomitant with high toxicity. Furthermore, positive results were shown in γH2AX in HepG2 cells at 500μM (Tsamou et al., 2012), and Maralhas et al. demonstrated that eugenol inhibited topoisomerase II activity (Maralhas et al., 2006). Assuming this effect, the absence of DNA damage in the comet assay supports what we already suggested for the results with etoposide. Eugenol is considered to be an antioxidant but could also be pro-oxidative at high concentrations due to the formation of catechols after metabolic reactions. MN formation in HepaRG cells induced by eugenol at 1.5mM could then be attributed to both inhibition of topoisomerase and pro-oxidative potential.

Estragole was not mutagenic in the Ames test either with or without rat or hamster liver S9 (Bristol, 2011; Drinkwater et al., 1976; Sekizawa and Shibamoto, 1982; Swanson et al., 1979; Zeiger et al., 1987). Estragole induced UDS in rat hepatocytes both in vitro and in vivo (Müller et al., 1994; Nesslany et al., 2010) as well as DNA adducts in HepG2 cells and in the liver of mice and rats after oral administration. Moreover, gpt mutations in rat liver were also increased, suggesting that estragole could be a possible genotoxic hepatocarcinogen (Nesslany et al., 2010; Suzuki et al., 2012; Zhou et al., 2007). Estragole is bioactivated by P450 to the carcinogen 1-hydroxyestragole, mainly by P4501A2 and 2A6 in human liver (Jeurissen et al., 2007). Our results are the first demonstration...
of micronucleus formation in the human hepatoma cell line with estragole, supporting the hypothesis that this natural compound used as an additive, flavoring agent or fragrance in a variety of food, cleaning and cosmetic products may be carcinogenic in humans and require further investigation for risk assessment.

The main objective of this paper was to determine the specificity and the sensitivity of both comet and micronucleus genotoxic tests performed on metabolic competent human HepaRG cells, for predicting in vivo genotoxicity. Our data suggest that the MN test performed better than the comet assay as previously described with other cell models (Kimura et al., 2013). Detection of only 8/11 (72%) in vitro genotoxins is no doubt due to the absence of CYP2E1, and the failure to bioactivate styrene and 2,4-DAT, which decreases the specificity of the test. However, the sensitivity of MN on HepaRG cells (72%) is satisfactory and close to other genotoxicity tests performed in other cell lines. Moreover, these data have been estimated only from a small number of compounds tested on HepaRG cells and it may vary when a larger sample of genotoxic and nongenotoxic carcinogens will be tested.

The best in vitro model for accurate human genotoxicity prediction would certainly not the one which will give positive results for low doses of mutagens but would rather be able not only to bioactivate promutagens but also to express well-balanced phase I, II, and III activities (as well as antioxidant and repair capacities), mimicking the integrated response as expected from human hepatocytes. For this purpose, the HepaRG cell line is certainly one of the best candidates.

In conclusion, rat liver S9 mimicking liver metabolism is currently the system used for human genotoxicity risk characterization although discrepancies in metabolic capabilities compared to humans have been highlighted (e.g., higher CYP1A1 and 2B levels, CYP substrate specificity between rat and human, underestimation of phase II activities, metabolites produced in the extracellular medium (Hashizume and Oda, 2012; Ku et al., 2007). Competent human liver cell models are an alternative in genetic toxicology to enhance extrapolation from in vitro to human effects. Although additional data on a wider panel of substances are needed, our results showed that the new human hepatoma cell line HepaRG could be one of those predictive models.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

AAP Bretagne-Critt-Santé grant number 02212.

REFERENCES


Swanson, A. B., Chambliss, D. D., Blomquist, J. C., Miller, E. C., and Miller, J. A. (1979). The mutagenicities of safrole, estragole, eugenol, trans-anethole, 4, 4-di-


