Performance of in vitro γH2AX assay in HepG2 cells to predict in vivo genotoxicity

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The γH2AX assay has recently been suggested as a new in vitro assay for detecting genotoxic (GTX) properties of chemicals. This assay is based on the phosphorylation of H2AX histone in response to DNA damage (e.g., induction of double-strand breaks (DSBs)). Quantification of γH2AX foci using flow cytometry can rapidly detect DNA damage induced by chemicals that cause DNA DSBs. Up to now, only few compounds have been tested with this assay. The main goal of this study was to compare the performance of this automated γH2AX assay with that of standard in vitro genotoxicity assays in predicting in vivo genotoxicity. HepG2 cells were exposed to 64 selected compounds with known GTX properties and subsequently analysed for induction of γH2AX foci. The results of this assay were compared with public data from standard in vitro genotoxicity tests. Accuracy, sensitivity and specificity in predicting in vivo genotoxicity, using the γH2AX assay alone or in combinations with conventional assays, were calculated. Both the γH2AX assay and the bacterial mutagenicity test (Ames) were highly specific for in vitro GTX, whereas chromosomal aberration/micronucleus test (CA/MN) resulted in highest sensitivity. The currently widely used in vitro genotoxicity test battery—Ames test, mouse lymphoma assay (MLA) and CA/MN test—resulted in low accuracy (55–65%) to predict in vivo genotoxicity. Interestingly, the inclusion of γH2AX assay in the standard battery, instead of MLA assay, resulted in higher accuracy (62–70%) compared with other combinations. Advantage of the γH2AX assay in HepG2 cells is its high sensitivity to detect DNA-reactive GTX compounds, although the reduced sensitivity for compounds that require metabolic activation needs to be improved. In conclusion, the automated γH2AX assay can be a useful, fast and cost-effective human cell–based tool for early screening of compounds for in vivo genotoxicity.

Introduction

Genotoxicity testing is designed to detect substances that are capable of directly or indirectly inducing genetic damage and can be defined as in vitro or in vivo testing, depending on the test system that is used. Any new substance must be tested for its genotoxic (GTX) properties before acquiring the regulatory approval to place it on the market. The induction of genotoxicity can be caused by gene mutation, clastogenicity (i.e., structural chromosome aberrations) or aneugenicity (i.e., numerical chromosomal aberrations (CA) (1–3)). Therefore, the measurement of these three genetic endpoints seems most informative in predicting in vivo GTX potential of an agent (2,4).

Tests for in vitro genotoxicity screening consist of a bacterial gene mutation test (Ames test) (OECD TG 471), a gene mutation test on mammalian cells [mostly done by the mouse lymphoma assay (MLA)] (OECD TG 476), a chromosomal breakage test by CA (OECD TG 473) and the micronucleus test (MN) (OECD TG 487) in mammalian cells (3). For drugs, further in vivo testing is required, which can be an analysis either of micronuclei in erythrocytes in blood or bone marrow or of chromosome aberrations in metaphase cells in bone marrow; whereas for other chemicals (food additives, pesticides or industrial chemicals) in vivo follow-up studies are only required in cases where a positive response in the in vitro tests is found, for the purpose of confirming of the GTX risk of the substance (1,5–8). In case of cosmetics after the European ban in November 2009 (EU Regulation 1223/2009) on animal-based acute testing of cosmetic ingredients for all human health effects, the in vivo genotoxicity testing is not allowed anymore (9).

According to the European report in 2005 on the statistics of animal use for scientific purposes, approximately 77 000 rodents were used annually for genotoxicity and carcinogenicity tests (10). Furthermore, it has been estimated that about 140 000 preregistered chemicals under REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulations need to be evaluated, which may require more than 300 000 animals to be sacrificed (11–14). In order to avoid these large numbers of sacrificed animals, the development of reliable and efficient in vitro testing is of major importance in the genotoxicity testing.

The reliability of in vitro genotoxicity testing represents a problem. Particularly, Kirkland et al. (15,16), have shown that the current in vitro tests (Ames, MLA, MN and CA assays) produce high false-positive (thus low specificity) rates in prescreening for the prediction of carcinogenicity while sensitivity is high. Therefore, an increase in the specificity of the current in vitro genotoxicity test battery is urgently needed by replacing, by optimising the conventional tests or by including new short-term tests into the test battery. This may lead to a considerable reduction of animal studies, ideally will avoid the unnecessary in vivo follow-up studies and may even lead to a replacement of in vivo studies for genotoxicity tests, thereby contributing to the promotion of 3Rs (reduction, replacement and refinement) in the regulatory safety testing (17). Another disadvantage of the current in vitro tests is that either they are time-consuming, they require a lot of test compounds or they are not amenable to automated high-throughput screening methods. Recently, however, methods have been developed for automatic scoring of MN in cultured cells, which can enhance the throughput of this assay (18).

Several assays have been proposed as suitable alternative methods for rapid testing of genotoxicity. High- or
medium-throughput screening methods, such as RadarScreen, Vitotox or GreenScreen HC, have been suggested as efficient in vitro methods for the detection of genotoxicity, resulting in high-prediction accuracies (19,20). However, most of these assays are limited either by the use of non-mammalian cells, e.g. bacteria or yeast (i.e. in RadarScreen and Vitotox assays) or by human lymphoblastoid TK6 cells (i.e. in GreenScreen HC), which lack endogenous metabolism. When cells lack metabolism, the addition of an exogenous metabolic activation system is required for those compounds that are activated in order to become GTX, which requires animals to generate.

Recently, Watters et al. (21) have suggested a new in vitro genotoxicity assay, the γH2AX assay, as an in vitro biomarker for detecting GTX properties of chemicals. The γH2AX assay is based on the total phosphorylation of H2AX histone in response to DNA damage by induction of double-strand breaks (DSBs). DSBs can occur not only due to two single-strand breaks (SSBs) in each vicinity (SSBs can be caused by DNA adducts) but also due to blocked DNA replication forks caused by bulky DNA adducts (22). In fact, the H2AX histone is a substrate for several phosphoinositide-3-kinase-related protein kinases, such as ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3 related) and DNA-dependent protein kinases. ATM is considered as the main mediator of H2AX phosphorylation in response to DSBs formation, whereas ATR was responsible for phosphorylated H2AX in response to single-stranded (SSBs) DNA breaks and during replication stress (23–25). This new assay not only is simple but also allows fluorescent visualisation and the physical localisation of the DSBs (26). Rapid analyses using fluorescence-activated cell sorter (FACS) provides an automated assay for the quantification of nuclear γH2AX foci induced by DNA damage (21). Moreover, flow cytometry is more cost-effective than immunofluorescent microscopy, which was used by Zhou et al. (27) for the detection of γH2AX foci formation. The simplicity, ease and sensitivity of this assay to detect immediate γH2AX accumulation as a measure for DNA damage increase its value compared with the existing time-consuming assays (8).

Therefore, the automated γH2AX assay may be a powerful, fast and cost-effective human cell–based tool for the early screening of new compounds for in vitro GTX properties. So far, however, this assay has been evaluated for only five compounds and using mouse lymphoma cells (LS178Y (21)). The aim of our study was to compare the performance of the automated γH2AX assay with that of standard in vitro genotoxicity assays in predicting in vivo genotoxicity for a large set of compounds and in a metabolically competent cell line. In the present study, HepG2 human cells were exposed to 64 agents with known in vitro and in vivo GTX properties, and γH2AX phosphorylation was analysed by flow cytometric analysis. HepG2 cells were chosen as in vitro cell model since it has been described before as a suitable model for the detection of GTX properties for environmental and dietary chemicals (28,29). HepG2 cells possess a liver-like enzyme pattern (30), expressing many phase I, II and III drug metabolising enzymes; thereby they are capable of regulating these enzymes by ligand-activated transcription factors (31–35). Thus, their metabolising capacities are evident (36), though less than that for human livers or primary human hepatocytes (37). Furthermore, and most important for genotoxicity, HepG2 cells are p53-competent. Finally, we calculated accuracies in predicting in vivo genotoxicity, using individual or combinations of the conventional assays with or without the γH2AX assay.

Materials and methods

Cell culture

Human hepatoma cells, HepG2, were cultured in the presence of an Eagle’s minimum essential medium plus Glutamax, supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin (all from Gibco BRL, Breda, the Netherlands), as previously described by Jennew et al. (37). The cells were incubated at 37°C and 5% CO₂.

Compounds

In this study, 64 compounds were tested, whereas DMSO (0.5% v/v final dose), Ethanol (0.5% v/v final dose) and medium were used as negative, solvent and controls, respectively, and Etoposide (Sigma–Aldrich, Zwijndrecht, Netherlands; CAS: 33419-42-0) was used as positive control. The compounds were selected based on publicly available toxicological data regarding their GTX properties. Information about in vitro and in vivo genotoxicity testing have been collected from National Toxicology Program (NTP (38)), International Agency for Research on Cancer (IARC (39)), Toxnet (40), Environmental Protection Agency (41) or from literature. In supplementary data, Table A (available at Mutagenesis Online) provides all data about in vivo and in vitro genotoxicity tests, CAS numbers, concentrations, solvents and suppliers.

Dose selection

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed in order to identify the proper, marginally cytotoxic, dose of the selected compounds for the γH2AX assay (42). Concentrations were selected where ca 80% of the cells were viable after 72-h exposure to the test compound (37). In order to avoid interference of cytotoxic effects in induction of γH2AX phosphorylation, the γH2AX was conducted after 24-h exposure. If a compound was not cytotoxic, the maximum dose was 2 mM, thereby taking into account the currently growing opinion that 10 mM is too high for in vitro genotoxicity tests (43). Accidentally, di(2-ethylhexyl)phthalate was tested at 10 mM.

Flow cytometric analysis–γH2AX assay

HepG2 cells [1.4 × 10⁵ cells/well (2 ml/well)] were plated in 6-well plates (D₄) and incubated for 72h, up to near confluency (~80%) on day 4 (D₄), when medium was refreshed and cells were incubated for another 24h. On the 5th day (D₅), the cells were exposed to fresh medium containing the test compounds and were incubated for another 24h at 5% CO₂ and 37°C. A single treatment of 24h was chosen in order to eliminate the interference of potentially immediate early stress responses of cells exposed to toxic stimuli (44).

The procedure for γH2AX staining was done according to Watters et al. (21), with some small modifications due to the different cell system (45). Methanol-fixed cells were washed with phosphate-buffered saline (PBS), containing 1 mg/ml bovine serum albumin (BSA), and incubated for 1 h at ambient temperature with the primary antibody mouse anti-phospho-histone H2AX (Ser-139) Clone JBW 301 (Upstate Biotechnology, Lake Placid, NY) diluted 1:1000 in PBS/BSA. Cells were washed and incubated for 1 h with the secondary antibody Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen, Leek, the Netherlands). Cells were resuspended in 0.5 ml PBS containing 20 µg/ml propidium iodide (Sigma–Aldrich) and 100 µg/ml RNAse A (Sigma–Aldrich). As positive and negative controls, cells were incubated with 5 µM etoposide and the correspondent solvent controls, respectively (21). A FACS, FACSsort (Becton Dickinson, Sunnyvale, CA, USA), was used for measuring the γH2AX staining. An excitation wavelength of 488 nm and emission filters of 515–545 nm band pass and 600 nm low pass were used (46). Ten thousands events were collected and data recorded using Cell Quest software (version 3.1, Becton Dickinson, Sunnyvale, CA). Cells with significant amounts of anti-phospho-Histone H2AX-positive signals were displayed as a percentage of total cells. Each compound was investigated in three independent replicate experiments (21). A value for γH2AX assay was considered significant in comparison to control cells when the P-value <0.05 based on paired t-test in the three independent experiments.

Prediction for in vivo genotoxicity by the in vitro assays

In accordance with Kirkland et al. (16), ‘Sensitivity’, ‘Specificity’ and ‘Accuracy’ are calculated as the following:

\[
\text{Sensitivity} = \frac{A}{B}, \quad \text{Specificity} = \frac{C}{D}, \quad \text{Accuracy} = \frac{(A + C)}{(B + D)}.
\]
where \( A \) is the number of in vivo GTX compounds tested as positives in the tested assay or in combination of assays, \( B \) the total number of in vivo positive GTX compounds, \( C \) the number of in vivo non-genotoxic (NGTX) compounds tested as negatives in the investigated assay (or combination of assays) and \( D \) the total number of in vivo NGTX compounds. Inconsistent or unclear results derived from different or same studies within the same test are referred to as equivocals \((E)\).

False-negative and false-positive rates can be calculated as follows:

- False negative \((\%) = 100\% - \) Sensitivity\(\%\).
- False positive \((\%) = 100\% - \) Specificity\(\%\).

### Results

In this study, the induction of \( \gamma \)H2AX phosphorylation in nuclei of human HepG2 cells was measured following exposure to 64 compounds. The obtained results were compared with data of standard in vitro genotoxicity tests, with respect to their ability to correctly predict in vivo genotoxicity. These 64 compounds comprise 24 in vitro GTX agents and 40 in vivo NGTX agents. The in vitro genotoxicity data were mainly based on result of the most commonly used in vivo tests, MN and CA, using rodent hematopoietic cells or additional in vivo assays. All genotoxicity data for all tested compounds are obtained from internationally recognised databases at NTP (38), IARC (39) and review papers, such as from Kirkland et al. (15,16), and are presented in supplementary Table A, available at Mutagenesis Online. The result for each genotoxicity test can either be positive (+), negative (−) or equivocal (E). Since the in vitro MN assay database is limited and the MN assay detects mainly the same endpoints as the CA assay, the results of both assays have been combined (CA/MN (15)).

Sensitivity, specificity and accuracy were calculated for each performance. These values are presented in ranges, since the equivocal results are considered as either positive or negative for the calculations.

### Single assays

The in vitro \( \gamma \)H2AX assay and the standard in vitro genotoxicity tests were individually used for the prediction of in vivo genotoxicity (Table I). The \( \gamma \)H2AX assay produced the lowest sensitivity but a relatively high specificity (78%), while the CA/MN test produced the highest sensitivity (92–96%) and the Ames assay gave the highest specificity (78–83%). In addition, the highest accuracy was derived from the CA/MN test (71–79%), followed by Ames (78%), the \( \gamma \)H2AX assay (69%) and MLA (60–66%), the latter thus being least accurate.

#### Combinations of two assays

Next we investigated the predictions of in vivo genotoxicity by combining two in vitro assays (Table II). The most accurate prediction when including the \( \gamma \)H2AX assay was by combining with the CA/MN test, which resulted in the highest accuracy (65–71%) among all combinations of two assays. The combination with MLA produced the least accurate prediction (58–60%).

#### Combinations of three assays

In the current regulatory in vitro testing for genotoxicity frequently three tests are required. Therefore, we also evaluated all possible combinations of three in vitro genotoxicity tests and calculated their prediction values (accuracy, sensitivity, specificity) (Table III). Sensitivities were high for all combinations (>83%), whereas the specificities were lower than those for most of the individual tests (<56%). The inclusion of the \( \gamma \)H2AX assay within the test battery (Ames, CA/MN and \( \gamma \)H2AX), instead of MLA assay, resulted in higher accuracy (62–70%) by improving the specificity (41–56%) compared with the other combinations. Using the standard test battery (Ames, MLA and CA/MN), the accuracy was 55–65%.

### Discussion

#### Overview of all performances

Most of the conventional in vitro genotoxicity assays have been described as time-consuming, laborious and not readily

<table>
<thead>
<tr>
<th>Results</th>
<th>Ames(^a)</th>
<th>MLA(^b)</th>
<th>CA/MN(^c)</th>
<th>( \gamma )H2AX(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of in vivo GTX(^e) tested (B)</td>
<td>24</td>
<td>18</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>No. (%) of in vivo GTX tested as clear positives (A) [sensitivity (A/B)]</td>
<td>17 (71%)</td>
<td>15 (83%)</td>
<td>22 (92%)</td>
<td>13 (54%)</td>
</tr>
<tr>
<td>No. of in vivo GTX with equivocal(^f) results (E1)</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Sensitivity (%) if equivocal results (E1) considered as positive [(A + E1)/B]</td>
<td>19 (79%)</td>
<td>17 (94%)</td>
<td>23 (96%)</td>
<td>13 (54%)</td>
</tr>
<tr>
<td>No. of in vivo NGTX(^e) tested (D)</td>
<td>40</td>
<td>32</td>
<td>39</td>
<td>40</td>
</tr>
<tr>
<td>No. (%) of in vivo NGTX tested as clear negatives (C) [specificity (C/D)]</td>
<td>31 (78%)</td>
<td>13 (41%)</td>
<td>22 (56%)</td>
<td>31 (78%)</td>
</tr>
<tr>
<td>No. of in vivo NGTX with equivocal results (E2)</td>
<td>2</td>
<td>5</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Specificity (%) if equivocals (E2) considered as negative [(C + E2)/D]</td>
<td>33 (83%)</td>
<td>18 (56%)</td>
<td>28 (72%)</td>
<td>31 (78%)</td>
</tr>
<tr>
<td>Accuracy (%) (A + E1 + C)/(B + D), E = positive</td>
<td>78%</td>
<td>60%</td>
<td>71%</td>
<td>69%</td>
</tr>
<tr>
<td>Accuracy (%) (A + C + E2)/(B + D), E = negative</td>
<td>78%</td>
<td>66%</td>
<td>79%</td>
<td>69%</td>
</tr>
</tbody>
</table>

\(^a\)Ames: in vitro bacterial assay (Salmonella).
\(^b\)MLA: in vitro mouse lymphoma assay (L5178Y mouse lymphoma cell line).
\(^c\)CA/MN: combination of in vitro chromosomal aberration and micronucleus test.
\(^d\)\( \gamma \)H2AX: phosphorylation of H2AX on HepG2 cells.
\(^e\)GTX is a compound which produces at least one positive response in its performed genotoxicity testing.
\(^f\)Equivocal: within the same test, contradictory or unclear results.
applicable to large numbers of compounds (8,47–49). The need for a fast in vitro test with high specificity for predicting in vivo genotoxicity has been stipulated by Kirkland et al. (50). Predicting in vivo genotoxicity using exclusively in vitro assays is also a key element for the development and promotion of the 3Rs concept. Therefore, improving the accuracy (balance between sensitivity and specificity) of the current in vitro genotoxicity testing battery is a main target. Because of this urgent need, the performance of the new and automatable γH2AX assay was evaluated.

All prediction calculations are summarised in Table IV. With respect to reporting false results, the new γH2AX assay performed comparable to Ames test, since both assays had the highest specificity and thus resulted in low false-positive rates (22%). Both assays also showed the lowest sensitivity with therefore high false-negative rates (29–46%), meaning wrongly considering GTX compounds as NGTX. For all combinations of two or three in vitro assays, the predictions for in vivo genotoxicity are less accurate than that by the individual assays, which is due to a decrease of specificity. Noteworthy, a recent re-evaluation of the NTP MLA data during the Mouse Lymphoma Expert Workshop of the International Workshop for Genotoxicity Tests (IWGT) led to the conclusion that the top concentration for the MLA assay for testing non-pharmaceuticals should be lowered, in order to minimise the high false rates of this assay due to the current results (51). To what extend this will affect the data presented in our paper is unknown.
The performance of the γH2AX assay in predicting in vivo genotoxicity indicated that not all GTX compounds are capable of inducing γH2AX phosphorylation, as 46% of the GTX compounds is missed. An example of clear false negative are diethylnitrosamine (DEN) and dimethylnitrosamine (DMN), which induce only small (m)ethyl-DNA adducts (38,40,52–55), that probably do not block replication forks (56) and that apparently induce only small SSBs to be detectable by the γH2AX assay. Several cytochrome P450 (CYP) enzymes, including CYP1A2, 2B6, 2E1 and 3A4 are expressed at low-expressed levels in HepG2 (34,57). This low activity of some CYP enzymes might be responsible for the inability of some GTX compounds to be detected by the γH2AX assay, as CYP enzymes are required for their metabolism. For example, the above mentioned GTX compounds, DEN and DMN, it is known that they are predominantly activated by CYP2E1 (57,58). Also, cyclophosphamide is known to be activated by CYP3A4 in human liver (59) and by CYP2B6 (60), while heterocyclic aromatic amine 2-amino-3-methylimidazo[4,5-f]quinoline by CYP1A2 (61). Additionally, CYP2E1 plays a key role in the activation of acetalaminophen (APAP) (62). These are all examples that may explain the weak and negative results obtained by γH2AX assay for these compounds with HepG2 cells.

Noteworthy, many of the false-negative GTX compounds for the γH2AX assay, APAP, phenol (Ph), L-ascorbic acid, tributylinoxide and diethylbestrol, were also false negative in the Ames test. These compounds may be non-DNA reactive GTX, where their initial target is not DNA (63). The Ames test is known to be especially sensitive for DNA-reactive GTX compounds but insensitive for non-DNA-reactive GTX compounds (64).

Another explanation for the false positives may be the induction of oxidative stress leading to the generation of reactive oxygen species (ROS), which could damage DNA (63); this is considered one of the most common sources of induced SSBs (65). Approximately the 99% of these SSBs is normally repaired (66), but the remaining 1% unrepaired SSBs can result in potentially clastogenic DSBs during DNA replication (65). Many compounds, including pesticides, solvents such as chloroform, carbon tetrachloride and phenols, catechols and catecholamines and oestrogens and metals, are believed to induce DNA damage via ROS production (63). Subsequently, DNA damage via ROS production is not detected by the γH2AX assay, which stresses its limitation.

Only few NGTX compounds have been shown to cause induction of γH2AX phosphorylation, namely ethyl acrylate (EA), diazinon (DZN) and eugenol (EuG), which thus may indicate false-positive responses. Some of these NGTX compounds have previously been described in literature to cause DSBs in vitro and/or in vivo (39) For instance, EA, which is generally considered a NGTX compound (38), induced in vitro DSBs at high concentrations on mouse lymphoma cells, which was performed by pulsed-field gel electrophoresis of DNA (67). Also, DZN and EuG have been reported previously as inducers of DNA strand breaks in vitro (68–71). A plausible explanation has been given by Bradley et al. (72), who have referred to a close correlation of compounds that induce DNA DSBs only at high concentrations and that induce cytotoxicity, which may be underlying their possible misclassification. In our study we only investigated one dose for each compound, which maximally is only slightly toxic since we treated cells for 24h with the IC20 for 72h as cut-off for cytotoxicity. Thus, it is not likely that excessive cytotoxicity affected our data and therewith caused false-positive results.

We cannot exclude, however, that some compounds that currently appear as false negatives in the H2AX assay would have generated positive results if higher doses were used. For further improvement, attention should be paid to dose settings, methodologies, criteria, other treatment periods and the incorporation of a cytotoxicity parameter into the assay itself (i.e. into FACS analyses), in order to increase both sensitivity and specificity and thus minimising the false-positive or false-negative results. With respect to dose and the related chance of finding false positives, it is relevant to mention that recommendations for lower testing doses on mammalian cells have already been suggested in a draft International Conference of Harmonisation guideline for genotoxicity testing of pharmaceuticals (2), by reducing the current maximum limit from 10 mM to 1 mM (73,74). Additionally, according to Mouse Lymphoma Expert Workgroup of the IWGT

**Table IV.** Overview of the performance of in vitro test batteries for prediction of in vivo genotoxicity. The range indicates results in case equivocals are considered as negative or positive

<table>
<thead>
<tr>
<th>Test batteries</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)</th>
<th>No. of compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ames</td>
<td>71–79</td>
<td>78–83</td>
<td>78</td>
<td>64</td>
</tr>
<tr>
<td>MLA</td>
<td>83–94</td>
<td>41–56</td>
<td>60–66</td>
<td>50</td>
</tr>
<tr>
<td>CA/MN</td>
<td>92–96</td>
<td>56–72</td>
<td>71–79</td>
<td>63</td>
</tr>
<tr>
<td>γH2AX</td>
<td>54</td>
<td>78</td>
<td>69</td>
<td>64</td>
</tr>
<tr>
<td>Combination of two assays</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ames and CA/MN</td>
<td>94–100</td>
<td>29–52</td>
<td>55–67</td>
<td>49</td>
</tr>
<tr>
<td>Ames and γH2AX</td>
<td>75–79</td>
<td>63–68</td>
<td>69–70</td>
<td>64</td>
</tr>
<tr>
<td>Ames and MLA</td>
<td>83–94</td>
<td>41–53</td>
<td>60–64</td>
<td>50</td>
</tr>
<tr>
<td>γH2AX and CA/MN</td>
<td>92–96</td>
<td>46–59</td>
<td>65–71</td>
<td>63</td>
</tr>
<tr>
<td>γH2AX and MLA</td>
<td>83–94</td>
<td>37–47</td>
<td>58–60</td>
<td>50</td>
</tr>
<tr>
<td>MLA and CA/MN</td>
<td>94–100</td>
<td>29–52</td>
<td>55–67</td>
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<tr>
<td>Combination of three assays</td>
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<tr>
<td>Ames, MLA and CA/MN</td>
<td>94–100</td>
<td>29–48</td>
<td>55–65</td>
<td>49</td>
</tr>
<tr>
<td>Ames, MLA and γH2AX</td>
<td>83–94</td>
<td>38–44</td>
<td>58</td>
<td>50</td>
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<tr>
<td>Ames, CA/MN and γH2AX</td>
<td>92–96</td>
<td>41–56</td>
<td>62–70</td>
<td>63</td>
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<tr>
<td>MLA, CA/MN and γH2AX</td>
<td>94–100</td>
<td>26–42</td>
<td>53–61</td>
<td>49</td>
</tr>
</tbody>
</table>
there is general agreement that the top concentration for non-pharmaceuticals should be re-evaluated and likely lowered in comparison to currently recommended levels (51). By testing lower concentrations, definitely, GTX responses related to high cytotoxicity will be avoided.

Finally, mechanisms other than DNA damage, such as kinase inhibition specifically of PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) family enzymes, i.e. by wortmannin, have been reported to influence the phosphorylation of H2AX (27). So far, only quercetin (Que) from the studied compounds in this report is known for its inhibitory activity against PI3K enzymes (75), which may be linked to the positive result in γH2AX assay. It has been reported that apart from SSBs causing drugs, enzyme inhibiting drugs do not directly damage the DNA, thus causing γH2AX formation (26). Therefore, all possible aspects related to γH2AX phosphorylation should be considered in the interpretation of the results.

### Conclusion

Taking into account the ability of the automated γH2AX assay to predict genotoxicity in vitro to the same accuracy as currently used in vitro assays, while its use of human metabolic competent cells, and its automated scoring, its limited use of test compound since small volumes are needed and its simple and rapid applicability to study large numbers of chemicals as it is amenable to robotised procedures, there are many arguments in favor of its usefulness as in vitro genotoxicity test. Especially its high sensitivity to detect DNA-reactive GTX compounds is a positive asset, though the reduced sensitivity for compounds that require metabolic activation needs to be improved. Implementing in vitro test batteries is promising and may also lead to reducing the unnecessary animal follow-up testing. New in vitro developments focusing on improving sensitivity (i.e. toxicogenomics) may be combined with this automated assay, producing a more efficient in vitro testing battery for genotoxicity.

Conflict of interest statement: None declared.

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γH2AX assay in HepG2


