Complementarity of phosphorylated histones H2AX and H3 quantification in different cell lines for genotoxicity screening

Laure Khoury, Daniel Zalko, Marc Audebert

► To cite this version:

Laure Khoury, Daniel Zalko, Marc Audebert. Complementarity of phosphorylated histones H2AX and H3 quantification in different cell lines for genotoxicity screening. Archives of Toxicology, 2016, 90 (8), pp.1983-1995. 10.1007/s00204-015-1599-1 . hal-01604521

HAL Id: hal-01604521

<https://hal.science/hal-01604521>

Submitted on 27 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - ShareAlike| 4.0 International License

Complementarity of phosphorylated histones H2AX and H3 quantification in different cell lines for genotoxicity screening

Laure Khoury, Daniel Zalko & Marc Audebert

Archives of Toxicology

ISSN 0340-5761

Volume 90

Number 8

Arch Toxicol (2016) 90:1983-1995

DOI 10.1007/s00204-015-1599-1

Your article is protected by copyright and all rights are held exclusively by Springer-Verlag Berlin Heidelberg. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your article, please use the accepted manuscript version for posting on your own website. You may further deposit the accepted manuscript version in any repository, provided it is only made publicly available 12 months after official publication or later and provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The final publication is available at link.springer.com".

Complementarity of phosphorylated histones H2AX and H3 quantification in different cell lines for genotoxicity screening

Laure Khoury^{1,2} · Daniel Zalko^{1,2} · Marc Audebert^{1,2}

Received: 15 April 2015 / Accepted: 10 September 2015 / Published online: 24 September 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract The in vitro micronucleus assay is broadly used, but is not per se able to discriminate aneugenic from clastogenic compounds, and cytotoxicity can be a confounding factor. In vitro genotoxicity assays generally rely on cell lines with limited metabolic capabilities. Recently, the use of histone H2AX and H3 phosphorylation markers (γ H2AX and p-H3) was proposed to discriminate aneugenic from clastogenic chemicals. The aim of the present study was to develop a new genotoxic screening strategy based on the use of the γ H2AX and p-H3 biomarkers in combination with cell lines with distinct biotransformation properties. First, we tested a training set of 20 model chemicals comprised of 10 aneugens, five clastogens and five cytotoxics on three human cell lines (HepG2, LS-174T and ACHN). Our data confirm the robustness of these two biomarkers to discriminate efficiently clastogens, aneugens and misleading cytotoxic chemicals in HepG2 cells. Aneugenic compounds induced either an increase or a decrease in p-H3 depending on their mode of action. Clastogens induced γ H2AX, and cytotoxic compounds generated a marked decrease in these two biomarkers. Moreover, the use of different cell lines permits to discriminate direct from bioactivated genotoxins without the need of an

exogenous metabolic activation system. Finally, we further evaluated this strategy using a test set of 13 chemicals with controversial genotoxic potential. The resulting data demonstrate that the combined analysis of γ H2AX and p-H3 is an efficient strategy. Notably, we demonstrated that three compounds (fisetin, hydroquinone and okadaic acid) display both aneugenic and clastogenic properties.

Keywords Genotoxicity · H2AX · H3 · HepG2 · Metabolism

Introduction

Chemicals are routinely screened for genotoxicity in vitro as a means of predicting carcinogenicity in humans. However, the specificity of in vitro mammalian genotoxicity assays is low, as they yield a high incidence of “misleading” or “irrelevant” positive results that are not observed in in vivo studies (Kirkland 2011). Although most human genotoxic carcinogens require metabolic activation, most of the cell lines currently used in in vitro genotoxicity assays express only limited metabolic phase I and II functional activities. Efforts have been made to develop more predictive genotoxicity assays, notably by the addition of an exogenous metabolic activation system (rat liver S9 fractions) (Ku et al. 2007). However, the biotransformation capacity, which conditions the production of metabolites and reactive intermediates, broadly varies among species (Kurebayashi et al. 2010; Mazur et al. 2010). Understanding the genotoxic mechanism (aneugen or clastogen) of a chemical is an important component of cancer risk assessment (Hernandez et al. 2013). One of the most widely used genotoxicity assays is the in vitro micronucleus (MN) assay. However, the MN technique does not provide insight into which aneugen or

Electronic supplementary material The online version of this article (doi:10.1007/s00204-015-1599-1) contains supplementary material, which is available to authorized users.

✉ Marc Audebert
marc.audebert@toulouse.inra.fr

¹ INRA, UMR1331, Toxalim, Research Centre in Food Toxicology, 180 chemin de Tournefeuille, BP 93173, 31027 Toulouse Cedex 3, France

² Université de Toulouse, INPT, UPS, UMR1331, 31062 Toulouse, France

clastogen mechanism is responsible for MN induction without complementary experiments. Kinetochore and centromere staining assays are available and can be used to characterize the genotoxic mechanism of chemicals (Hashimoto et al. 2010; Zacharaki et al. 2013; Hernandez et al. 2013), but these techniques have low throughput and are time-consuming. Moreover, apoptosis can be a confounding factor in the interpretation of the MN result (Meintieres et al. 2001). It is thus important to develop and assess new reliable genotoxicity methods aiming for a more rapid and effective determination of genotoxic MoA.

Aneugenic agents act primarily on non-DNA targets such as spindle fibers or kinases, resulting in a dysfunction and/or reduced function of the various steps of cell division, leading to improper chromosome segregation. Aneugens follow thresholded concentration–response relationships (Parry et al. 1994). Histone H3, which is phosphorylated at serine 10 during mitosis by the Aurora kinase family to permit chromosome condensation and segregation, is a biomarker of mitotic cells (Hans and Dimitrov 2001; Prigent and Dimitrov 2003). It has been demonstrated that aneugenic compounds induce phosphorylation of the histone H3 (p-H3), making p-H3 a potential aneugenic biomarker because of the mitotic delay induced by these chemicals (Muehlbauer and Schuler 2005; Ando et al. 2014; Bryce et al. 2014; Cheung et al. 2015). In contrast to aneugens, clastogenic compounds induce DNA breaks. Phosphorylation of histone H2AX, the resulting product being named γ H2AX, is now clearly established as a biomarker of genotoxicity induced by DNA damaging compounds (Banerjee and Chakravarti 2011) and has been used in different genotoxicity assays (Watters et al. 2009; Matsuzaki et al. 2010; Tsamou et al. 2012; Khoury et al. 2013; Nikolova et al. 2014; Khoury et al. 2015), notably to differentiate aneugens from clastogens in conjunction with the p-H3 biomarker (Ando et al. 2014; Bryce et al. 2014; Cheung et al. 2015). We previously developed and validated a high-throughput genotoxicity assay, named γ H2AX in-cell western (ICW) technique, in human hepatic HepG2 cells. The assay demonstrated high sensitivity and specificity, but failed to discriminate aneugenic from clastogenic compounds (Khoury et al. 2013, 2015).

The aim of the present work was to develop a new genotoxicity screening strategy combining the study of γ H2AX and p-H3 biomarkers to differentiate between aneugenic and clastogenic compounds, in a human cell line context, using cell lines with poor versus high biotransformation capacities permitting to discriminate between direct and bioactivated genotoxins. For this purpose, we used the ICW technique, which allows cytotoxicity and genotoxicity to be determined in a single experiment (Graillot et al. 2012a, b; Khoury et al. 2013). In the present study, first we worked with a panel of 20 model compounds identified as

being aneugens, clastogens or cytotoxics, in three human cell lines (HepG2, LS-174T and ACHN). Both HepG2 and LS-174T cell lines have notable bioactivation capabilities, whereas ACHN cells are very poor metabolizers (Audebert et al. 2010, 2011; Guo et al. 2011; Lin et al. 2012; Khoury et al. 2015). We further evaluated this strategy by testing a set of 13 chemicals with controversial genotoxic MoA.

Materials and methods

Chemicals and reagents

All stock solutions of the compounds were prepared in 100 % dimethyl sulfoxide (DMSO). From the stocks, 10-fold dilution series were prepared for all the compounds, except cytotoxic compounds for which threefold dilution series were prepared. All the compounds were of analytical grade and were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France), excepted for alisertib, tozasertib and volasertib, which were purchased from Selleckchem (Euromedex, France). Penicillin, streptomycin, trypsin, PBS, RNase A and Triton X-100 were purchased from Sigma-Aldrich. The phosphatase inhibitor cocktail tablets (“PHOSSTOP”) were purchased from Roche, and the blocking solution (MAXblock Blocking Medium) was purchased from Active Motif (Belgium). CF770 antibody (goat antibody anti-rabbit and anti-mouse) and RedDot2 were purchased from Biotium (Hayward, CA, USA).

Selection of compounds

The identities of the 20 training set and 13 test set chemicals are listed in Table 1, along with current knowledge of their toxic mode of action. All the compounds were selected from the literature and represent a broad range of genotoxic activities that are generally characterized in both in vitro and in vivo assays. The cytotoxic apoptosis-inducing compounds were selected for their diverse range of activities/toxic mechanisms. The highest concentration tested for most of the compounds was 1 mM, in accordance with the concentration currently used in in vitro genotoxicity tests (Moore et al. 2011). For chemicals with low solubility in DMSO, the highest concentrations tested were 100 μ M (monastrol, econazole, PFOS, vinblastine, citalopram, fisetin, chlorambucil, lomefloxacin, griseofulvin and phenolphthalein), 10 μ M (alisertib and tozasertib) and 0.1 μ M (okadaic acid).

Cell culture

HepG2 human hepatoblastoma cells (ATCC N° HB-8065), ACHN human renal adenocarcinoma cells (ATCC N° CRL

Table 1 Chemicals studied with their proposed mode of toxic action

Name of compound	CAS no.	Mode of toxic action	
Carbendazim	10605-21-7	Aneugen	Spindle poison
Colchicine	64–86-8	Aneugen	Spindle poison
Griseofulvin	126-07-8	Aneugen	Spindle poison
Nocodazole	31430-18-9	Aneugen	Spindle poison
Monastrol	254753-54-3	Aneugen	Spindle poison
Taxol	33069-62-4	Aneugen	Spindle poison
Vinblastine	143-67-9	Aneugen	Spindle poison
BI6727 (Volasertib)	755038-64-4	Aneugen	PLK1 inhibitor
MLN8237 (Alisertib)	1028486-01-2	Aneugen	Aurora A inhibitor
VX-680 (Tozasertib)	639089-54-6	Aneugen	Aurora A inhibitor
Benzo[a]pyrene (BaP)	50-32-8	Clastogen	DNA adducts
Chlorambucil	305-03-3	Clastogen	DNA alkylation
Etoposide (ETO)	33419-42-0	Clastogen	Topoisomerase II inhibitor
Mitomycin C (MMC)	50-07-7	Clastogen	DNA cross link
Methyl methane sulfonate (MMS)	66-27-3	Clastogen	DNA alkylation
Bortezomib	179324-69-7	Cytotoxic	26S proteasome inhibitor
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	555-60-2	Cytotoxic	Mitochondrial uncoupler
Nutlin-3	548472-68-0	Cytotoxic	Inhibitor of Mdm2/p53 interaction
Thapsigargin (TG)	67526-95-8	Cytotoxic	ER stress-induced apoptosis
Tributyltin methoxide (TBT)	1067-52-3	Cytotoxic	20S proteasome inhibitor
Citalopram	59729-33-8	Equivocal	Unknown
Chlorothalonil	1897-45-6	Equivocal	DNA adducts
Daidzein	486-66-8	Equivocal	Unknown
Econazole	27220-47-9	Equivocal	Unknown
Fludioxonil	131341-86-1	Equivocal	Unknown
Fisetin	528-48-3	Equivocal	Aurora B kinase inhibitor/spindle poison
Formaldehyde	50-00-0	Equivocal	Spindle poison/DNA adducts
Hydroquinone	123-31-9	Equivocal	Spindle poison/DNA adducts
Lomefloxacin	98079-51-7	Equivocal	Spindle poison
Okadaic acid	78111-17-8	Equivocal	PP2A phosphatase inhibitor/DNA adducts
Perfluorooctane sulfonate (PFOS)	1763-23-1	Equivocal	Unknown
Phenolphthalein (PHT)	77-09-8	Equivocal	Spindle poison
Phthalic anhydride	85-44-9	Equivocal	Unknown

1611) and LS-174T human epithelial colorectal adenocarcinoma cells (ATCC N° CL-188) were grown in α MEM medium supplemented with 10 % FBS, 100 U mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin. Cultures were maintained in a humidified atmosphere with 5 % CO₂ at 37 °C, and the medium was refreshed every 2–3 days during subculturing.

In-cell western (ICW) assay

The in-cell western technique was performed as previously described (Graillot et al. 2012a, b; Khoury et al. 2013, 2015). Briefly, HepG2, ACHN and LS-174T cell lines (3.2×10^4 , 3×10^4 and 3.6×10^4 cells per well,

respectively), were grown in 96-well plates containing 200 μ L medium per well. Sixteen hours later, the cells were treated in duplicate with the model compounds or vehicle in serum-free medium. For each plate, DMSO (0.2 % v/v final concentration) was used as negative control. The positive controls used in each treatment for γ H2AX studies were 1 μ M benzo[a]pyrene (BaP) for HepG2 and LS-174T cell lines, and 1 μ M etoposide for ACHN cells. The positive control used for p-H3 studies in each treatment was 1 μ M nocodazole for the three cell lines. After 24 h of treatment, the cells were washed and fixed with paraformaldehyde (Electron Microscopy Science). The paraformaldehyde was then neutralized with 20 mM NH₄Cl. Cells were permeabilized with 0.2 % Triton X-100 in PBS, and

washed with PBS supplemented with 2 % fetal calf serum, and 0.2 % Triton X-100 (PST buffer). Cells were blocked with MAXblock Blocking Medium supplemented with phosphatase inhibitor PHOSSTOP and RNase A. Cells were incubated with the primary antibody of interest in PST buffer: rabbit monoclonal anti- γ H2AX (Clone 20E3, Cell Signaling) or rabbit monoclonal anti-p-H3 (Active Motif). After three washes, secondary detection was carried out using an infrared fluorescent dye conjugated to goat antibody (CF770, Biotium). For DNA labeling, RedDot2 (Biotium) was used in conjugation with the secondary antibody. After the incubation and three washes, DNA and the biomarker of interest (γ H2AX or p-H3) were simultaneously visualized using an Odyssey Infrared Imaging Scanner (LiCor ScienceTec, Les Ulis, France). Raw absorbance data were averaged for the duplicate, corrected for background; the relative fluorescence units from the scanning enabled quantitative analysis. The ICW technique allows cytotoxicity and genotoxicity to be determined in a single experiment (Graillot et al. 2012a, b; Khoury et al. 2013). To determine cytotoxicity, the DNA content (related to the number of cells) recorded in the different treated cells was compared to the DNA content in control cells and was expressed as relative cell count (% RCC). For the determination of genotoxicity, relative fluorescent units for γ H2AX (or for p-H3) per cell (as determined by γ H2AX (or p-H3) divided by DNA content) were divided by the respective controls (vehicle only) to determine the change in the level of phosphorylation of histone compared with the control. The same method was used for the global histone H2AX and H3, to quantify both histone levels compared with the control. The primary antibodies used were rabbit monoclonal anti-H2AX (Active Motif) and mouse monoclonal anti-H3 (Active Motif). All experiments were performed at least three times independently on different days (biological triplicates) to calculate mean and SEM.

Data analysis

Genotoxicity was considered positive only if three criteria were achieved: (1) The compound induced a 1.3-fold increase in histone phosphorylation (γ H2AX or p-H3) or a 0.7-fold decrease in p-H3, (2) a statistically significant difference with the control and (3) a level of cytotoxicity below 50 % compared to the control. These parameters are based on our previous studies (Khoury et al. 2013, 2015) and are similar to those used by other groups which use γ H2AX quantification (Smart et al. 2011; Garcia-Canton et al. 2013a, b; Ando et al. 2014; Bryce et al. 2014). Statistically significant increases or decreases in the phosphorylation of H2AX or H3, or in histones nonphosphorylated (H2AX, H3 and H1), were compared with controls using Student's test with Excel 2010 software ($*p < 0.05$;

$**p < 0.01$). Error bars represent SEM (the standard error of the mean). For concentration–response relationships analysis, we have done an ANOVA test between the different positive concentrations tested (ns, not significant; a, $p < 0.05$; b, $p < 0.01$).

Genotoxic signature determination

Based on the positive genotoxicity criteria detailed above for the two biomarkers tested, we characterized a genotoxic signature. In a first step, we built our analysis on data from HepG2 cell assays, in which cytotoxic compounds induced a concomitant γ H2AX and p-H3 decrease. Aneugenic compounds induced an increase or decrease in p-H3 depending of the aneugen's mode of action, as well as an increase in γ H2AX at high concentrations. Clastogens induced γ H2AX and a decrease in p-H3 at high concentrations. To confirm the genotoxic signature classification based on HepG2 cells, and with the aim to discriminate between direct and bioactivated genotoxins, the results obtained using the other cell lines were in a second step taken into account. In both LS-174T and ACHN cell lines, similarly to HepG2 cells, cytotoxic compounds induced a concomitant γ H2AX and p-H3 decrease, and clastogens induced γ H2AX and a decrease in p-H3 at high concentrations. However, bioactivation-dependent clastogens did not induce γ H2AX in ACHN cells. Aneugenic compounds induced an increase or a decrease in p-H3 depending on the aneugen's MoA in LS-174T and ACHN cell lines, but this without any effect on γ H2AX contrary to HepG2 cells.

Results

Results for aneugenic compounds

A set of 10 aneugenic model compounds (Table 1 and S1) was evaluated for their effects on histone H2AX and H3 phosphorylation in three human cell lines (HepG2, LS-174T and ACHN). Results are shown in Table 2 and in Fig. 1 and S1. These 10 aneugenic model compounds had the same effect on the phosphorylation of histone H3 in the three cell lines used, with an increase in p-H3 for the seven spindle poison aneugens (carbendazim, colchicine, griseofulvin, nocodazole, monastrol, taxol and vinblastine) and the PLK1 inhibitor (volasertib), and a decrease in p-H3 for the two Aurora A kinase inhibitors (alisertib and tozasertib) (Fig. 1 and S1). To confirm that the observed decrease in the p-H3 was not related to a decrease in the quantity of total histone H3, we performed supplementary experiments to quantify total histone H3. For the alisertib and tozasertib, which induced a marked decrease in p-H3 (Fig. 1), no variations in total histone H3 were detected in the HepG2 cell

Table 2 Phosphorylation of histones H2AX (γ H2AX) and H3 (p-H3) in HepG2, LS-174T and ACHN cell lines treated with chemicals with known mode of toxic action (color table online)

Name of compound	HepG2		LS-174T		ACHN	
	γ H2AX	p-H3	γ H2AX	p-H3	γ H2AX	p-H3
Aneugen						
Carbendazim	$\nearrow$ 1000	$\nearrow$ 10	(1000)	$\nearrow$ 10	$\searrow$ 100	$\nearrow$ 10
Colchicine	(1000)	$\nearrow$ 0.1	(0.01)	$\nearrow$ 0.01	$\searrow$ 0.1	$\nearrow$ 0.1
Griseofulvin	(100)	$\nearrow$ 1	(100)	$\nearrow$ 10	$\searrow$ 100	$\nearrow$ 100
Nocodazole	$\nearrow$ 1000	$\nearrow$ 1	(1)	$\nearrow$ 1	(1000)	$\nearrow$ 1
Taxol	$\nearrow$ 1	$\nearrow$ 0.01	(0.1)	$\nearrow$ 0.1	$\searrow$ 0.1	$\nearrow$ 0.1
Vinblastine	$\searrow$ 100	$\nearrow$ 0.001	$\searrow$ 0.01	$\nearrow$ 0.01	$\searrow$ 0.1	$\nearrow$ 0.1
Monastrol	$\nearrow$ 100	$\nearrow$ 100	(100)	(100)	(100)	$\nearrow$ 100
Volasertib	$\nearrow$ 0.1	$\nearrow$ 0.1	$\nearrow$ 0.1	$\nearrow$ 0.1	(10)	$\nearrow$ 0.1
Alisertib	(10)	$\searrow$ 1	$\searrow$ 1	$\searrow$ 1	(10)	$\searrow$ 1
Tozasertib	(10)	$\searrow$ 0.1	$\searrow$ 1	$\searrow$ 0.1	(10)	$\searrow$ 0.1
Clastogen						
Benzo[a]pyrene	$\nearrow$ 0.1	(1)	$\nearrow$ 0.1	(1)	(1)	(1)
Chlorambucil	$\nearrow$ 10	$\searrow$ 100	$\nearrow$ 10	$\searrow$ 100	$\nearrow$ 10	$\searrow$ 10
Etoposide	$\nearrow$ 0.1	$\searrow$ 10	$\nearrow$ 1	$\searrow$ 1	$\nearrow$ 1	$\searrow$ 1
MMC	$\nearrow$ 0.1	$\searrow$ 1	$\nearrow$ 0.01	$\searrow$ 0.1	$\nearrow$ 0.1	$\searrow$ 0.1
MMS	$\nearrow$ 10	(1000)	$\nearrow$ 100	(100)	$\nearrow$ 1000	(1000)
Cytotoxic						
Bortezomib	$\searrow$ 0.01	(0.03)	(0.01)	(0.01)	$\searrow$ 0.01	$\searrow$ 0.01
CCCP	$\searrow$ 30	(100)	$\searrow$ 30	$\searrow$ 100	(100)	(100)
Nutlin-3	$\searrow$ 1	$\searrow$ 1	$\searrow$ 3	$\searrow$ 10	$\searrow$ 1	$\searrow$ 10
Thapsigargin	$\searrow$ 0.03	$\searrow$ 0.03	(0.03)	$\searrow$ 0.1	$\searrow$ 0.1	$\searrow$ 0.1
TBT	$\searrow$ 0.3	$\searrow$ 0.3	(0.3)	(0.3)	(0.3)	(0.3)

Orange and upward arrows represent an increase in histone phosphorylation compared to the control. Green and downward arrows represent a decrease in histone phosphorylation compared to the control. Concentrations (in μ M) correspond to the lowest effective concentration (LEC) observed. Clear boxes indicate no biomarker variation, and the highest concentration tested with a level of cytotoxicity below 50 % is indicated in brackets

line (Fig. S2). As observed in our previous studies (Khouri et al. 2013, 2015), aneugens in HepG2 cells induced γ H2AX, always less than twofold compared to controls (Table 2). An increase in γ H2AX was also observed with volasertib in HepG2 and LS-174T cell lines. Conversely, a decrease in γ H2AX was observed for vinblastine in the three cell lines. For all these 10 aneugenic compounds, the genotoxic effect obtained on p-H3 was observed at lower concentrations than for γ H2AX (Table 2). The result observed with aneugens was a nonlinear-type concentration–response relationship for p-H3 fold induction, with a plateau of the curve (Fig. 1 and S1).

Results for clastogenic compounds

We tested five clastogenic model compounds (Table 1), in the three cell lines selected for both biomarkers (γ H2AX and p-H3). Results are summarized in Table 2 and in Fig. 2. These five compounds (benzo[a]pyrene (BaP), chlorambucil, etoposide (ETO), mitomycin C (MMC) and methyl methane sulfonate (MMS)) induced an increase in γ H2AX in the three cell lines, except (as expected) BaP in the

ACHN cells, due to the absence of BaP bioactivation in these cells, as previously described (Audebert et al. 2010, 2012; Khouri et al. 2013, 2015). In the three cell lines, at high concentrations, we observed a decrease in p-H3 for three compounds (chlorambucil, ETO and MMC). The genotoxic effects observed for clastogenic agents were an increase in γ H2AX at low concentrations and a decrease in p-H3 at high concentrations (Table 2). We also observed that clastogens induced a continuously increasing concentration–response curve of γ H2AX (Fig. 2).

Results for cytotoxic compounds

We tested cytotoxic compounds that are frequently responsible for irrelevant in vitro MN genotoxic profile (Bryce et al. 2014). We studied five agents (bortezomib, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), nutlin-3, thapsigargin (TG) and tributyltin methoxide (TBT)), each inducing the apoptosis process by different mechanisms (Table 1 and S1). For all these compounds, we observed a decrease in γ H2AX and/or p-H3 in the three cell lines (Table 2; Fig. 3). No variations in total histone H2AX or

Fig. 1 In vitro cytotoxicity and genotoxicity of three aneugenic compounds (carbendazim, taxol and tozasertib) tested for γ H2AX and p-H3 in the HepG2 cell line. Cytotoxicity is represented by the % RCC. Each value represents the mean $\pm$ SEM ($n \geq 3$) after 24 h of treatment. Significant differences were observed between controls and matched groups (* $p \leq 0.05$; ** $p \leq 0.01$), as well as between positive groups (ns not significant; a, $p < 0.05$; b, $p < 0.01$)

H3 were observed for these five cytotoxic compounds in the HepG2 cell line (Fig S2).

Results for equivocal compounds

Thirteen compounds whose genotoxic potential is not clearly defined were then investigated (Table 1 and S1). We tested these compounds for both histones phosphorylation in the three selected cell lines, and we compared these data with the genotoxic signature obtained with model compounds to classify them (Table 3). Three compounds (citalopram, daidzein and phenolphthalein) induced a decrease in γ H2AX and p-H3 in HepG2 cells (Fig. 4) and were classified as cytotoxic compounds. Three

Fig. 2 In vitro cytotoxicity and genotoxicity of three clastogenic compounds (benzo[a]pyrene, etoposide and chlorambucil) tested for γ H2AX and p-H3 in the HepG2 cell line. Cytotoxicity is represented by the % RCC. Each value represents the mean $\pm$ SEM ($n \geq 3$) after 24 h of treatment. Significant differences were observed between controls and matched groups (* $p \leq 0.05$; ** $p \leq 0.01$), as well as between positive groups (ns not significant; a, $p < 0.05$; b, $p < 0.01$)

compounds (chlorothalonil, formaldehyde and phthalic anhydride) induced an increase in γ H2AX in HepG2 cell line (Fig. 5). At high concentrations, formaldehyde caused also a decrease in p-H3 in these cells as observed for some clastogenic compounds (Table 2). Consequently, we classified these three compounds as clastogens. Four compounds (econazole, fludioxonil, lomefloxacin and perfluorooctane sulfonate (PFOS)) were classified as aneugens (Fig. 6 and Table 2). Econazole induced a decrease in p-H3 in HepG2 and ACHN cells, similarly to Aurora kinase inhibitors volasertib and tozasertib, without any concomitant γ H2AX or H1 decrease, contrary to cytotoxic compounds (Fig. S3). Fludioxonil induced an increase in p-H3 in the three tested cell lines. Lomefloxacin and PFOS also induced

Fig. 3 *In vitro* cytotoxicity and genotoxicity of three cytotoxic compounds (nutlin-3, thapsigargin and tributyltin methoxide) tested for γ H2AX and p-H3 in the HepG2 cell line. Cytotoxicity is represented by the % RCC. Each value represents the mean $\pm$ SEM ($n \geq 3$) after 24 h of treatment. Significant differences were observed between controls and matched groups (* $p \leq 0.05$; ** $p \leq 0.01$)

an increase in p-H3 in HepG2 cells. The last three tested compounds (fisetin, hydroquinone and okadaic acid) demonstrated different effects on histone phosphorylation compared to the model compounds tested. Fisetin presented an unusual genotoxic signature and was highly cytotoxic in LS-174T cells (Fig. S4). In HepG2 cells, at 10^{-5} M, it produced an increase in p-H3 as other aneugenic compound did (Fig. 7). However, at 10^{-4} M an important increase in γ H2AX and, conversely, a decrease in p-H3 were observed, in accordance with the results obtained with other clastogens at high concentration. The same clastogen signature (high γ H2AX increase + p-H3 decrease) was also observed in ACHN cells (Fig. S5). Hydroquinone induced

a concentration-dependent increase in γ H2AX in the three cell lines (Fig. 7, S4 and S5) like the other clastogenic compounds tested (Fig. 2). However, hydroquinone also induced an increase in p-H3 in HepG2 cells, similarly to the other aneugenic model compounds tested (Fig. 1). Okadaic acid induced an important concentration-dependent increase in γ H2AX only in metabolic competent cell lines (HepG2 and LS-174T cells) (Fig. 7, S3 and S4) like the clastogenic agent BaP (Fig. 2). Nonetheless, this compound also induced an increase in p-H3 in the three cell lines (Fig. 7, S4 and S5), like the aneugenic model compounds tested (Fig. 1). Consequently, for these three compounds (fisetin, hydroquinone and okadaic acid), we propose an aneugen and a clastogen genotoxic potential (Table 3).

Discussion

The aim of the present study was to develop a new *in vitro* genotoxicity screening strategy based on the use of the γ H2AX and p-H3 biomarkers in combination with cell lines with distinct biotransformation properties, in a high-throughput manner. First, we successfully classified 20 model compounds as clastogens, aneugens or cytotoxics based on the γ H2AX and p-H3 biomarkers in the three cell lines used. We observed with the ICW technique the specificity of p-H3 for aneugens with an increase in this biomarker with spindle poisons, thereby confirming recent reports (Ando et al. 2014; Bryce et al. 2014; Cheung et al. 2015) and a decrease in p-H3 with Aurora kinase inhibitors (Harrington et al. 2004; Michaelis et al. 2014). We also observed a threshold-type concentration–response in p-H3 for aneugens, as previously reported with the MN test (Elhajouji et al. 1997). Consequently, not only the p-H3 biomarker made it possible to efficiently distinguish between aneugenic and clastogenic chemicals, contrary to the MN FISH staining technique, but we also hypothesize that it could be used to characterize the aneugen MoA. We also noted that at high concentrations, aneugens with a spindle poison MoA induced γ H2AX in HepG2 cells, confirming the results of our previous studies (Khouri et al. 2013, 2015). This result may be linked to the chromothripsis phenomenon (Janssen et al. 2011; Crasta et al. 2012; Orth et al. 2012). As observed in our previous studies (Audebert et al. 2012; Khouri et al. 2013, 2015) and by other groups (Watters et al. 2009; Smart et al. 2011; Tsamou et al. 2012; Ando et al. 2014; Bryce et al. 2014; Nikolova et al. 2014), clastogens induced a continuously increasing concentration–response curve of γ H2AX. With high concentrations of some clastogenic compounds, we also observed a decrease in p-H3. This observation is in agreement with a recent study (Sharma et al. 2015) and may reflect a global chromatin compaction that occurs to

Table 3 Phosphorylation of histones (γ H2AX) and H3 (p-H3) in HepG2, LS-174T and ACHN cell lines treated with chemicals with unknown or equivocal mode of genotoxic action (color table online)

Name of compound	HepG2		LS-174T		ACHN		Proposed classification
	γ H2AX	p-H3	γ H2AX	p-H3	γ H2AX	p-H3	
Citalopram	↘ 100	↘ 100	↘ 100	↘ 100	↘ 100	↘ 100	Cytotoxic
Daidzein	↘ 1000	↘ 1000	↘ 1000	(1000)	(1000)	↘ 1000	Cytotoxic
Phenolphthalein	↘ 100	↘ 100	↘ 10	(10)	(10)	(10)	Cytotoxic
Chlorothalonil	↗ 10	(10)	(1)	(1)	(1)	(1)	Clastogen
Formaldehyde	↗ 1000	↘ 1000	(100)	(100)	(1000)	↘ 1000	Clastogen
Phthalic anhydride	↗ 1000	(1000)	(1000)	(1000)	(1000)	(1000)	Clastogen
Econazole	(10)	↘ 10	↘ 10	↘ 10	(10)	↘ 10	Aneugen
Fludioxonil	↗ 10	↗ 10	(100)	↗ 10	(100)	↗ 10	Aneugen
Lomefloxacin	(100)	↗ 100	(100)	(100)	(100)	(100)	Aneugen
PFOS	↗ 10	↗ 10	↘ 100	↘ 100	(100)	(100)	Aneugen
Fisetin	↗ 100	↗ 10	(10)	(10)	↗ 100	↘ 10	Clastogen + Aneugen
Hydroquinone	↗ 10	↗ 10	↗ 10	↘ 100	↗ 10	(10)	Clastogen + Aneugen
Okadaic acid	↗ 0.01	↗ 0.001	↗ 0.001	↗ 0.01	(0.01)	↗ 0.01	Clastogen + Aneugen

Orange and upward arrows represent an increase in histone phosphorylation compared to the control. Green and downward arrows represent a decrease in histone phosphorylation compared to the control. Concentrations (in μ M) correspond to the lowest effective concentration (LEC) observed. No-color boxes indicate no biomarker variation, and the highest concentration tested with a level of cytotoxicity below 50 % is indicated in brackets

repress transcription to facilitate DNA repair and mitotic delay. The five model cytotoxic compounds induced a concomitant loss of γ H2AX and p-H3 biomarkers. This observation was not reported in other studies using the same biomarkers (Bryce et al. 2014; Cheung et al. 2015). This difference may be explained by the difference in the techniques used. With FACS analysis with cells in suspension, no rigorous washing steps are required (Bryce et al. 2014; Cheung et al. 2015). Conversely, with the ICW technique we used in our studies on adherent cells, numerous washing steps were performed using detergent (Triton X100). Consequently, apoptotic and damaged cells were discarded before the final analysis, but possibly some proteins from live cells as well. The observed decrease in γ H2AX and p-H3 biomarkers (and also H1 linker histone, Fig S2) with cytotoxic compounds, without a marked decrease in RCC, is probably linked to the loss of higher-order chromatin compaction and corresponding histone dissociation resulting from general toxicity (Wojcik et al. 2013).

Because the proposed genotoxic screening strategy with model compounds was particularly encouraging, we analyzed a test set of 13 chemicals with controversial genotoxic potential. Three compounds (citalopram, daidzein and phenolphthalein) displayed cytotoxic properties (decrease in γ H2AX and/or p-H3 biomarkers) in our study. So far, citalopram was not reported to be genotoxic in vitro (Brambilla and Martelli 2009; Brambilla et al. 2009), and only one in vivo study reported aneugenic and clastogenic properties of citalopram at high doses (Attia et al. 2013). All available studies on phenolphthalein genotoxicity have

reported negative results, except one in vitro study that reported aneugenic and clastogenic properties of phenolphthalein but only at concentrations associated with striking cytotoxicity (Heard et al. 2013). It is possible that for these two compounds, the apparent discrepancy between the results of these studies and our own results relies on the high cytotoxicity reported in the former experiments. Daidzein's genotoxicity was previously stated to be equivocal (Stopper et al. 2005), and some studies reported both aneugenic and clastogenic properties in the V79 cell line (Di Virgilio et al. 2004; Dorn et al. 2007). The nature of the cell line used could explain the observed differences. Notably, V79 cells are more susceptible to cytotoxicity than p53-competent cells (like the cell lines used in this study) and are therefore more likely to produce misleading MN-positive results (Fowler et al. 2012).

Because three compounds (chlorothalonil, formaldehyde and phthalic anhydride) displayed γ H2AX induction in HepG2 cells, they were classified as clastogens. Chlorothalonil is considered to be a nongenotoxic carcinogen because of negative in vitro genotoxic data (Vinken et al. 2008). However, its carcinogenicity may be linked with the formation of a specific metabolite (Rakitsky et al. 2000). Reports on the genotoxicity of formaldehyde are controversial. In addition to its well-documented clastogenic activity (Lu et al. 2010), an aneugenic activity of formaldehyde cannot completely be ruled out (Kumari et al. 2012; Kuehner et al. 2013). However, in our assay, formaldehyde induced γ H2AX/p-H3 in HepG2 cells, in agreement with a recently published study (Yoshida and Ibuki 2014). Phthalic

Fig. 4 In vitro cytotoxicity and genotoxicity of citalopram, daidzein and phenolphthalein tested for γH2AX and p-H3 in the HepG2 cell line. Cytotoxicity is represented by the % RCC. Each value represents the mean ± SEM ($n \geq 3$) after 24 h of treatment. Significant differences were observed between controls and matched groups (* $p \leq 0.05$; ** $p \leq 0.01$)

anhydride was considered to be a false-positive compound (Kirkland et al. 2008). However, it has been reported to induce MN at noncytotoxic concentrations in different cell lines (Fowler et al. 2012) and was found to be genotoxic in our previous study (Khoury et al. 2013). For all these compounds, a particular metabolism in HepG2 cells may explain our results in the different cell lines tested.

The aneugenic mode of action of econazole is not clearly understood (Bellincampi et al. 1980). This compound did not display a spindle poison property (Brunner et al. 1991), but induced MN specific to aneugenic chemicals (Antocchia et al. 1991). Because in our assay econazole induced a decrease in p-H3 in HepG2 and ACHN cells without any effect on the other histones tested, we propose that this

Fig. 5 In vitro cytotoxicity and genotoxicity of chlorothalonil, formaldehyde and phthalic anhydride tested for γH2AX and p-H3 in the HepG2 cell line. Cytotoxicity is represented by the % RCC. Each value represents the mean ± SEM ($n \geq 3$) after 24 h of treatment. Significant differences were observed between controls and matched groups (* $p \leq 0.05$; ** $p \leq 0.01$)

compound could act through an Aurora kinase inhibitor mode of action. Several studies showed that fludioxonil is not genotoxic in vitro, but fludioxonil has been reported to increase the incidence of hepatocellular tumors in the rat (EFSA 2007). We previously reported that fludioxonil induced γH2AX in HepG2 cells (Graillot et al. 2012a). Here, we confirm this observation and also demonstrated p-H3 inductions in the three cell lines tested and hence categorize this compound explicitly as aneugen. This result is in agreement with the conclusions of a very recent study that demonstrated the aneugenic property of fludioxonil in the CHO-K1 cell line (Kligerman et al. 2015). Lomefloxacin genotoxicity in vitro was equivocal (Kersten et al. 1999; Snyder and Cooper 1999) and was possibly mediated

Fig. 6 In vitro cytotoxicity and genotoxicity of econazole, fludionil, lomefloxacin and PFOS tested for γH2AX and p-H3 in the HepG2 cell line. Cytotoxicity is represented by the % RCC. Each

value represents the mean ± SEM ($n \geq 3$) after 24 h of treatment. Significant differences were observed between controls and matched groups (* $p \leq 0.05$; ** $p \leq 0.01$)

through a clastogen topoisomerase inhibition MoA (Perone et al. 2002; Williams et al. 2013). *In vivo*, its clastogenic properties were also reported (Singh et al. 2003; Attia 2009). In our study, lomefloxacin demonstrated p-H3 induction in HepG2 cells, and we categorized this chemical as aneugen. PFOS is carcinogenic in rodents, presumably through a nongenotoxic mechanism (Jacquet et al. 2012) because no in vitro evidence for genotoxicity has yet been put forward and because previous oxidative stress data for this compound are conflicting (Eriksen et al. 2010; Florentin et al. 2011). In our assay, PFOS was found to be genotoxic in HepG2 cells, with low but significant increase in H2AX and H3 phosphorylation and was thus categorized as aneugen.

Fisetin genotoxicity was reported to act through a clastogen MoA by topoisomerase II inhibition, but also as an aneugen through Aurora kinase inhibition (Gollapudi et al. 2014). Hydroquinone was reported to induce MN with aneugenic and clastogenic properties (Antocchia et al. 1991; Hashimoto et al. 2010) and also to induce oxidative stress (Peng et al. 2013). Okadaic acid has been recognized as a model aneugenic compound in numerous studies (Hashimoto et al. 2010; Bryce et al. 2014) through the inhibition of PP1 and PP2A phosphatases that alter centrosome replication leading to aberrant spindle formation (Valdiglesias et al. 2013). However, okadaic acid was also reported to

induce DNA adducts (Fessard et al. 1996), and its genotoxicity is enhanced by bioactivation processes (Le Hegarat et al. 2003). Moreover, results may depend on the type of cell used (Souid-Mensi et al. 2008; Valdiglesias et al. 2010). All these reports are in full agreement with our results and with the classification of these three compounds (fisetin, hydroquinone and okadaic acid) as chemicals with both clastogenic and aneugenic properties, which demonstrates the efficiency of our genotoxicity screening strategy.

In conclusion, the present study strongly supports the combined use of γH2AX/p-H3 biomarkers and of different cell lines for genotoxicity screening. This strategy made it possible to rapidly classify compounds as clastogen, aneugen or cytotoxic in a high-throughput manner. HepG2 cells were found to be sensitive to all types of genotoxins tested. Combining with γH2AX/p-H3 biomarkers, they enabled a rapid screening of the genotoxic potential of chemicals without the need of an exogenous metabolic activation system. Moreover, as demonstrated in this study, the use of complementary cell lines with distinct bioactivation capacity can provide additional key information about the involvement of metabolic activation processes in genotoxicity induction. The use of this genotoxicity screening strategy should thus improve in vitro genotoxicity testing, with a possible concomitant reduction in the number of animals used for genotoxicity assessment.

Fig. 7 In vitro cytotoxicity and genotoxicity of fisetin, hydroquinone and okadaic acid tested for γH2AX and p-H3 in the HepG2 cell line. Cytotoxicity is represented by the % RCC. Each value represents the mean ± SEM ($n \geq 3$) after 24 h of treatment. Significant differences were observed between controls and matched groups ($*p \leq 0.05$; $**p \leq 0.01$), as well as between positive groups (ns not significant; a , $p < 0.05$; b , $p < 0.01$)

Acknowledgments This work benefited from fruitful discussions with many genotoxicity experts who generously shared their experience, special thanks to Drs. David Kirkland, Richard Walmsley, Steven Bryce and Stephen Dertinger.

Funding This work was supported in part by the French ANSES MYCO-TOX EST-13-214 and ANR “Contreperf” Grants.

References

Ando M, Yoshikawa K, Iwase Y, Ishiura S (2014) Usefulness of monitoring gamma-H2AX and cell cycle arrest in HepG2 cells for estimating genotoxicity using a high-content analysis system. J Biomol Screen 19:1246–1254

- Antoccia A, Degraffi F, Battistoni A, Ciliutti P, Tanzarella C (1991) In vitro micronucleus test with kinetochore staining: evaluation of test performance. Mutagenesis 6:319–324
- Attia SM (2009) Use of centromeric and telomeric DNA probes in in situ hybridization for differentiation of micronuclei induced by lomefloxacin. Environ Mol Mutagen 50:394–403
- Attia SM, Ashour AE, Bakheet SA (2013) Comet-FISH studies for evaluation of genetic damage of citalopram in somatic cells of the mouse. J Appl Toxicol 33:901–905
- Audebert M, Riu A, Jacques C, Hillenweck A, Jamin EL, Zalko D, Cravedi JP (2010) Use of the gammaH2AX assay for assessing the genotoxicity of polycyclic aromatic hydrocarbons in human cell lines. Toxicol Lett 199:182–192
- Audebert M, Dolo L, Perdu E, Cravedi JP, Zalko D (2011) Use of the gammaH2AX assay for assessing the genotoxicity of bisphenol A and bisphenol F in human cell lines. Arch Toxicol 85:1463–1473
- Audebert M, Zeman F, Beaudoin R, Pery A, Cravedi JP (2012) Comparative potency approach based on H2AX assay for estimating the genotoxicity of polycyclic aromatic hydrocarbons. Toxicol Appl Pharmacol 260:58–64
- Banerjee T, Chakravarti D (2011) A peek into the complex realm of histone phosphorylation. Mol Cell Biol 31:4858–4873
- Bellincampi D, Gualandi G, la Monica E, Poley C, Morpurgo GP (1980) Membrane-damaging agents cause mitotic non-disjunction in *Aspergillus nidulans*. Mutat Res 79:169–172
- Brambilla G, Martelli A (2009) Update on genotoxicity and carcinogenicity testing of 472 marketed pharmaceuticals. Mutat Res 681:209–229
- Brambilla G, Mattioli F, Martelli A (2009) Genotoxic and carcinogenic effects of antipsychotics and antidepressants. Toxicology 261:77–88
- Brunner M, Albertini S, Wurgler FE (1991) Effects of 10 known or suspected spindle poisons in the in vitro porcine brain tubulin assembly assay. Mutagenesis 6:65–70
- Bryce SM, Bemis JC, Mereness JA, Spellman RA, Moss J, Dickinson D, Schuler MJ, Dertinger SD (2014) Interpreting in vitro micronucleus positive results: simple biomarker matrix discriminates clastogens, aneugens, and misleading positive agents. Environ Mol Mutagen 55:542–555
- Cheung JR, Dickinson DA, Moss J, Schuler MJ, Spellman RA, Heard PL (2015) Histone markers identify the mode of action for compounds positive in the TK6 micronucleus assay. Mutat Res Genet Toxicol Environ Mutagen 777:7–16
- Crasta K, Ganem NJ, Dagher R, Lantermann AB, Ivanova EV, Pan Y, Nezi L, Protopopov A, Chowdhury D, Pellman D (2012) DNA breaks and chromosome pulverization from errors in mitosis. Nature 482:53–58
- Di Virgilio AL, Iwami K, Watjen W, Kahl R, Degen GH (2004) Genotoxicity of the isoflavones genistein, daidzein and equol in V79 cells. Toxicol Lett 151:151–162
- Dorn SB, Degen GH, Muller T, Bonacker D, Joosten HF, van der Louw J, van Acker FA, Bolt HM (2007) Proposed criteria for specific and non-specific chromosomal genotoxicity based on hydrophobic interactions. Mutat Res 628:67–75
- EFSA (2007) Conclusion regarding the peer review of the pesticide risk assessment of the active substance fludioxonil. http://www.efsa.europa.eu/en/efsajournal/doc/praper_concl_sr110_fludioxonil_en.pdf?ssbinary=true
- Elhajoui A, Tibaldi F, Kirsch-Volders M (1997) Indication for thresholds of chromosome non-disjunction versus chromosome lagging induced by spindle inhibitors in vitro in human lymphocytes. Mutagenesis 12:133–140
- Eriksen KT, Raaschou-Nielsen O, Sorensen M, Roursgaard M, Loft S, Moller P (2010) Genotoxic potential of the perfluorinated chemicals PFOA, PFOS, PFBS, PFNA and PFHxA in human HepG2 cells. Mutat Res 700:39–43

- Fessard V, Grosse Y, Pfohl-Leszkowicz A, Puiseux-Dao S (1996) Okadaic acid treatment induces DNA adduct formation in BHK21 C13 fibroblasts and HESV keratinocytes. *Mutat Res* 361:133–141
- Florentin A, Deblonde T, Diguio N, Hautemaniere A, Hartemann P (2011) Impacts of two perfluorinated compounds (PFOS and PFOA) on human hepatoma cells: Cytotoxicity but no genotoxicity? *Int J Hyg Environ Health* 214:493–499
- Fowler P, Smith K, Young J, Jeffrey L, Kirkland D, Pfuhler S, Carmichael P (2012) Reduction of misleading (“false”) positive results in mammalian cell genotoxicity assays. I. Choice of cell type. *Mutat Res* 742:11–25
- Garcia-Canton C, Anadon A, Meredith C (2013a) Assessment of the in vitro gammaH2AX assay by high content screening as a novel genotoxicity test. *Mutat Res* 757:158–166
- Garcia-Canton C, Anadon A, Meredith C (2013b) Genotoxicity evaluation of individual cigarette smoke toxicants using the in vitro gammaH2AX assay by high content screening. *Toxicol Lett* 223:81–87
- Gollapudi P, Hasegawa LS, Eastmond DA (2014) A comparative study of the aneugenic and polyploidy-inducing effects of fisetin and two model Aurora kinase inhibitors. *Mutat Res, Genet Toxicol Environ Mutagen* 767:37–43
- Graillot V, Takakura N, Hegarat LL, Fessard V, Audebert M, Cravedi JP (2012a) Genotoxicity of pesticide mixtures present in the diet of the French population. *Environ Mol Mutagen* 53:173–184
- Graillot V, Tomasetti F, Cravedi JP, Audebert M (2012b) Evidence of the in vitro genotoxicity of methyl-pyrazole pesticides in human cells. *Mutat Res* 748:8–16
- Guo L, Dial S, Shi L, Branham W, Liu J, Fang JL, Green B, Deng H, Kaput J, Ning B (2011) Similarities and differences in the expression of drug-metabolizing enzymes between human hepatic cell lines and primary human hepatocytes. *Drug Metab Dispos* 39:528–538
- Hans F, Dimitrov S (2001) Histone H3 phosphorylation and cell division. *Oncogene* 20:3021–3027
- Harrington EA, Bebbington D, Moore J, Rasmussen RK, Ajose-Adeogun AO, Nakayama T, Graham JA, Demur C, Hercend T, Diu-Hercend A, Su M, Golec JM, Miller KM (2004) VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo. *Nat Med* 10:262–267
- Hashimoto K, Nakajima Y, Matsumura S, Chatani F (2010) An in vitro micronucleus assay with size-classified micronucleus counting to discriminate aneugens from clastogens. *Toxicol In Vitro* 24:208–216
- Heard PL, Rubitski EE, Spellman RA, Schuler MJ (2013) Phenolphthalein induces centrosome amplification and tubulin depolymerization in vitro. *Environ Mol Mutagen* 54:308–316
- Hernandez LG, van Benthem J, Johnson GE (2013) A mode-of-action approach for the identification of genotoxic carcinogens. *PLoS ONE* 8:e64532
- Jacquet N, Maire MA, Landkocz Y, Vasseur P (2012) Carcinogenic potency of perfluorooctane sulfonate (PFOS) on Syrian hamster embryo (SHE) cells. *Arch Toxicol* 86:305–314
- Janssen A, van der Burg M, Szuhai K, Kops GJ, Medema RH (2011) Chromosome segregation errors as a cause of DNA damage and structural chromosome aberrations. *Science* 333:1895–1898
- Kersten B, Zhang J, Brendler-Schwaab SY, Kasper P, Muller L (1999) The application of the micronucleus test in Chinese hamster V79 cells to detect drug-induced photogenotoxicity. *Mutat Res* 445:55–71
- Khoury L, Zalko D, Audebert M (2013) Validation of high-throughput genotoxicity assay screening using gammaH2AX in-cell western assay on HepG2 cells. *Environ Mol Mutagen* 54:737–746
- Khoury L, Zalko D, Audebert M (2015) Evaluation of four human cell lines with distinct biotransformation properties for genotoxic screening. *Mutagenesis*. doi:10.1093/mutage/gev058
- Kirkland D (2011) Improvements in the reliability of in vitro genotoxicity testing. *Expert Opin Drug Metab Toxicol* 7:1513–1520
- Kirkland D, Kasper P, Muller L, Corvi R, Speit G (2008) Recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests: a follow-up to an ECVAM workshop. *Mutat Res* 653:99–108
- Kligerman AD, Young RR, Stankowski LF, Jr, Pant K, Lawlor T, Aardema MJ, Houck KA (2015) An evaluation of 25 selected Toxic-Cast chemicals in medium-throughput assays to detect genotoxicity. *Environ Mol Mutagen* 2014 Dec 23. doi:10.1002/em.21934
- Ku WW, Bigger A, Brambilla G, Glatt H, Gocke E, Guzzie PJ, Hakura A, Honma M, Martus HJ, Obach RS, Roberts S (2007) Strategy for genotoxicity testing—metabolic considerations. *Mutat Res* 627:59–77
- Kuehner S, Holzmann K, Speit G (2013) Characterization of formaldehyde’s genotoxic mode of action by gene expression analysis in TK6 cells. *Arch Toxicol* 87:1999–2012
- Kumari A, Lim YX, Newell AH, Olson SB, McCullough AK (2012) Formaldehyde-induced genome instability is suppressed by an XPF-dependent pathway. *DNA Repair (Amst)* 11:236–246
- Kurebayashi H, Okudaira K, Ohno Y (2010) Species difference of metabolic clearance of bisphenol A using cryopreserved hepatocytes from rats, monkeys and humans. *Toxicol Lett* 198:210–215
- Le Hegarat L, Puech L, Fessard V, Poul JM, Dragacci S (2003) Aneugenic potential of okadaic acid revealed by the micronucleus assay combined with the FISH technique in CHO-K1 cells. *Mutagenesis* 18:293–298
- Lin J, Schyschka L, Muhl-Benninghaus R, Neumann J, Hao L, Nussler N, Dooley S, Liu L, Stockle U, Nussler AK, Ehnert S (2012) Comparative analysis of phase I and II enzyme activities in 5 hepatic cell lines identifies Huh-7 and HCC-T cells with the highest potential to study drug metabolism. *Arch Toxicol* 86:87–95
- Lu K, Collins LB, Ru H, Bermudez E, Swenberg JA (2010) Distribution of DNA adducts caused by inhaled formaldehyde is consistent with induction of nasal carcinoma but not leukemia. *Toxicol Sci* 116:441–451
- Matsuzaki K, Harada A, Takeiri A, Tanaka K, Mishima M (2010) Whole cell-ELISA to measure the gammaH2AX response of six aneugens and eight DNA-damaging chemicals. *Mutat Res* 700:71–79
- Mazur CS, Kenneke JF, Hess-Wilson JK, Lipscomb JC (2010) Differences between human and rat intestinal and hepatic bisphenol-A glucuronidation and the influence of alamethicin on in vitro kinetic measurements. *Drug Metab Dispos* 38:2232–2238
- Meintieres S, Biola A, Pallardy M, Marzin D (2001) Apoptosis can be a confusing factor in in vitro clastogenic assays. *Mutagenesis* 16:243–250
- Michaelis M, Selt F, Rothweiler F, Loschmann N, Nusse B, Dirks WG, Zehner R, Cinatl J Jr (2014) Aurora kinases as targets in drug-resistant neuroblastoma cells. *PLoS ONE* 9:e108758
- Moore MM, Honma M, Clements J, Awogi T, Douglas GR, van Goethem F, Gollapudi B, Kimura A, Muster W, O’Donovan M, Schoeny R, Wakuri S (2011) Suitable top concentration for tests with mammalian cells: mouse lymphoma assay workgroup. *Mutat Res* 723:84–86
- Muehlbauer PA, Schuler MJ (2005) Detection of numerical chromosomal aberrations by flow cytometry: a novel process for identifying aneugenic agents. *Mutat Res* 585:156–169
- Nikolova T, Dvorak M, Jung F, Adam I, Kramer E, Gerhold-Ay A, Kaina B (2014) The gammaH2AX assay for genotoxic and non-genotoxic agents: comparison of H2AX phosphorylation with cell death response. *Toxicol Sci* 140:103–117
- Orth JD, Loewer A, Lahav G, Mitchison TJ (2012) Prolonged mitotic arrest triggers partial activation of apoptosis, resulting in DNA damage and p53 induction. *Mol Biol Cell* 23:567–576

- Parry JM, Fielder RJ, McDonald A (1994) Thresholds for aneuploidy-inducing chemicals. Advisory Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment of the UK Department of Health. *Mutagenesis* 9:503–504
- Peng C, Arthur D, Liu F, Lee J, Xia Q, Lavin MF, Ng JC (2013) Genotoxicity of hydroquinone in A549 cells. *Cell Biol Toxicol* 29:213–227
- Perrone CE, Takahashi KC, Williams GM (2002) Inhibition of human topoisomerase IIalpha by fluoroquinolones and ultraviolet A irradiation. *Toxicol Sci* 69:16–22
- Prigent C, Dimitrov S (2003) Phosphorylation of serine 10 in histone H3, What for? *J Cell Sci* 116:3677–3685
- Rakitsky VN, Koblyakov VA, Turusov VS (2000) Nongenotoxic (epigenetic) carcinogens: pesticides as an example. A critical review. *Teratog Carcinog Mutagen* 20:229–240
- Sharma AK, Bhattacharya S, Khan SA, Khade B, Gupta S (2015) Dynamic alteration in H3 serine 10 phosphorylation is G1-phase specific during ionization radiation induced DNA damage response in human cells. *Mutat Res* 773:83–91
- Singh AC, Kumar M, Jha AM (2003) Genotoxicity of lomefloxacin—an antibacterial drug in somatic and germ cells of Swiss albino mice in vivo. *Mutat Res* 535:35–42
- Smart DJ, Ahmedi KP, Harvey JS, Lynch AM (2011) Genotoxicity screening via the gammaH2AX by flow assay. *Mutat Res* 715:25–31
- Snyder RD, Cooper CS (1999) Photogenotoxicity of fluoroquinolones in Chinese hamster V79 cells: dependency on active topoisomerase II. *Photochem Photobiol* 69:288–293
- Souid-Mensi G, Moukha S, Mobio TA, Maaroufi K, Creppy EE (2008) The cytotoxicity and genotoxicity of okadaic acid are cell-line dependent. *Toxicol* 51:1338–1344
- Stopper H, Schmitt E, Kobras K (2005) Genotoxicity of phytoestrogens. *Mutat Res* 574:139–155
- Tsamou M, Jennen DG, Claessen SM, Magkouloupoulou C, Kleijnans JC, van Delft JH (2012) Performance of in vitro gammaH2AX assay in HepG2 cells to predict in vivo genotoxicity. *Mutagenesis* 27:645–652
- Valdiglesias V, Mendez J, Pasaro E, Cemeli E, Anderson D, Laffon B (2010) Assessment of okadaic acid effects on cytotoxicity, DNA damage and DNA repair in human cells. *Mutat Res* 689:74–79
- Valdiglesias V, Prego-Faraldo MV, Pasaro E, Mendez J, Laffon B (2013) Okadaic acid: more than a diarrhetic toxin. *Mar Drugs* 11:4328–4349
- Vinken M, Doktorova T, Ellinger-Ziegelbauer H, Ahr HJ, Lock E, Carmichael P, Roggen E, van Delft J, Kleijnans J, Castell J, Bort R, Donato T, Ryan M, Corvi R, Keun H, Ebbels T, Athersuch T, Sansone SA, Rocca-Serra P, Stierum R, Jennings P, Pfaller W, Gmuender H, Vanhaecke T, Rogiers V (2008) The carcinoG-ENOMICS project: critical selection of model compounds for the development of omics-based in vitro carcinogenicity screening assays. *Mutat Res* 659:202–210
- Watters GP, Smart DJ, Harvey JS, Austin CA (2009) H2AX phosphorylation as a genotoxicity endpoint. *Mutat Res* 679:50–58
- Williams GM, Brunnemann KD, Smart DJ, Molina D, Jeffrey AM, Duan JD, Krebsfaenger N, Kampkoetter A, Schmuck G (2013) Relationship of cellular topoisomerase IIalpha inhibition to cytotoxicity and published genotoxicity of fluoroquinolone antibiotics in V79 cells. *Chem Biol Interact* 203:386–390
- Wojcik K, Zarebski M, Cossarizza A, Dobrucki JW (2013) Daunomycin, an antitumor DNA intercalator, influences histone-DNA interactions. *Cancer Biol Ther* 14:823–832
- Yoshida I, Ibuki Y (2014) Formaldehyde-induced histone H3 phosphorylation via JNK and the expression of proto-oncogenes. *Mutat Res* 770:9–18
- Zacharaki P, Stephanou G, Demopoulos NA (2013) Comparison of the aneugenic properties of nocodazole, paclitaxel and griseofulvin in vitro. Centrosome defects and alterations in protein expression profiles. *J Appl Toxicol* 33:869–879