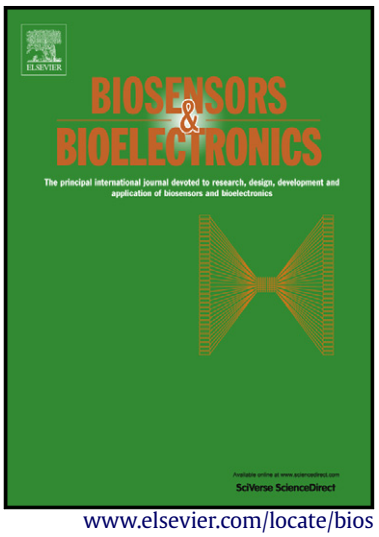

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The cell-based biosensor system HepG2CDKN1A-DsRed for rapid and simple detection of genotoxic agents

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Abstract

The regulatory requirements for genotoxicity testing rely on a battery of genotoxicity tests, which generally consist of bacterial and mammalian cell assays for detection of gene mutations and chromosomal aberrations. However, for rapid screening, these methods are not appropriate. We have developed a new cell-based biosensor system that provides rapid and simple detection of genotoxic substances. This is based on stable transfection of human hepatoma HepG2 cells with a plasmid that encodes the red fluorescent protein DsRed2 under the control of the *CDKN1A* promoter (HepG2CDKN1A-DsRed cells). As the major downstream target gene of activated TP53, the tumour-suppressor gene *CDKN1A* is responsible for cell-cycle arrest following DNA damage, and it has been shown to be specifically up-regulated by genotoxic carcinogens. The assay is optimised for a 96-well microplate format and spectrofluorimetric quantification of induced *DsRed* expression. The assay was evaluated by testing direct-acting and indirect-acting genotoxic compounds with different mechanisms of action, along with non-genotoxic compounds. Of 25 compounds that are known to be genotoxic *in vitro* and *in vivo*, 21 (84%) were detected as positive at non-cytotoxic doses, whereas of 12 compounds not considered genotoxic, 11 (92%) were negative. These data indicate the high sensitivity and specificity of our biosensor system. Based on its simplicity and sensitivity, this biosensor developed with HepG2CDKN1A-DsRed cells has the potential to become a valuable tool for genotoxicity screening for chemical safety evaluation, as well as for environmental and occupational monitoring of exposure to genotoxic agents and their complex mixtures.

Keywords: whole-cell biosensor system, CDKN1A promoter, DsRed reporter protein, genotoxicity, spectrofluorimetry

Introduction

Exposure to genotoxic compounds is a major health concern, as these compounds can induce damage to genetic material that is associated with elevated risk of cancer and other DNA-damage-associated diseases. Therefore, legislation has been established that demands testing of new compounds and products for their potential genotoxicity, such as pharmaceuticals, pesticides, food additives and cosmetics. The regulatory requirements for genotoxicity testing rely on a battery of genotoxicity tests, which generally consist of *in-vitro* tests for gene mutations in bacteria and mammalian cells, and *in-vivo* tests for chromosomal damage in rodent haematopoietic cells (Elespuru et al., 2009). However, these methods are time consuming and expensive, and they require relatively high quantities of the test material. Thus, they are not suitable for screening purposes when large numbers of samples have to be tested in a short period of time; i.e., for new drug discovery and environmental monitoring.

To overcome these problems, rapid, high-throughput biological assays that measure cell responses to induced genetic damage are being developed to provide 'early warning' of genotoxicity. Many of these assays are based on the promoter of a DNA-damage-responsive gene as the sensor, which can be fused to a reporter gene that codes for a product that can be measured. Bacterial systems in which genotoxic effects are identified based on changes in expression of SOS response genes were developed more than three decades ago (Biran et al., 2010). The same principle was later used for the development of *Saccharomyces cerevisiae* yeast reporter assays in which the *RAD54*, *RAD51* and *RNR2* promoters were fused to a reporter gene (Afanassiev et al., 2000; Billinton et al., 1998; Walmsley et al., 1997).

In mammalian cells, the tumour-suppressor TP53 has a central role in cell responses to DNA damage through its activation of the transcription of several essential genes that control cell-cycle arrest/ DNA repair, and cell senescence, differentiation and apoptosis (Vogelstein et al., 2000). Several mammalian-cell-based reporter systems that use TP53-regulated promoters have been developed as sensors of genotoxic stress, including *GADD45a* (Hastwell et al., 2006; Liu et al., 2009), *GADD153* (Zhang et al., 2009), and *p53R2* (Mizota et al., 2011; Ohno et al., 2005, 2008; Westerink et al., 2010).

In the present study, we describe the development and validation of a genotoxicity biosensor system that is based on human hepatocellular carcinoma HepG2 cells that are stably transformed with a red fluorescence protein (*DsRed2*) gene under the control of the cyclin-dependent kinase 1A inhibitor (*CDKN1A*) promoter. HepG2 cells were chosen as the host cells because they have retained the inducibility and activities of several phase I and phase II

xenobiotic metabolising enzymes, and they have been shown to be a suitable model for detection of different classes of indirect-acting genotoxic agents (Knasmuller et al., 2004). HepG2 cells also express the wild-type tumour suppressor TP53 (Bressac et al., 1990), which makes them an appropriate model for detecting P53-regulated responses to DNA damage at the level of gene transcription and translation (van Delft et al., 2004). *CDKN1A* (*p21, WAF1, CIP1*) is the major downstream target gene of activated TP53, and it is responsible for cell-cycle arrest and inhibition of DNA replication (Moldovan et al., 2007; Vogelstein et al., 2000). Numerous *in-vitro* and *in-vivo* studies have demonstrated that exposure to genotoxic agents and irradiation can up-regulate the expression of *CDKN1A* (Ellinger-Ziegelbauer et al., 2005; Hreljac et al., 2008; Petkovic et al., 2011; Straser et al., 2011; Zegura et al., 2008). These studies have indicated that up-regulation of *CDKN1A* expression is a sensitive indicator of cell responses to DNA damage, although the *CDKN1A* promoter has not been used in reporter assays for genotoxicity testing so far. In our preliminary study, we transfected HepG2 cells with a plasmid containing enhanced green fluorescence protein (*eGFP*) under the control of the *CDKN1A* promoter, and we demonstrated that in response to DNA damage induced by direct-acting (i.e., methylmethane sulphonate [MMS], cisplatin [CDDP]) and indirect-acting (benzo(a)pyrene [BaP]) genotoxic compounds, transcription of the *CDKN1A* promoter was activated, which led to concurrent accumulation of eGFP (Zager et al. 2010). However, we noted difficulties in the quantification of the GFP fluorescence that was measured spectrofluorimetrically. This was due to the overlap of the fluorescence spectrum of GFP with endogenous autofluorescence that can arise from cell and growth media components, and also from the compounds tested (e.g., with BaP). This is not a problem when the fluorescence signal can be measured using fluorescence microscopy or flow cytometry. However, when the GFP fluorescence is measured spectrofluorimetrically, this can lead to low signal-to-noise ratios and restrictions to the detection sensitivity (Billinton and Knight, 2001). Therefore, for the present study, we transfected HepG2 cells with a plasmid in which we replaced the *eGFP* reporter gene with that of *DsRed2*, which has excitation/ emission in the red region of the spectrum that does not overlap with cell autofluorescence. The performance of this system was then evaluated by testing a series of non-genotoxic and genotoxic compounds.

Materials and methods

Cell line

The human hepatocellular carcinoma HepG2 cell line was obtained from the European Collection of Animal Cell Cultures (Wiltshire, UK). The HepG2 cells were grown in Minimal Essential Medium (MEM, Life Technologies, Gibco, Paisley, Grand Island, USA) without phenol red and supplemented with 10% heat-inactivated foetal bovine serum (Life Technologies), 1% non-essential amino-acid solution, and 2 mM L-glutamine (Sigma, St. Louis, USA). The cells were routinely sub-cultured twice per week, and were maintained in a humidified atmosphere with 5% CO_2 at 37 °C.

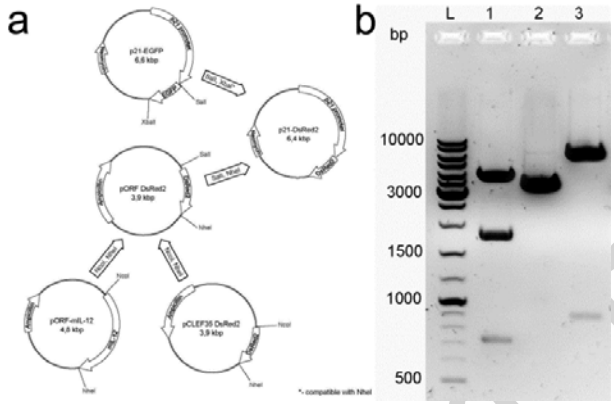
Plasmid construction and cell transfection

The eukaryotic expression plasmid that encodes the *DsRed* reporter gene under the transcriptional control of the *CDKN1A* promoter (CDKN1A-DsRed) was prepared using standard molecular biology techniques of restriction and ligation. The source for the coding sequence of the *CDKN1A* promoter was the WWP-LUC plasmid, which was generously provided by Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, Maryland, USA) and was previously cloned into the pEGFPN1 vector for the pp21-EGFP plasmid, as described in our earlier study (Zager et al., 2010). The pCLEF35DsRed2 plasmid (InVivoGen, San Diego, USA) was the source of the *DsRed2* sequence. Additionally, the pORF-mIL12 plasmid (InVivoGen) was used for the sub-cloning, to provide the appropriate restriction sites on each side of the *DsRed2* sequence. This was achieved by replacing the *mIL-12* coding sequence in pORF-mIL12 with *DsRed2*, to create the pORF-DsRed plasmid. Finally, the CDKN1A-DsRed plasmid was constructed by replacing the *eGFP* sequence in pp21-EGFP with the *DsRed2* sequence excised from the pORF-DsRed2 plasmid (Fig. 1a). The newly constructed CDKN1A-DsRed plasmid was cloned into the DH5α *Escherichia coli* strain and isolated using Qiagen Maxi Endo-Free kits (Qiagen, Hilden, Germany), according to the manufacturer instructions.

The purified plasmid DNA was subjected to quality control using agarose gel electrophoresis (Fig. 1b), and quantity determination using spectrophotometry. The newly constructed CDKN1A-DsRed plasmid was cut with three combinations of restriction enzymes that have restriction sites in different parts of the construct (within the vector and the insert), and the identity of the plasmid was confirmed by comparing the pattern of bands on electrophoresis gels with the expected bands. The CDKN1A-DsRed plasmid was cut with the NcoI restriction enzyme (expected bands: 4055, 1748, 703 bp), the XhoI and MfeI restriction enzymes (expected bands: 3257, 3249 bp), and the SalI and MfeI restriction enzymes (expected bands: 5679, 827 bp). All of the restriction enzymes were obtained from Fermentas

(Thermo Scientific, Vilnius, Lithuania). The transfection of HepG2 cells with the CDKN1A-DsRed plasmid was performed by electroporation, as described in recent studies (Towhidi et al., 2012; Zager et al., 2010). The selection of stably transfected clones with low basal *DsRed* expression and high inducibility was performed using MMS (Supplementary data, Fig. S1), and the selected clone was named as HepG2CDKN1A-DsRed. The stability of the expression of CDKN1A promoter has been confirmed also in our previous study in which we showed that it was not inactivated by methylation (Kamensek et al., 2011). The stably transfected cell line was grown in media supplemented with the geneticin selective antibiotic (G418 sulphate; Life Technologies).

Figure 1



Test compounds and stock solutions

The initial experiments were performed with MMS, CDDP, BaP and vinblastin (VLB). Further validation was performed with an additional 37 compounds that are either known not to be genotoxic, or that belong to different groups with known genotoxicity and carcinogenicity (Table 1). Fifteen of the selected compounds are on the European Centre for the Validation of Alternative Methods list of compounds recommended for evaluation of new genotoxicity assays (Kirkland et al., 2008), while others were selected from the Chemical Carcinogenesis Research Information System (CCRIS) database and from literature data. According to the solubilities of these compounds, the stock solutions were prepared in bi-distilled water, dimethylsulphoxide or methanol. Further dilutions were performed in MEM, with the final concentration of the vehicle adjusted to 0.5% at all compound concentrations. The highest tested concentration of the genotoxic compounds was set at 1 mM, when this was not limited by solubility in the solvent or in MEM, or by cytotoxicity (ICH, 2011). The highest tested concentration of the soluble non-genotoxic compounds was set at 10 mM.

Cell treatment

The assays were performed in black, clear-bottomed, 96-well microtitre plates (Greiner Bio-One, Nuernberg, Germany). Aliquots of HepG2CDKN1A-DsRed cell suspensions (80 µL; 3.75 $\times 10^5$ cells mL^{-1}) were distributed into the 96 wells of the microtitre plates. The cells were incubated for 2 h to allow them to attach, and then 20 µL of the test compounds at the appropriate concentrations (5-fold higher than the final concentration) were added to each well, with the plates then incubated at 37 ^oC and in 5% CO_2. The DsRed fluorescence was determined after 24 h and 48 h of exposure, considering DsRed maturation time and accumulation in the cytoplasm (Jakobs et al., 2000). The cell viability was determined after 48 h of exposure. For each microtitre plate, the tests included two test compounds at five different concentrations as six parallel analyses, with a positive control (MMS- or BaP-treated cells) and a negative control (vehicle-treated cells). The scheme of the microplate layout is illustrated in the Supplementary data (Fig. S2).

Determination of DsRed fluorescence intensity

The induction of the DsRed fluorescence intensity was determined according to the following three analysis systems.

Spectrofluorimetry: The fluorescence intensity of the DsRed protein was measured using a fluorescence microplate reader with a monochromator (Synergy, BioTek, Winooski, USA) or a filter system (Tecan, Männedorf, Switzerland), set at 535 nm (excitation) and 590 nm (emission).

Fluorescence microscopy: The expression of the DsRed protein was followed by fluorescence microscopy using an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan), equipped with a DP72 digital camera (Olympus). Images of each sample were acquired and additionally processed with the FIJI software (Schindelin et al., 2012).

Flow cytometry: The cells were grown in Petri dishes (10 cm) and were treated with MMS, CDDP, BaP or VLB for 48 h. The cells were then harvested by trypsinisation, washed, re-suspended in phosphate-buffered saline to a concentration of 1 $\times 10^5$ cells mL^{-1}, and transferred to 5-mL polystyrene round-bottomed tubes (BD Biosciences, San Jose, CA, USA). The DsRed fluorescence intensity was measured on at least 30,000 cells per sample, using a FACSCanto II flow cytometer (BD Biosciences) equipped with a 488-nm laser (aircooled, 20 mW solid state) and a 585/42-nm band-pass filter. First, the cell population gate was determined from the bi-parametric logarithmic plot defined by the forward and side scatter to

eliminate debris. Secondly, the histograms of the gated cells against their fluorescence intensity were recorded and the median of the DsRed fluorescence intensity of the gated cells was determined, using the FACSDiva V6.1.2 software (BD Biosciences).

Determination of cell viability

The cell viability was determined after 48 h exposure (immediately after measurement of the DsRed fluorescence) using the colorimetric (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS; Sigma, St. Louis, USA) assay, as the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega, Madison, USA), according to the manufacturer instructions. Here, 20 µL MTS solution was added to each well of the 96-well microtitre plates. After 2 h of incubation, the absorbance of the resulting formazan solution was measured at 492 nm, using a microplate reader.

Data collection, statistical analysis and definition of a positive result

After the 48 h treatments, the DsRed fluorescence and cell viability data were collected for the microtitre plates. The fluorescence data of each well was divided by its formazan absorbance data to obtain the mean DsRed fluorescence per viable cell. These values were then normalised to the vehicle-treated control, as a measure of relative DsRed induction (RDI). The relative cell viability was calculated by dividing the formazan absorbance of the treated cells by the absorbance of the control cells. Where the DsRed induction was measured also after 24 h of exposure, the cell viability data determined after the 48 h exposure were used for the normalisation of the DsRed fluorescence readout.

Statistical analysis was performed using the SigmaStat software (Systat Software, Inc., Richmond CA, USA). All of the data were first tested for normality with the Kolmorogov-Smirnov normality test. The significance between control and treated cells was determined using one-way analysis of variance (ANOVA) and two-tailed Students' t-tests. The significance of the differences in the RDI between the control and treated cells were determined within each experiment (n = 6), and between repeated independent experiments (n = 3). Within the experiments $p < 0.001$ and between the experiments $p < 0.05$ were considered significant.

A threshold for the determination a positive genotoxicity result for our test system was established based on the calculations of the means and standard deviations of the DsRed fluorescence of the vehicle and the actively treated cells, as described by Hastwell et al.

(2006). The threshold for a positive genotoxicity result was set at RDI ≥1.5, which was greater than three standard deviations of the vehicle mean DsRed fluorescence.

Another threshold for data acceptance was set for the reduction in the cell viability caused by the tested compounds. At cytotoxic doses, the breakdown of cell integrity can lead to non-specific DNA damage. In addition, in our test system, reduced cell density can interfere with the optical measurements, due to the differences in background reflectance and microplate absorbance, and this might give unreliable results for the fluorescence measurements. To avoid possible misinterpretations of the genotoxicity results, we set the threshold for genotoxicity data rejection as when the relative cell viability was reduced by >25%.

Results and discussion

In this study, we aimed to optimise a newly developed biosensor system that uses HepG2 cells that were stably transfected with *CDKN1A*-dependent *DsRed* reporter gene (HepG2CDKN1A-DsRed cells) for the detection of genotoxic agents, and to determine the applicability of this biosensor system to the detection of genotoxic agents with different mechanisms of action.

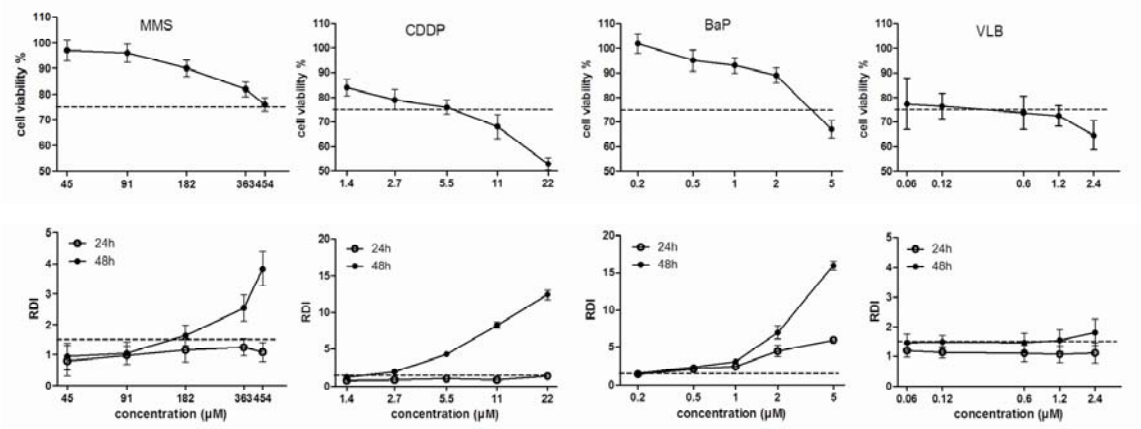
Optimisation of the HepG2CDKN1A-DsRed biosensor system

Four genotoxic compounds with different mechanisms of action were used to optimise the performance of the assay. MMS is an *sn-2* alkylating agent that forms methyl adducts in DNA (Beranek, 1990). CDDP is platinum-based alkylating agent that forms DNA-DNA and DNA-protein cross-links that block DNA replication and transcription (Corda et al., 1993). BaP is an indirect-acting genotoxic carcinogen that is metabolised by cytochrome P450 enzymes to the diol epoxide, which forms bulky DNA adducts (Perlow et al., 2002). VLB is a mitotic spindle inhibitor that does not directly interact with the DNA molecule, but prevents polymerisation of microtubules during mitosis (Owellen et al., 1976). These four compounds have also been shown to activate TP53 and its downstream regulated genes (Jaiswal and Narayan, 2002; Pabla et al., 2008; Sadikovic and Rodenhiser, 2006; Tishler et al., 1995).

To determine the optimal exposure time for measurement of the induction of the DsRed signal, we measured DsRed fluorescence intensity 24 h and 48 h after exposure, using a spectrofluorimeter. During the 24-h exposure, only BaP induced a significant increase in DsRed fluorescence, while in cells exposed to MMS, CDDP and VLB, an increase in DsRed

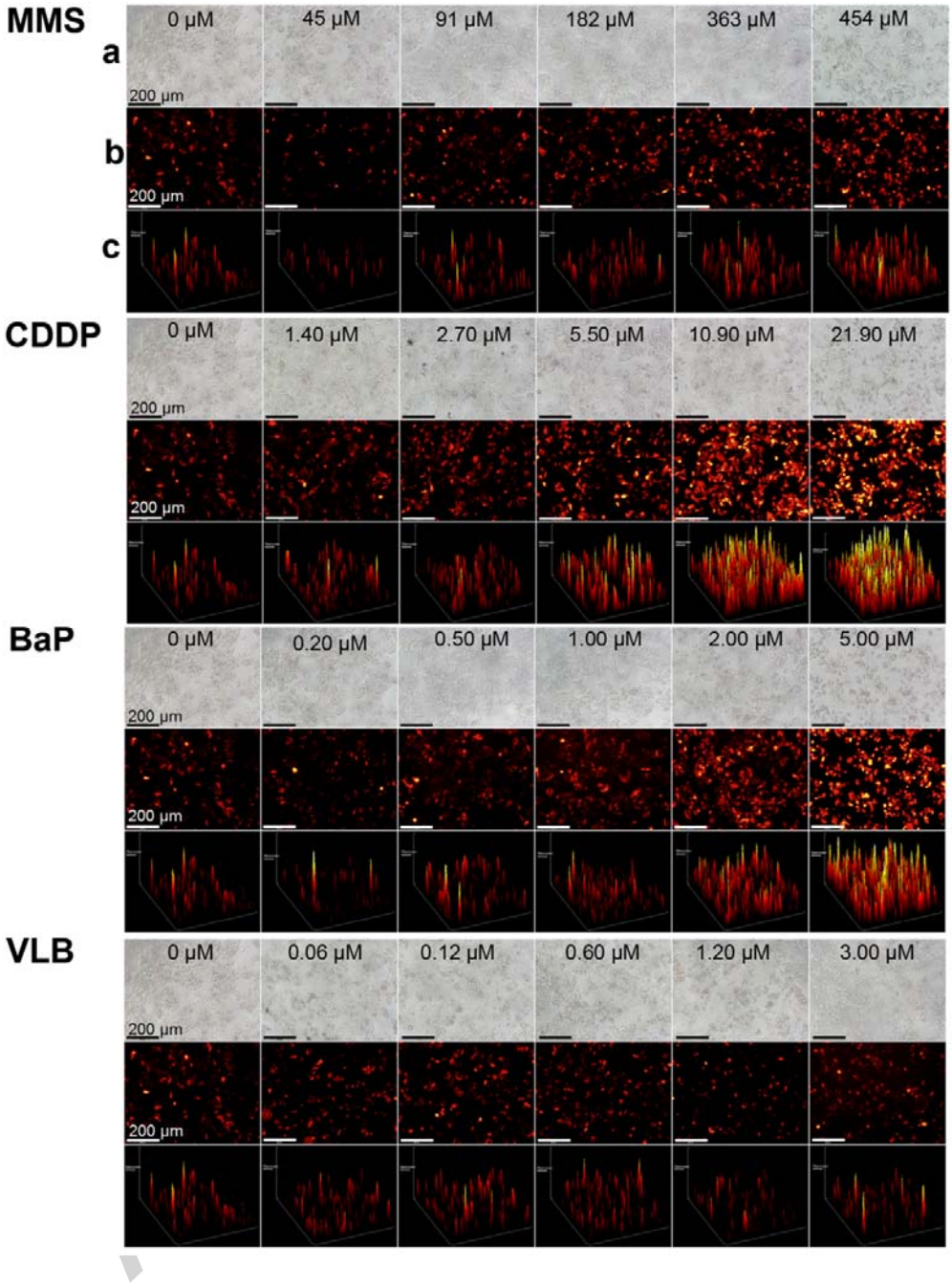
fluorescence was detected after 48 h of exposure (Fig. 2). The most potent inducer of *CDKN1A*-dependent *DsRed* expression was BaP, with the lowest effective concentration (LOEC) of 0.5 μM. At the highest non-cytotoxic concentration of BaP (2 μM), there was a nearly 5-fold increase in DsRed fluorescence over the control (RDI = 4.9). The LOEC of CDDP was 2.7 μM, and at its highest non-cytotoxic concentration (5.5 μM), the RDI was 5.05. MMS showed much lower potential, with the LOEC at 182 μM and an RDI of 6.3 at the highest tested concentration (454 μM). VLB was negative according to the criteria we set for considering a tested compound as genotoxic: an RDI ≥1.5 was detected at concentrations that reduced cell viability by more than 25%.

Figure 2



Like these spectrofluorimetric measurements of DsRed fluorescence intensity, fluorescence microscopic images also showed dose-dependent increases in DsRed fluorescence in cells exposed to MMS, CDDP and BaP, while in the cells exposed to VLB there was no increase in DsRed fluorescence (Fig. 3). This lack of induction of the *CDKN1A* reporter by VLB might be explained by a recent study that demonstrated that VBL can down-regulate the expression of *CDKN1A* via the C-Jun pathway (Kolomeichuk et al., 2008).

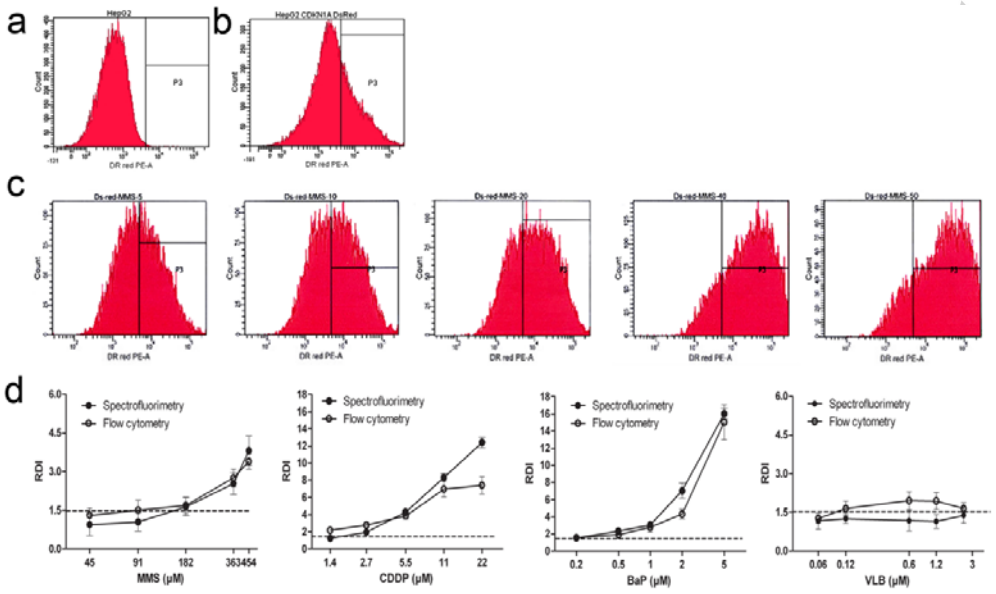
Figure 3



The results obtained using the spectrofluorimetric quantification of the induction of *DsRed* expression in these HepG2CDKN1A-DsRed cells were validated also using flow cytometry (Fig. 4). The fluorescence intensity histogram of the parent HepG2 cells (Fig. 4a) compared to HepG2CDKN1A-DsRed cells (Fig. 4b) shows low background fluorescence in the range of DsRed protein fluorescence spectrum, which was represented by the low number of fluorescence events. In the HepG2CDKN1A-DsRed cells exposed to graded doses of MMS, a dose-dependent increase in the fluorescence events can be seen as a displacement of

the peak to a higher fluorescence intensity (Fig 4c). Figure 4d shows that the measurements of the DsRed fluorescence intensity with flow cytometry and spectrofluorimetry provided comparable results for BaP, MMS and VLB, while for the fluorescence intensity of the CDDP-exposed cells, the higher measurements at the highest concentrations were measured using spectrofluorimetry. At this dose of CDDP, the cell viability was reduced by 45%, and so the difference here can be explained by the overestimation of the RDI using the normalisation to viable cells.

Figure 4



Determination of the sensitivity and specificity of the HepG2CDKN1A-DsRed cell-based biosensor system

For determination of the sensitivity and specificity of this HepG2CDKN1A-DsRed biosensor system, an additional 37 compounds were tested. Among all of the 41 compounds tested, the gathered information from previous sources indicated that 25 compounds were genotoxic *in vitro* and *in vivo*, four compounds were genotoxic *in vitro* with *in vivo* data on genotoxicity either negative or not available, and 12 of the compounds were not genotoxic (Table 1; Supplementary data, Figs. S3, S4, S5).

In the test system of the present study, positive genotoxicity results were obtained with the compounds that were known to be *in-vitro* and *in-vivo* genotoxins, which included polyaromatic hydrocarbons and aromatic amines that form bulky adducts, alkylating agents, an antimetabolite, a topoisomerase II inhibitor, DNA cross-linkers, and chromium(VI) salts,

and the three cyanobacterial toxins (microcystin-LR, nodularin, cylindrospermopsin). Four compounds that have been shown to be genotoxic *in vitro* and *in vivo* and to be rodent carcinogens were not detected as genotoxic here: 2-acetylaminofluorene, o-toluidine, cadmium chloride and VLB.

8-hydroxyquinoline that induces DNA and chromosomal damage in vitro, but not in vivo (Kirkland et al., 2005) was negative in our test system. Of the three compounds that are positive *in vitro* for which *in vivo* genotoxicity data are not available, positive response was induced by the intercalating agent propidium iodide, by a model bacterial mutagen sodium azide and by tyrosine kinase inhibitor imatinib mesylate.

Of the 12 compounds that were selected as being considered not to be genotoxic, only EDTA was positive (LOEC = 350 μM). More detailed explanation on the observed positive and negative responses is given in the Supplementary data.

The data in Table 1 show that out of the 25 tested compounds that were reported to be genotoxic *in vitro* and *in vivo*, 21 (84%) were seen as positive in the present study, and of the 12 compounds that were reported to be non-genotoxic, 11 (92%) were negative. Similar sensitivities and specificities have been reported for several other reporter test systems. The GreenScreenHT assay that employs human lymphoblastoid TK6 cells expressing the GADD45a-GFP reporter showed 96% sensitivity and 76% specificity (Birell et al., 2010), while TK6 cells transfected with p53*R*2-dependent luciferase reporter gene showed 85% sensitivity and 93% specificity (Mizota et al., 2011). Drawback of test systems employing TK6 cells is their metabolic incompetence, thus the use of exogenous metabolic activation is required for detection of indirect acting genotoxicants. Westerink et al. (2010) developed a test system with metabolically competent HepG2 cells. The cells were stably transfected with luciferase gene under the control of promoter regions of RAD51C, Cystatin A, and t responsive element of the p53 protein that are indicators of genotoxic stress and Nrf2 responsive elements as an indicator of oxidative stress. The system showed lower sensitivity (74%) and specificity (81%) compared to our test system (84% and 92%, respectively).

Bacterial vs mammalian cell based reporter biosensors for genotoxicity testing

Numerous genotoxicity assays based on genetically engineered bacteria have been developed over years (Biran et al., 2010). The advantages of bacterial cells for the development of whole cell biosensors are their rapid growth, ease of culturing and easy genetic manipulation to construct genetically engineered microorganisms ‘tailored’ to generate quantifiable signal that reflects genotoxic potency of the tested sample. Due to their

robustness and sufficient shelf life genetically modified bacteria are successfully used for the development of devices consisting of immobilized bacteria coupled to the appropriate transducer for signal measurement (Biran et al., 2010). Using these technologies progress has been recently focused on development of microbial cell array biochips with multiple reporter cells incorporated into single platform that enables simultaneous detection of a broad spectrum of pollutants (Melamed et al., 2012; Ahn and Gu, 2012; Elad and Belkin, 2013). Bacterial biosensors are highly sensitive, rapid and cost effective, which are the desired properties of biosensors, particularly in the environmental monitoring for the detection of genotoxic pollutants.

On the other hand, mammalian cells are compared to bacteria much more demanding in terms of cultivation, growth, robustness as well as genetic manipulation. Consequently the mammalian cell based biosensors are more time consuming and less cost effective. However, mammalian cells reflect toxicological, physiological and cellular responses relevant for humans and animals, which circumvent the limitations compared to bacterial biosensors. Thus, when the genotoxicity data are used for the prediction of *in vivo* genotoxicity and potential carcinogenicity of chemicals for humans, biosensors based on engineered human cell are more appropriate. Therefore, the strengths and weaknesses of human cell and bacterial cell based biosensors for genotoxicity detection should be regarded in terms of their applications. The performance of biosensors for the applications in environmental monitoring are judged by their high sensitivity and rapid acquisition of the results, therefore for such purposes bacterial biosensor are more appropriate. On the other hand, the performance of biosensors applied for the prediction of *in vivo* genotoxicity and potential carcinogenicity for humans should be judged by their accurate predictability, hence human cells biosensor should be used for such applications.

Table 1. Data from the 41 compounds assayed using the HepG2CDKN1A-DsRed biosensor system. A response was considered positive when the relative DsRed induction (RDI) was ≥1.5 and the cell viability was not reduced by ≥25%.

Compound	CAS	MW	Viability reduction		CDKN1A-DsRed genotoxicity		Genotoxicity data[d]			Additional information	References
			Max. conc. (µM)	LOEC (µM)[a]	LOEC (µM)[b]	RDI[c]	Ames test	*In-vitro* tests	*In-vivo* tests		
Compounds that are genotoxic *in vitro* and *in vivo*											
Methyl methanesulphonate*	66-27-3	110.1	454	NT	**182**	**1.71**	+	+	+	Monofunctional alkylating agent (N7 alkylation); strong clastogen; probable human carcinogen (IARC group 2A)	Kirkland et al., 2008
Cyclophosphamide*	50-18-0	261.1	1000	NT	**0.1**	**2.19**	+	+	+	Alkylating agent, anticancer drug, requires metabolic activation; human carcinogen (IARC group 1)	Kirkland et al., 2008
Benzo(a)pyrene*	50-32-8	252.3	5	5	**0.5**	**1.82**	+	+	+	Polyaromatic hydrocarbon, induces DNA adduct formation, requires metabolic activation; human carcinogen (IARC	Kirkland et al., 2008

										group 1)	
2-Acetylaminofluorene (2-AF)*	53-96-3	223.3	100	100	**Neg.**	/	+	+	+	Derivative of fluorene, induces DNA adduct formation, requires metabolic activation; rodent carcinogen	Kirkland et al., 2008; CCRIS, 2014
2-Aminoanthracene	613-13-8	193.2	10	NT	**0.5**	**2.13**	+	+	+	Aromatic amine, induces DNA adduct formation, requires metabolic activation, rodent carcinogen	Kirkland et al., 2005; CCRIS, 2014
Aflatoxin B1*	1162-65-8	312.3	16	NT	**3.2**	**1.62**	+	+	+	Micotoxin, induces DNA adduct formation, requires metabolic activation; human carcinogen (IARC group 1)	Kirkland et al., 2008
2-Amino-3-methylimidazo [4,5-f]quinoline – IQ*	76180-96-6	198.2	200	100	**100**	**1.59**	+	+	+	Heterocyclic aromatic amine, induces DNA adduct formation, requires metabolic activation; probable human carcinog	Kirkland et al., 2008

										en (IARC group 2A)	
2-Amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)*	105650-23-5	224,3	200	100	**12.5**	**1.98**	+	+	+	Heterocyclic aromatic amine, induces DNA adduct formation, requires metabolic activation; possible human carcinogen (IARC group 2A)	Kirkland et al., 2008
2-Amino-3,8-dimethylimidazo [4,5-f]quinoxaline (MeIQx)	77500-04-0	213.3	200	200	**12.5**	**4.35**	+	+	+	Heterocyclic aromatic amine, induces DNA adduct formation, requires metabolic activation; possible human carcinogen (IARC group 2A)	Kirkland et al., 2005
2-amino-3,4,8-dimethylimidazo [4,5-f]quinoxaline (DiMeIQx)	95896-78-9	227.3	200	100	**50**	**1.51**	+	+	+	Heterocyclic aromatic amine, induces DNA adduct formation, requires metabolic activation	Pezdirc et al., 2013
Cisplatin*	15663-27-1	300.1	21.9	10.9	**2.7**	**2.16**	+	+	+	Platinum complex, anticance	Kirkland et al., 2008

										r drug, induces DNA-DNA and DNA-protein cross-links; probable human carcinogen (IARC group 2A)	
5-Fluorouracil	51-21-8	130.1	100	NT	**10**	**3.46**	+	+	+	Antimetabolite, anticancer drug; inadequate results for rodent carcinogenicity; not classified as human carcinogen (IARC group 3)	Kirkland et al., 2005; CCRIS, 2014
Cadmium chloride*	10108-64-2	183.3	10	1.25	**Neg.**	/	-	+	+	Inorganic carcinogen, oxidant, DNA repair inhibition; human carcinogen (IARC group 1)	Kirkland et al., 2008; Fatur et al, 2002; Filipic, 2012
Potassium dichromate (CrVI)	7778-50-9	294.2	10	2.5	**2.5**	**3.77**	+	+	+	Inorganic carcinogen, oxidant, DNA repair inhibition; human carcinogen (IARC group 1)	Kirkland et al., 2011
Sodium chromate (CrVI)	18540-29-9	162.0	1	0.25	**0.125**	**1.78**	+	+	+	Inorganic carcinogen, oxidant, DNA repair inhibitio	Kirkland et al., 2011

										n; human carcinogen (IARC group 1)	
4-Nitroquinoline N-oxide	56-57-5	190.2	1	0.5	**0.06**	**1.64**	+	+	+	Quinoline derivative, induces DNA adduct formation and oxidative DNA damage; rodent carcinogen	Kirkland et al., 2005; CCRIS, 2014
Etoposide*	33419-42-0	588.6	100	NT	**10**	**6.40**	-	+	+	Topoisomerase II inhibitor, anticancer drug; human; human carcinogen (IARC group 1)	Kirkland et al., 2008
Paclitaxel*	33069-62-4	853.9	20	2.5	**2.5**	**1.55**	-	+	+	Aneugen, mitotic inhibitor; stabilises microtubule formation	Kirkland et al., 2008
Vinblastine	865-21-4	811.0	3	0.6	**Neg.**	/	-	+	+	Aneugen, mitotic inhibitor; inhibits tubulin polymerisation	Kirkland et al., 2011
Mitomycin-C	50-07-7	334.3	100	NT	**20**	**2.26**	-	+	+	DNA cross-linker, anticancer drug; possible human carcinogen (IARC group 2A)	Kirkland et al., 2005; CCRIS, 2014
Bleomycin	11056-06-7	141.6	10	NT	**1**	**1.77**	-	+	+	Inhibitor of DNA synthesis	Kirkland et al., 2011

										, anticancer drug; possible human carcinogen (IARC group 2A)	
O-Toluidine	95-53-4	107.2	100	NT	**Neg.**	/	-	+	+	Monocyclic aromatic amine, induces DNA adduct formation, requires metabolic activation; human carcinogen (IARC group 1)	Kirkland et al., 2005; CCRIS, 2014
Microcystin LR	101043-37-2	995.2	1	NT	**1**	**1.75**	-	+	+*	Cyanobacterial toxin, induces oxidative DNA damage; possible human carcinogen (IARC group 2A)	Kirkland et al., 2011; Zegura et al., 2008, 2011
Nodularin	118399-22-7	825.0	1.2	NT	**0.01**	**1.86**	-	+	+*	Cyanobacterial toxin, induces oxidative DNA damage; not classified as human carcinogen (IARC group 3)	Zegura et al., 2011
Cylindrospermopsin	143545-90-8	415.5	1.2	NT	**0.12**	**1.53**	-	+	+*	Cyanobacterial toxin, induces DNA adduct formatio	Straser et al., 2011

										n, requires metabolic activation	
Compounds that are genotoxic *in vitro*, with negative or no data on *in vivo* genotoxicity											
8-Hydroxyquinoline	148-24-3	145.16	100	10	**Neg.**	/	+	+	-	Antioxidant, ion chelator; not classified as human carcinogen (IARC group 3)	Kirkland et al., 2005; CCRIS, 2014
Propidium iodide	25535-16-4	668.4	200	NT	**12.5**	**3.26**	+	+	N/A	Intercalating agent; widely used laboratory reagent	GENETOX, 2014
Sodium azide	26628-22-8	650.1	615	NT	**615**	**3.78**	+	-	N/A	Azide, specific bacterial and plant mutagen	Owais and Kleinhofs, 1988; CCRIS, 2014
Imatinib mesilate	220127-57-1	493.6	20.2	20.2	**1.02**	**1.64**	+	N/A	N/A	Protein kinase inhibitor, anticancer drug	Fabarius et al., 2007; Pichler et al., 2014
Compounds that are considered as non-genotoxic											
Mannitol*	69-65-8	182.2	10000	NT	**Neg.**	/	-	-	-	Six-carbon sugar alcohol or polyol; used as sugar replacement; negative in rodent carcinogenicity studies	Kirkland et al., 2008
EDTA*	60-00-4	292.2	10000	2800	**350**	**2.10**	-	-	N/A	Polyamine carboxylic acid; acts as ion chelator	Kirkland et al., 2008

Ampicilin*	69-53-4	349.4	22.9	NT	**Neg.**	/	-	-	-	Beta lactam antibiotic; not classified as human carcinogen (IARC group 3)	Kirkland et al., 2008
Urea*	57-13-6	60.1	10000	NT	**Neg.**	/	-	-	N/A	Negative in rodent carcinogenicity studies	Kirkland et al., 2008
Xanthohumol	6754-58-1	354.4	20	NT	**Neg.**	/	-	-	N/A	Prenylated flavonoid, antioxidant, antigenotoxic activity	Plazar et al., 2007
Boric acid	10043-35-3	61.8	10000	10000	**Neg.**	/	-	-	-	Weak acid of boron, antiseptic, insecticide; negative in rodent carcinogenicity studies	EFSA, 2013; CCRIS, 2014
Sucrose	57-50-1	342.3	10000	NT	**Neg.**	/	-	-	N/A		Kirkland et al., 2011
N,N-Dimethylformamide	68-12-2	73.1	10000	NT	**Neg.**	/	-	-	-	Aliphatic amine; used as solvent; hepatotoxin; not classified as human carcinogen (IARC group 3)	CCRIS, 2014
NaCl	7647-14-5	58.4	10000	NT	**Neg.**	/	-	-	N/A	Acts as tumour promoter	CCRIS, 2014
Ethanol	67-17-5	46.1	10000	NT	**Neg.**	/	-	-	N/A	Acts as tumour promoter	Kirkland et al., 2005; CCRIS, 2014
Chromic nitrate	13548-38-4	400.2	100	NT	**Neg.**	/	N/A	-	N/A	Chromium(III) compoun	Beyersmann and Hartwig, 2008

(CrIII)										ds did not induce genotoxic effects in the majority of studies with intact cells and are not carcinogenic	
Chromic nitrate/ EDTA complex	NA	340.2	100	NT	**Neg.**	/	N/A	N/A	N/A	Chromium(III) compound complexed with EDTA, no data on genotoxicity	Beyersmann and Hartwig, 2008

[a] Lowest effective concentrations (LOEC) that reduce cell viability by ≥25%, compared to the solvent-treated control.

[b] Lowest effective concentrations (LOEC) that induce ≥1.5-fold increase in relative DsRed fluorescence, over the solvent-treated control.

[c] Relative DsRed fluorescence induction (RDI) detected at LOEC.

[d] Published data from bacterial reverse mutation assay (Ames test), genotoxicity *in vitro* (chromosomal aberrations, micronucleus and/or mouse lymphoma assay), genotoxicity *in vivo* (chromosomal aberrations and/or micronucleus).

*Compounds listed by the European Centre for the Validation of Alternative Methods in their recommended list of genotoxic and non-genotoxic chemicals for assessment of new genotoxicity tests (Kirkland et al. 2008).

** *in vivo* comet assay data

NT, Non-toxic: within the tested concentration range, cell viability was not reduced by ≥25%, compared to the solvent-treated control.

N/A, data not available.

Neg., negative (RDI < 1.5 at all tested concentrations)

Conclusions

The data from the present study show that this new biosensor system with HepG2 cells stably transfected with the *DsRed2* red fluorescent protein reporter gene under the control of the *CDKN1A* promoter efficiently detects different types of genotoxic agents. The main advantages of this biosensor system are two-fold: (i) the use of metabolically competent human cells that allow the detection of most of the indirect-acting genotoxic compounds without the use of exogenous metabolic activation; and (ii) the use of spectrofluorimetric measurements of the fluorescent DsRed reporter protein in microplates, which provides easy handling and data acquisition. The validation of the test carried out here demonstrates the high sensitivity and specificity of this biosensor system. We believe that this genotoxicity assay can become a valuable tool with potential applications in the fields of chemicals safety evaluation and environmental and occupational monitoring of exposure to genotoxic agents and their complex mixtures.

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Figures legends

Figure 1. Schematic representation of the CDKN1A-DsRed plasmid construction (a), and confirmation of the successful construction of the plasmid using restriction analysis (b). L, GeneRulertm DNA ladder mix (Fermentas); restriction carried out with the restriction enzymes as: lane 1, NcoI; lane 2, XhoI and MfeI; and lane 3, SalI and MfeI.

Figure 2. Relative cell viability after 48 h exposure (top panels) and relative DsRed (reporter protein) induction (RDI) after 24 h and 48 h exposure (bottom panels) of HepG2CDKN1A-DsRed cells to the initial four genotoxic compounds with different mechanisms of action. MMS, methyl methanesulfonate; CDDP, cisplatin; BaP, benzo(a)pyrene; VLB, vinblastine. Dashed lines, 25% cell viability reduction threshold (top) and RDI 1.5 genotoxicity threshold (bottom). Data are means ± standard deviations from three independent experiments. Logarithmic scale is used to display data on the x-axis.

Figure 3. Representative images of DsRed fluorescence induction after exposure of HepG2CDKN1A-DsRed cells to different genotoxic compounds. The cells were plated into 96-well microtitre plates and exposed to different concentrations (as indicated) of the initial four genotoxic compounds with different mechanisms of action. MMS, methyl methanesulfonate; CDDP, cisplatin; BaP, benzo(a)pyrene; VLB, vinblastine. After 48 h exposure, images of the cells were taken with light microscopy (a) and fluorescence microscopy (b), and surface plots of the fluorescence intensities were created using the Fiji software (c).

Figure 4. Representative fluorescence intensity histograms of parental HepG2 cells (a), non-treated HepG2CDKN1A-DsRed cells (b), and HepG2CDKN1A-DsRed cells exposed to increasing concentrations of MMS for 48 h (c). (d) Comparisons of flow cytometry and spectrofluorimetry measurements of DsRed fluorescence of HepG2CDKN1A-DsRed cells exposed to different concentrations (as indicated) of the initial four genotoxic compounds with different mechanisms of action. MMS, methyl methanesulfonate; CDDP, cisplatin; BaP, benzo(a)pyrene; VLB, vinblastine. Data are means ±standard deviation from three independent experiments. Logarithmic scale is used to display data on the x-axis.

Table 1. Data from the 41 compounds assayed using the HepG2CDKN1A-DsRed biosensor system. A response was considered positive when the relative DsRed induction (RDI) was ≥1.5 and the cell viability was not reduced by ≥25%.

Compound	CAS	MW	Viability reduction		CDKN1A-DsRed genotoxicity		Genotoxicity data[d]			Additional information	References
			Max. conc. (µM)	LOEC (µM)[a]	LOEC (µM)[b]	RDI[c]	Ames test	*In-vitro* tests	*In-vivo* tests		
Compounds that are genotoxic *in vitro* and *in vivo*											
Methyl methanesulphonate*	66-27-3	110.1	454	NT	**182**	**1.71**	+	+	+	Monofunctional alkylating agent (N7 alkylation); strong clastogen; probable human carcinogen (IARC group 2A)	Kirkland et al., 2008
Cyclophosphamide*	50-18-0	261.1	1000	NT	**0.1**	**2.19**	+	+	+	Alkylating agent, anticancer drug, requires metabolic activation; human carcinogen (IARC group 1)	Kirkland et al., 2008
Benzo(a)pyrene*	50-32-8	252.3	5	5	**0.5**	**1.82**	+	+	+	Polyaromatic hydrocarbon, induces DNA adduct formation, requires metabolic activation; human carcinogen (IARC group 1)	Kirkland et al., 2008
2-Acetylaminofluorene (2-AF)*	53-96-3	223.3	100	100	**Neg.**	/	+	+	+	Derivative of fluorene, induces DNA adduct formation, requires	Kirkland et al., 2008; CCRIS, 2014

										metabolic activation ; rodent carcinogen	
2-Aminoanthracene	613-13-8	193.2	10	NT	**0.5**	**2.13**	+	+	+	Aromatic amine, induces DNA adduct formation, requires metabolic activation, rodent carcinogen	Kirkland et al., 2005; CCRIS, 2014
Aflatoxin B1*	1162-65-8	312.3	16	NT	**3.2**	**1.62**	+	+	+	Micotoxin , induces DNA adduct formation, requires metabolic activation ; human carcinogen (IARC group 1)	Kirkland et al., 2008
2-Amino-3-methylimidazo [4,5-f]quinoline – IQ*	76180-96-6	198.2	200	100	**100**	**1.59**	+	+	+	Heterocyclic aromatic amine, induces DNA adduct formation, requires metabolic activation ; probable human carcinogen (IARC group 2A)	Kirkland et al., 2008
2-Amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)*	105650-23-5	224,3	200	100	**12.5**	**1.98**	+	+	+	Heterocyclic aromatic amine, induces DNA adduct formation, requires metabolic activation ; possible human carcinogen (IARC group 2A)	Kirkland et al., 2008

2-Amino-3,8-dimethylimidazo [4,5-f]quinoxaline (MeIQx)	77500-04-0	213.3	200	200	**12.5**	**4.35**	+	+	+	Heterocyclic aromatic amine, induces DNA adduct formation, requires metabolic activation; possible human carcinogen (IARC group 2A)	Kirkland et al., 2005
2-amino-3,4,8-dimethylimidazo [4,5-f]quinoxaline (DiMeIQx)	95896-78-9	227.3	200	100	**50**	**1.51**	+	+	+	Heterocyclic aromatic amine, induces DNA adduct formation, requires metabolic activation	Pezdirc et al., 2013
Cisplatin*	15663-27-1	300.1	21.9	10.9	**2.7**	**2.16**	+	+	+	Platinum complex, anticancer drug, induces DNA-DNA and DNA-protein cross-links; probable human carcinogen (IARC group 2A)	Kirkland et al., 2008
5-Fluorouracil	51-21-8	130.1	100	NT	**10**	**3.46**	+	+	+	Antimetabolite, anticancer drug; inadequate results for rodent carcinogenicity; not classified as human carcinogen (IARC group 3)	Kirkland et al., 2005; CCRIS, 2014
Cadmium chloride*	10108-64-2	183.3	10	1.25	**Neg.**	/	-	+	+	Inorganic carcinogen, oxidant, DNA repair	Kirkland et al., 2008; Fatur et al, 2002; Filipic, 2012

										inhibition; human carcinogen (IARC group 1)	
Potassium dichromate (CrVI)	7778-50-9	294.2	10	2.5	**2.5**	**3.77**	+	+	+	Inorganic carcinogen, oxidant, DNA repair inhibition; human carcinogen (IARC group 1)	Kirkland et al., 2011
Sodium chromate (CrVI)	18540-29-9	162.0	1	0.25	**0.125**	**1.78**	+	+	+	Inorganic carcinogen, oxidant, DNA repair inhibition; human carcinogen (IARC group 1)	Kirkland et al., 2011
4-Nitroquinoline N-oxide	56-57-5	190.2	1	0.5	**0.06**	**1.64**	+	+	+	Quinoline derivative, induces DNA adduct formation and oxidative DNA damage; rodent carcinogen	Kirkland et al., 2005; CCRIS, 2014
Etoposide*	33419-42-0	588.6	100	NT	**10**	**6.40**	-	+	+	Topoisomerase II inhibitor, anticancer drug; human; human carcinogen (IARC group 1)	Kirkland et al., 2008
Paclitaxel*	33069-62-4	853.9	20	2.5	**2.5**	**1.55**	-	+	+	Aneugen, mitotic inhibitor; stabilises microtubule formation	Kirkland et al., 2008
Vinblastine	865-21-4	811.0	3	0.6	**Neg.**	/	-	+	+	Aneugen, mitotic inhibitor; inhibits	Kirkland et al., 2011

										tubulin polymerisation	
Mitomycin-C	50-07-7	334.3	100	NT	**20**	**2.26**	-	+	+	DNA cross-linker, anticancer drug; possible human carcinogen (IARC group 2A)	Kirkland et al., 2005; CCRIS, 2014
Bleomycin	11056-06-7	141.6	10	NT	**1**	**1.77**	-	+	+	Inhibitor of DNA synthesis, anticancer drug; possible human carcinogen (IARC group 2A)	Kirkland et al., 2011
O-Toluidine	95-53-4	107.2	100	NT	**Neg.**	/	-	+	+	Monocyclic aromatic amine, induces DNA adduct formation, requires metabolic activation; human carcinogen (IARC group 1)	Kirkland et al., 2005; CCRIS, 2014
Microcystin LR	101043-37-2	995.2	1	NT	**1**	**1.75**	-	+	+*	Cyanobacterial toxin, induces oxidative DNA damage; possible human carcinogen (IARC group 2A)	Kirkland et al., 2011; Zegura et al., 2008, 2011
Nodularin	118399-22-7	825.0	1.2	NT	**0.01**	**1.86**	-	+	+*	Cyanobacterial toxin, induces oxidative DNA damage; not classified as human carcinogen (IARC	Zegura et al., 2011

										group 3)	
Cylindrospermopsin	143545-90-8	415.5	1.2	NT	**0.12**	**1.53**	-	+	+*	Cyanobacterial toxin, induces DNA adduct formation, requires metabolic activation	Straser et al., 2011
Compounds that are genotoxic *in vitro*, with negative or no data on *in vivo* genotoxicity											
8-Hydroxyquinoline	148-24-3	145.16	100	10	**Neg.**	/	+	+	-	Antioxidant, ion chelator; not classified as human carcinogen (IARC group 3)	Kirkland et al., 2005; CCRIS, 2014
Propidium iodide	25535-16-4	668.4	200	NT	**12.5**	**3.26**	+	+	N/A	Intercalating agent; widely used laboratory reagent	GENETOX, 2014
Sodium azide	26628-22-8	650.1	615	NT	**615**	**3.78**	+	-	N/A	Azide, specific bacterial and plant mutagen	Owais and Kleinhofs, 1988; CCRIS, 2014
Imatinib mesilate	220127-57-1	493.6	20.2	20.2	**1.02**	**1.64**	+	N/A	N/A	Protein kinase inhibitor, anticancer drug	Fabarius et al., 2007; Pichler et al., 2014
Compounds that are considered as non-genotoxic											
Mannitol*	69-65-8	182.2	10000	NT	**Neg.**	/	-	-	-	Six-carbon sugar alcohol or polyol; used as sugar replacement; negative in rodent carcinogenicity studies	Kirkland et al., 2008
EDTA*	60-00-4	292.2	10000	2800	**350**	**2.10**	-	-	N/A	Polyamine carboxylic acid; acts as ion	Kirkland et al., 2008

										chelator	
Ampicilin*	69-53-4	349.4	22.9	NT	**Neg.**	/	-	-	-	Beta lactam antibiotic; not classified as human carcinogen (IARC group 3)	Kirkland et al., 2008
Urea*	57-13-6	60.1	10000	NT	**Neg.**	/	-	-	N/A	Negative in rodent carcinogenicity studies	Kirkland et al., 2008
Xanthohumol	6754-58-1	354.4	20	NT	**Neg.**	/	-	-	N/A	Prenylated flavonoid, antioxidant, antigenotoxic activity	Plazar et al., 2007
Boric acid	10043-35-3	61.8	10000	10000	**Neg.**	/	-	-	-	Weak acid of boron, antiseptic, insecticide; negative in rodent carcinogenicity studies	EFSA, 2013; CCRIS, 2014
Sucrose	57-50-1	342.3	10000	NT	**Neg.**	/	-	-	N/A		Kirkland et al., 2011
N,N-Dimethylformamide	68-12-2	73.1	10000	NT	**Neg.**	/	-	-	-	Aliphatic amine; used as solvent; hepatotoxin; not classified as human carcinogen (IARC group 3)	CCRIS, 2014
NaCl	7647-14-5	58.4	10000	NT	**Neg.**	/	-	-	N/A	Acts as tumour promoter	CCRIS, 2014
Ethanol	67-17-5	46.1	10000	NT	**Neg.**	/	-	-	N/A	Acts as tumour promoter	Kirkland et al., 2005; CCRIS, 2014
Chromic nitrate (CrIII)	13548-38-4	400.2	100	NT	**Neg.**	/	N/A	-	N/A	Chromium(III) compounds did not induce	Beyersmann and Hartwig, 2008

										genotoxic effects in the majority of studies with intact cells and are not carcinogenic	
Chromic nitrate/ EDTA complex	NA	340.2	100	NT	**Neg.**	/	N/A	N/A	N/A	Chromium(III) compound complexed with EDTA, no data on genotoxicity	Beyersmann and Hartwig, 2008

[a] Lowest effective concentrations (LOEC) that reduce cell viability by ≥25%, compared to the solvent-treated control.

[b] Lowest effective concentrations (LOEC) that induce ≥1.5-fold increase in relative DsRed fluorescence, over the solvent-treated control.

[c] Relative DsRed fluorescence induction (RDI) detected at LOEC.

[d] Published data from bacterial reverse mutation assay (Ames test), genotoxicity *in vitro* (chromosomal aberrations, micronucleus and/or mouse lymphoma assay), genotoxicity *in vivo* (chromosomal aberrations and/or micronucleus).

*Compounds listed by the European Centre for the Validation of Alternative Methods in their recommended list of genotoxic and non-genotoxic chemicals for assessment of new genotoxicity tests (Kirkland et al. 2008).

** *in vivo* comet assay data

NT, Non-toxic: within the tested concentration range, cell viability was not reduced by ≥25%, compared to the solvent-treated control.

N/A, data not available.

Neg., negative (RDI < 1.5 at all tested concentrations)

Highlights

- **We developed cell-based biosensor system for rapid genotoxicity screening.**
- **Advantage is the use of metabolically active hepatocellular carcinoma cells.**
- **Simple and reliable detection of genotoxicity is achieved with spectrofluorimetry.**
- **Our cell-based biosensor system is highly specific and sensitive.**