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**Author:** Schaap, M.M.  
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Proliferating primary hepatocytes from the pUR288 lacZ plasmid mouse are valuable tools for genotoxicity assessment in vitro

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Mirjam M. Schaap*
Edwin P. Zwart*
Myrthe W. van den Dungen
Hedwig M. Braakhuis
Paul A. White
Harry van Steeg
Jan van Benthem
Mirjam Luijten

* Contributed equally
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Abstract

Safety assessments of substances with regard to genotoxicity are generally based on a combination of *in vitro* and *in vivo* tests. These tests are performed according to a (tiered) test strategy whereby a positive result *in vitro* usually triggers further testing *in vivo*. A low specificity and high frequency of irrelevant positive results associated with most *in vitro* mammalian cell genotoxicity assays necessitates the design and validation of suitable alternatives. As such, we examined the feasibility of culturing primary hepatocytes from the pUR288 *lacZ* reporter mouse, and moreover, using established cultures to reliably assess genotoxic activity *in vitro*. Initial studies characterizing the metabolic capacity of proliferating *lacZ* primary hepatocytes indicated that these cells retained at least some activities important for xenobiotic metabolism: cytochrome P450 1A1 enzyme activities were markedly increased in the hepatocytes after exposure to benzo(a)pyrene, and also UDP-glucuronosyl transferase and glutathione-S-transferase activities, both Phase II enzymes, were detected. Increasing levels of phosphorylated p53 at residue serine 389 after UV treatment indicated a properly functioning p53, one of the criteria for an effective new test system. Four genotoxic substances with different mechanisms of genotoxicity, *i.e.*, benzo(a)pyrene, bleomycin, etoposide, and cyclophosphamide, were tested in the *lacZ* rescue assay. For etoposide and cyclophosphamide, the induction of mutant colonies was rather low. Exposure to benzo(a)pyrene and bleomycin, however, yielded a clear concentration-dependent induction of the *lacZ* mutant frequency. Based on our preliminary observations, proliferating *lacZ* primary hepatocytes are a promising new tool for the assessment of genotoxic hazard.
Introduction

Assessment of genotoxicity is an important step in the safety evaluation of both chemicals and pharmaceuticals. The aim of genotoxicity testing is to assess the potential of substances to cause DNA damage to humans. Generally, the standard approach is a tiered test strategy including short-term in vitro and in vivo genotoxicity tests. A positive in vitro genotoxicity result usually triggers further in vivo testing.

The sensitivity of the most commonly used in vitro mammalian genotoxicity tests for the identification of carcinogens is rather high, but their specificity is very low (reviewed by Kirkland et al., 2005). Thus, several researchers have noted an unacceptably high occurrence of irrelevant positive results. Several (confounding) factors have been suggested to contribute to this high rate of misleading positive results. These include varying (insufficient) DNA repair capacity, p53 integrity, and lack of metabolism in various cellular systems leading to the reliance on exogenous metabolic activation systems such as rat liver S9 (Kirkland et al., 2007). As a consequence of these shortcomings, subsequent use of a large number of experimental animals is needed in order to confirm or reject positive in vitro results. In the case of misleading positive results, this has proved to be an unnecessary investment of time, effort and expense (Kirkland et al., 2005; Kirkland et al., 2007). Therefore, alternative in vitro assays that more reliably reflect the in vivo situation are in demand.

This study aimed to develop a new in vitro genotoxicity test based on primary hepatocytes isolated from pUR288 lacZ plasmid-based transgenic mice. These mice harbor about twenty chromosomally integrated copies of the pUR288 plasmid (per haploid genome), which contains a lacZ mutation target (Boerrigter et al., 1995; Vijg et al., 1997). This system has proven to be highly accurate with respect to the identification of mutagenic carcinogens without any tissue restriction (Gorelick, 1995; Lambert et al., 2005). Moreover, the pUR288 lacZ plasmid-based model has the advantage over other transgenic rodent models (e.g., the MutaMouse) in that it is able to detect size-change mutations, including large genome rearrangements with one breakpoint in lacZ and the other breakpoint elsewhere in the genome (van Steeg et al., 2000; Garcia et al., 2007).

In previous studies we used mouse embryonic fibroblasts derived from the same mouse model, and showed that activities of both clastogens and mutagens
could be detected in this system (Mahabir et al., 2009). Mouse embryonic fibroblasts are, however, prone to become aneuploid (Woo and Poon, 2004) and have, when compared to hepatocytes, a limited biotransformation capacity. These features make them less attractive as a test system for regulatory purposes. We, therefore, investigated whether primary hepatocytes could be more suitable for routine in vitro genotoxicity testing. Primary hepatocytes represent a well-accepted in vitro cell culture system. Cultured in a collagen sandwich configuration primary hepatocytes contain all the major enzyme pathways for drug and xenobiotic biotransformation. In addition, they retain their typical morphology and reorganize to form an architecture similar to that found in the intact liver (Dunn et al., 1991; Hengstler et al., 2000; Richert et al., 2002; Gebhardt et al., 2003). As such, hepatocytes have been adopted for use in a variety of pharmacological and toxicological settings (Gebhardt et al., 2003; Hewitt et al., 2007). Sandwich-cultured primary hepatocytes, however, are non-proliferating cells because of a G1-S arrest (de Smet et al., 2001; Fassett et al., 2003). For the detection of mutations (i.e. irreversible changes to DNA) proliferation is imperative, and several studies have now shown that primary hepatocytes can be induced to proliferate in vitro to a limited extent (Block et al., 1996; Eckl and Raffelsberger, 1997; Muller-Tegethoff et al., 1997; Chen et al., 2010). Since the aforementioned advantages of primary hepatocytes in a sandwich configuration are not necessarily equivalent for their proliferating counterparts, we present here a preliminary characterization of proliferating primary hepatocytes from the pUR288 lacZ plasmid-based transgenic mouse in terms of biotransformation capacity and p53 responsiveness. In addition, we tested four genotoxic compounds, and provide a proof-of-concept as a first step towards the evaluation of this promising in vitro genotoxicity test.

Materials and methods

Materials and chemicals
Benzo(a)pyrene (BaP; CAS No. 50-32-8) was obtained from Serva (Heidelberg, Germany). Bleomycin (BLM; CAS No. 11056-06-7), cyclophosphamide (CPA; CAS No. 6055-19-2), and etoposide (ETO; CAS No. 33419-42-0) were purchased from Sigma-Aldrich (Zwijndrecht, NL). Chemicals needed for the biotransformation assays (ethoxyresorufin-O-deethylase (EROD), UDP-glucuronosyl transferase (UGT), and glutathione-S-transferase (GST)) were obtained from Sigma-Aldrich.
Mice
CS7BL/6 pUR288 lacZ mice (Boerrigter et al., 1995; Vijg et al., 1997) were bred and maintained under specific pathogen-free conditions in a climate-controlled room with a 12 hour (h) on/off light cycle. Feed and water were available ad libitum. Each offspring was analyzed for the presence of the transgene by means of PCR as described previously (de Vries et al., 1997). The study was approved by RIVM’s Ethical Committee on Experimental Animals, in accordance with national legislation.

Isolation and culture of primary mouse hepatocytes
Primary hepatocytes were isolated from eight to fifteen-week old transgenic male mice by a two-step collagenase perfusion technique described by Mathijs et al. (2009). Cells were cultured according to Chen et al. (2010), with slight modifications. In brief, hepatocytes were plated at a density of \(1.3 \times 10^6\) cells/10 cm dish. After 4 h, the attachment medium was removed and replaced with 10 ml serum-free medium (William’s E medium supplemented with 10 mM HEPES, 2 mM L-glutamine, 10 mM pyruvate, 0.35 mM proline, 200 units/L insulin, 7 ng/ml glucagon, 100 units/ml penicillin G, 100 mg/ml streptomycin sulphate) containing 1 ng/ml murine epidermal growth factor (EGF). The cells were then incubated at 37 °C (95% relative humidity, 5% CO\(_2\)).

BrdU assay
Immediately after perfusion, hepatocytes were cultured in 6 cm culture dishes in the presence of bromodeoxyuridine (BrdU; 3 mg/ml Sigma-Aldrich). Cells were fixed for 10 minutes (min) with 4% paraformaldehyde every 24 h for five consecutive days. Cells were denatured by adding 2 ml 2M HCl for 1 h at 37 °C. Subsequently, cells were neutralized with borate buffer (pH 8.5) for 12 min at room temperature. After three washing steps with PBS containing 0.05% Tween20 (PBT), the cells were blocked for 1 h at 37 °C with PBT supplemented with 2% normal caprine serum. The blocking buffer was removed and the cells were incubated with anti-BrdU monoclonal antibody (250x dilution in PBT supplemented with 2% normal caprine serum; Abcam (Cambridge, UK)) for 1 h at 37 °C. After three washes with PBT the cells were incubated for 1 h at 37 °C in the dark with FITC conjugated leporine polyclonal secondary antibody to rat (200x dilution in PBT supplemented with 2% normal caprine serum; Abcam). Following incubation, cells were dehydrated for 3 min in ethanol (70%, 90%,
100%), embedded in ProLong Gold anti-fade (Life Technologies, Bleiswijk, NL), and visualized with fluorescence microscopy. For quantitative evaluation of the results, twenty microscopic fields (20× magnification) were randomly chosen. Within those fields, all nuclei were counted and classified as BrdU-positive or BrdU-negative. The proliferation rate was expressed as the ratio of BrdU-positive nuclei to the total number of counted cells.

**Ethoxyresorufin-O-deethylase (EROD) assay**
EROD activity was measured in intact hepatocytes as described previously (Donato et al., 1993). The hepatocytes were seeded in 6 cm culture dishes. The EROD assay was performed in duplicate at 24, 48, 72, 96 and 192 h after the start of culture. Prior to each EROD assay hepatocytes were exposed for 16 h to 3 µM BaP. The total amount of protein in each well was determined by a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) and enzyme activities were corrected for protein content. Activity is expressed as pmol resorufin formed per mg protein per minute (pmol/mg protein/min).

**UDP-glucuronosyl transferase and glutathione-S-transferase assay**
The overall activity of UGT and GST was measured in intact hepatocytes according to Westerink et al. (2007). 4-Methylumbelliferone and monochlorobimane were used as substrates for the UGT and GST assay, respectively. Thirty-two h after culture initiation, cells were exposed for 12 h to phenobarbital in concentrations ranging from 0.03-3 mM. Subsequently, the cells were scraped and centrifuged for 5 min at 50xg. Using a Branson sonifier (Branson Ultrasonics Corporation, Danbury, CT, USA), a cell suspension in 200 µl PBS was sonicated three times at 4 °C for 20 seconds (s) with a 40 s interval between the pulses. The assays were performed in duplicate. The decrease and increase in fluorescent signal for, respectively, the UGT and GST assay was measured every two min on a Fluostar Galaxy microplate reader (BMG Labtech, Ortenberg, Germany) at 37 °C. The excitation wavelength was set at 320 nm for the UGT assay and at 355 nm for the GST assay. For both assays, the emission was measured at 460 nm. The conversion rate was calculated from the linear part of the reaction curve of product concentration versus time.
Western blot analysis
To determine p53 function, phosphorylation of the p53 protein at residue serine 389 was measured. 72 h after start of culture, hepatocytes were washed with PBS and exposed to 10 and 20 J/m² UV-C light. Six and twenty-four h later, Western blot analysis was performed in duplicate as described previously (Bruins et al., 2004).

Determination of lacZ mutant frequency
Twenty-four h after the start of culture, cells were treated for 6 h with various concentrations of test chemicals in serum-free medium containing 1 ng/ml EGF. After treatment, the cells were washed with PBS (pH 7.4), incubated in serum-free medium containing 1 ng/ml EGF for an additional 48 h, and collected in 5 ml PBS. Finally, the cell suspension was centrifugated at 50 x g at 4 °C for 5 min. The cell pellet was stored at −20 °C until the lacZ rescue assay was performed using the procedure described by Dollé et al. (1996). The lacZ rescue assay was performed using two biological replicates.

Statistical analyses
Statistical procedures were performed using SPSS version 19.0.0.1 (SPSS, Chicago, IL, USA) software. All values were expressed as means ± SD when appropriate. Comparisons between multiple groups were performed with one-way ANOVA followed by Dunnett’s test or Bonferroni correction. P<0.05 was defined as the threshold for statistical significance.

Results

Growth characteristics of primary hepatocytes
Genotoxicity of chemicals can be detected using cells or intact tissues carrying reporter genes, like lacZ. Cell division is needed to fix DNA lesions into mutations. As such, proliferation of the primary mouse hepatocytes is essential. The specific culture conditions used to induce proliferation resulted in a strong perturbation of the cell morphology: hepatocytes spread out and adopted a fibroblast-like appearance (Figure 1). Cell proliferation was evaluated by means of BrdU incorporation on five consecutive days: 0, 24, 48, 72, and 96 hours after start of culture. The proliferation rate, expressed as the ratio of BrdU-positive nuclei to the total number of counted cells, is shown in Figure 2. We found
an increase in BrdU-positive cells as early as 24 hours after culture initiation; the percentage of BrdU-labeled nuclei was significantly elevated from 48 hours onwards, and increased further over time – at least until 96 hours after start of culture. Approximately 50% of the nuclei were stained with BrdU between 48 and 72 hours of culture, indicating that under our conditions the hepatocytes have a doubling time of approximately 50 hours.

Figure 1. Typical example of primary hepatocytes after 72 hours of culture (magnification, 40×).

Figure 2. Labeling index (BrdU) of proliferating cultures at different times after hepatocyte isolation. Each column represents the mean and standard deviation of three separate cultures. * P<0.01 compared with t = 0 and 24 hours; # P < 0.05 compared with t = 48 hours.
Biotransformation capacity – Phase I and Phase II enzymes
The added value of primary hepatocytes to assess genotoxicity is their metabolic activation capacity. In the present study, activities of both Phase I and Phase II biotransformation enzymes were measured. For Phase I, cytochrome P450 1A1 (CYP1A1)-associated 7-ethoxyresorufin-O-deethylase (EROD) activity was determined over time after exposure to benzo(a)pyrene (BaP; see Figure 3). In untreated cells EROD activity was virtually absent; however upon BaP exposure, the enzyme activity increased considerably, reaching a maximum enzyme induction after 72 hours culture. From 96 hours onwards, the EROD activity gradually declined.

Figure 3. EROD activity over time in primary mouse hepatocytes upon BaP treatment. Hepatocytes were treated for 16 hours with 3 µM BaP or vehicle, thereafter EROD activity was measured. Experiments were performed at 24, 48, 72, 96 and 192 hours after culture initiation. Results are presented as the mean activity ± SD of two separate cultures.

For Phase II metabolism, the overall activity of UGT and GST was measured 48 hours after the start of culture. Figure 4 shows that even without induction with phenobarbital, GST and UGT activities were detectable in the primary hepatocytes. Exposure to phenobarbital did not result in a substantial increase in enzyme activity, neither for UGTs nor for GSTs.
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Figure 4. Overall activity of glutathione-S-transferase (GST) and UDP-glucuronosyl transferase (UGT) with and without pre-treatment of phenobarbital. Experiments were performed after 48 hours of culture. Results are presented as the mean activity ± SD of two separate cultures.

Phosphorylation of p53 at residue serine 389 after UV treatment
The p53 status of cell lines currently used in genotoxicity assays is a factor that may be contributing to the occurrence of misleading positive results (Pfuhler et al., 2011; Fowler et al., 2012). Therefore, one of the criteria for an effective new test system is that the cells to be used are p53 proficient. We verified whether p53 in the proliferating hepatocytes is functioning properly by measuring phosphorylation of the p53 protein at residue serine 389 at 6 and 24 hours after exposure to a direct-acting genotoxicant, i.e., UV radiation (Bruins et al., 2008). Increasing levels of phosphorylated p53.S389 were observed in a dose-dependent manner after UV treatment (Figure 5). The increase in phosphorylated p53 was more pronounced at 6 hours after exposure, compared to 24 hours. At both time points, a clear increase in cell death was observed microscopically (data not shown). Cell death was, however, more pronounced at 24 hours after UV irradiation.
Figure 5. Phosphorylation of p53.S389 in primary mouse hepatocytes after exposure to UV radiation. Protein extracts were prepared at different time points after the treatment. Phosphorylation of p53.S389 was visualized by Western blotting after immunoprecipitation. The remnant was moved to an additional Western blot, which was incubated with actin antibody to control for loading.

Figure 6. LacZ mutant frequencies in primary mouse hepatocytes after exposure to cyclophosphamide, etoposide, benzo(a)pyrene or bleomycin. Asterisks indicate a significant difference between the lacZ mutant frequencies in the exposed groups compared to the unexposed groups. * P<0.05; ** P<0.01. Results are presented as the mean ± SD of two separate cultures.

LacZ mutant frequency analyses
Four genotoxicants were tested in the lacZ rescue assay: etoposide (ETO), bleomycin (BLM), benzo(a)pyrene (BaP), and cyclophosphamide (CPA). The latter two require metabolic activation in order to induce a genotoxic effect. The resulting lacZ mutant frequencies are presented in Figure 6. For CPA (Figure
6A) the induction of mutant colonies was rather low and statistically significant compared to background values only for the highest concentration tested (1000 \( \mu \)M). ETO exposure (Figure 6B) led to gene mutations only at the two highest concentrations tested. A slight, not statistically significant, increase in \( \textit{lacZ} \) mutant frequency was detected at 100 \( \mu \)M, whereas treatment with 300 \( \mu \)M ETO yielded a significant 2.5-fold increase in mutant frequency. For BaP (Figure 6C) and BLM (Figure 6D) a concentration-dependent increase in \( \textit{lacZ} \) mutant frequency was observed. This effect was most pronounced for BLM (Figure 6D).

**Discussion**

In this study, we examined the feasibility of culturing primary hepatocytes from the pUR288 \( \textit{lacZ} \) reporter mouse, and moreover, the ability to use established cultures to assess genotoxic activity \textit{in vitro}. Primary hepatocytes are considered one of the most relevant and practical tools for \textit{in vitro} toxicity assessment (Gebhardt et al., 2003; Hewitt et al., 2007). In most studies, however, primary hepatocytes are cultured in a collagen sandwich configuration since cells cultured under conventional conditions, \textit{i.e.}, on a collagen monolayer and in the presence of growth factors, have been reported to adopt a flat morphology that is accompanied by a rapid loss of essential hepatocyte features such as metabolic functions and bile canalicular transport (Rana et al., 1994; Elaut et al., 2006). The culture conditions used in the present study (without collagen monolayer) did, as expected, lead to morphological changes and cellular proliferation. These findings are consistent with previous studies, in which similar conditions were successfully employed to stimulate hepatocyte proliferation (Eckl and Raffelsberger, 1997; Muller-Tegethoff et al., 1997; Chen et al., 2010).

To determine whether this growth-associated dedifferentiation also included loss of metabolic competence, we measured cytochrome P450 CYP1A1, GST and UGT enzyme activities. The levels of CYP1A1 activity were very low without addition of any ligand of the aryl hydrocarbon receptor. It has been previously described that primary mouse hepatocytes, both freshly isolated and cryopreserved, exhibit low levels of P450 enzymes. For instance, the EROD activity in freshly isolated and cryopreserved hepatocytes equals 6.3 and 6.1 pmol/mg protein/minute, respectively (Gebhardt et al., 2003). P450 enzymes, however, will be induced in hepatocytes upon exposure to a toxic compound
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(Cervenkova et al., 2001). Using BaP as inducer, CYP1A1 enzyme activities were markedly increased in our proliferating hepatocytes, reaching maximum enzyme levels at 72 hours after culture initiation. These levels of CYP1A1 activity are comparable to those obtained upon induction using primary mouse hepatocytes cultured in a collagen sandwich (van Kesteren et al., 2011). Whether or not activities for other CYPs are constitutively present and/or can be induced in a similar manner in these proliferating hepatocytes will have to be determined.

For the Phase II enzymes GST and UGT, overall activities were measured in the proliferating primary hepatocytes after 48 hours of culture. The activities were, not surprisingly, much lower as compared to freshly isolated hepatocytes in suspension (Gebhardt et al., 2003). Treatment with the model inducer phenobarbital did not significantly increase GST or UGT activity. A similar lack of induction has been reported for UGT in OF-1 mouse hepatocyte monolayers (Viollon-Abadie et al., 2000). Nevertheless, both GST and UGT enzyme activities were detectable after 48 hours of culture. These findings show promise for the other Phase II enzymes (sulfotransferases, epoxide hydrolases, and acetyltransferases) since it is known that Phase II enzymes are better preserved in culture than Phase I enzymes, at least in rat hepatocytes (Rogiers and Vercruysse, 1993; Kern et al., 1997).

Taken together, our preliminary data suggest that the proliferating lacZ primary hepatocytes are metabolically competent. This is an important observation, because Phase I and II biotransformations are responsible for the activation of numerous carcinogens. For instance, benzo(a)pyrene is activated by CYP1A1/1B1 and epoxide hydrolase (Westerink and Schoonen, 2007). Usually, S9 is used exogenous metabolic activation system in in vitro genotoxicity tests. Metabolites produced by S9 may be quite different from those produced by normal liver metabolism and, as such, may contribute to the high rate of misleading positive results (Kirkland et al., 2007).

Another confounding factor recognized as possibly contributing to the high rate of false positives is an impaired p53 function. The p53 protein is a DNA damage sensor that prevents the accumulation of genetic lesions. It plays an active role in a variety of cellular processes, including cell cycle arrest, DNA repair, and apoptosis (Lu, 2005). Upon exposure to stress signals, p53 is activated by posttranslational modifications, of which phosphorylation is the most
commonly occurring. After UV irradiation, p53 is phosphorylated specifically at murine residue Ser389 (Bruins et al., 2008). The increase in phosphorylated p53 S389 in this study is consistent with p53 functioning properly upon genotoxic stress.

To offer a proof-of-concept for the lacZ hepatocytes as a new in vitro genotoxicity detection system we tested four genotoxic compounds. The selected compounds require metabolic activation (BaP, CPA) and/or induce DNA strand breaks (CPA, BLEO, ETO). CPA is a prodrug, which is suggested as positive control in OECD test guidelines (OECD, 1997). It requires metabolic activation by CYP2B6, yielding phosphoramidemustard capable of reacting with DNA molecules to form crosslinks (Zhang et al., 2005). Previous studies showed that CYP2B10 activity, the mouse ortholog of human CYP2B6, is present in primary mouse hepatocytes (Mathijs et al., 2009). Here we showed that a significant lacZ mutant frequency increase was only observed at the highest concentration tested, i.e., 1000 µM. This weak response could be due to the type of cells we used, since CPA has been shown to induce DNA damage in blood leukocytes, but not in livers of CPA-treated mice and rats (Recio et al., 2010).

ETO is a known topoisomerase II inhibitor, and as such, is commonly used as a antitumor drug (Anderson and Berger, 1994; Choudhury et al., 2004). The clastogenicity of topoisomerase inhibitors is believed to result from the transient stabilization of the topoisomerase IIα-DNA complex. This stabilized complex prevents re-ligation of DNA by topoisomerase IIα, which, in turn, may result in the formation of a DNA strand break. An important implication of an indirect (topoisomerase IIα-mediated) effect on DNA is the presence of a threshold for clastogenicity, which has been demonstrated for various topoisomerase inhibitors including ETO (Lynch et al., 2003; Smart et al., 2008). Our mutant frequency data for ETO are consistent with this threshold concept: at low concentrations no significant changes in mutant frequency were detected.

BaP is a polycyclic aromatic hydrocarbon and a well-studied promutagen. Upon biotransformation DNA adducts are formed that give rise to mutations. BLM is a chemotherapy drug that acts by induction of DNA strand breaks. Treatment with BaP or BLM yielded a clear concentration-dependent induction of the lacZ mutant frequency (up to 9.8-fold and 32.8-fold, respectively). These findings are consistent with previous in vivo and in vitro studies of cells from the lacZ plasmid-based transgenic mouse (de Vries et al., 1997; Mahabir et al., 2009).
Summarizing, we offered a proof of concept that (i) our lacZ model detects both mutagens and clastogens and (ii) addition of an exogenous metabolic activation system is not mandatory to detect compounds which need metabolic activation.

We have established a proof-of-concept for an in vitro genotoxicity detection system based on proliferating primary mouse hepatocytes from the pUR288 lacZ plasmid mouse. It justifies a more detailed assessment of the usefulness of this system. We plan to further evaluate this new assay by testing a large number of the chemicals recommended by Kirkland et al. (2008), including known irrelevant positives to demonstrate the reliability of this new approach. In addition, we will determine the activities of the major enzymes involved in xenobiotic transformation. Another aim is to investigate whether the test is still promising if cryopreserved hepatocytes, frozen immediately after perfusion, are used. In the frame of the “3R principle”, this will reduce the number of experimental animals and make the test more acceptable to the general public.

The main issue to be addressed, however, is cytotoxicity, which to date is not yet incorporated in our lacZ test system. A method to measure cytotoxicity is mandatory in any (in vitro) genotoxicity test. It is essential that exposure of the (target) cells, measured as a certain level of cytotoxicity, is demonstrated. The importance of cytotoxicity is shown in the OECD test guidelines where the required level of cytotoxicity is clearly described for every different genotoxicity test, e.g. 10-20% cell survival in in vitro gene mutation tests (OECD, 1997). Furthermore, the choice of concentrations to be tested often is limited by cytotoxicity, i.e., cell survival, cell viability, proliferation rate. Finally, very high cytotoxicity may lead to irrelevant positive results (Kirkland et al., 2007). In the present experiment, for instance, the positive result found for CPA at the highest concentration tested only may be the result of cytotoxicity. We, therefore, question the biological relevance of this particular finding. Obviously, a focus for further development of our lacZ test model is the implementation of a measure for cytotoxicity.

We conclude that lacZ primary mouse hepatocytes are promising tools for the assessment of genotoxicity. If we are able to accomplish all our aims and particularly if we succeed in the implementation of a cytotoxicity measure, this
new test may become a useful alternative to *in vitro* tests for both mutagens and clastogens with the big advantage that gene mutations and chromosome rearrangements can be determined simultaneously in one test system.

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References


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