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# Development of a panel of high-throughput reporter-gene assays to detect genotoxicity and oxidative stress☆

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ABSTRACT

The lack of toxicological information on many of the compounds that humans use or are exposed to, intentionally or unintentionally, poses a big problem in risk assessment. To fill this data gap, more emphasis is given to fast in vitro screening tools that can add toxicologically relevant information regarding the mode(s) of action via which compounds can elicit adverse effects, including genotoxic effects. By use of bioassays that can monitor the activation of specific cellular signalling pathways, many compounds can be screened in a high-throughput manner. We have developed two new specific reporter-gene assays that can monitor the effects of compounds on two pathways of interest: the p53 pathway (p53 CALUX) for genotoxicity and the Nrf2 pathway (Nrf2 CALUX) for oxidative stress. To exclude non-specific effects by compounds influencing the luciferase reporter-gene expression non-specifically, a third assay was developed to monitor changes in luciferase expression by compounds in general (Cytotox CALUX). To facilitate interpretation of the data and to avoid artefacts, all three reporter-gene assays used simple and defined reporter genes and a similar cellular basis, the human U2OS cell line. The three cell lines were validated with a range of reference compounds including genotoxic and non-genotoxic agents. The sensitivity (95%) and specificity (85%) of the p53 CALUX was high, showing that the assay is able to identify various types of genotoxic compound, while avoiding the detection of false positives. The Nrf2 CALUX showed specific responses to oxidants only, enabling the identification of compounds that elicit part of their genotoxicity via oxidative stress. All reporter-gene assays can be used in a high-throughput screening format and can be supplemented with other U2OS-based reporter-gene assays that can profile nuclear receptor activity, and several other signalling pathways.



## 1. Introduction

Humans are continuously exposed to complex mixtures of compounds and their breakdown products that are either intentionally produced or that are endogenously present in the environment, in food, medicines or consumer products. The presence of several types of compound and/or contaminant has been linked to adverse effects in both humans and the environment [1]. However, for many compounds there is not enough toxicological information available to assess their risk. To fill this information gap, more emphasis is put on gaining insight in the molecular mode(s) of action of compounds by use of in vitro technologies, as proposed for example by the US National Research Council (NRC) in their long-range vision for toxicity testing and risk assessment [2]. Approaches focus on how toxic compounds influence adverse outcome pathways [3] that can lead to adverse (health) effects. The idea behind this approach is that compounds cause adverse health effects because they can interfere in cell-signalling pathways at the molecular level. Therefore, there is a need for novel monitoring tools on pathways of interest that are cheap, fast and predictive for human health. These high-throughput assays should provide insight in the mode(s) of action of single compounds [2].

An important aspect of the toxicity of certain compounds is their ability to act as carcinogens, either by damaging the DNA directly (i.e. genotoxic carcinogens) or indirectly (non-genotoxic carcinogens) by acting through pathways associated with the modulation of reactive oxygen species, apoptosis, endocrine controls, cell proliferation or immune surveillance [4]. Toxicity assessment plays a pivotal role in the safety assessment of, e.g., pharmaceuticals [27] and cosmetics, which in the case of cosmetics ingredients has to be conducted without the use of animal studies, according to EU regulations [5]. Recently, assays with human cell lines have been

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developed, which focus on activation of promoter regions of target genes involved in genotoxic responses, e.g., GADD45a [6] or p53R2 [7]. While these genes are primarily regulated by the p53 tumour-suppressor gene, they are also influenced by other stress pathways, including those involving JNK, NF-kappaB and Nrf2 [8,9] and nuclear receptor pathways such as PPAR [10,11]. While these pathways are all clearly relevant in the cellular response to genotoxic stress or carcinogenicity, they do not allow differentiating between the various pathways that lead to the observed genotoxic activity.

We have developed several bioassays with human cell lines that can be used to determine whether compounds are influencing specific pathways. All these cell lines are based on the human U2OS (bone-derived) cell line in combination with highly specific reporter constructs containing only defined responsive elements and a minimal promoter linked to luciferase. By use of this minimal reporter construct, responses other than those related to the signalling pathway of interest are avoided. Until now, these assays focused on specific nuclear receptors [12] and several of these assays have been used successfully to study effects of single compounds or environmental extracts [13] on e.g., hormone receptors that are associated with endocrine disruption and reproductive effects [14–16].

In the present study, three new reporter-gene assays based on the human U2OS cell line, were developed and validated with an extensive panel of compounds as suggested by Kirkland et al. [25]. The U2OS cell line has several advantages over other frequently applied cell lines in that it contains wild type p53, is frequently utilized in genotoxicity research, is relatively easy to maintain in culture, has a relatively short doubling time, and contains a relatively intact cell cycle and apoptotic machinery [17,18] including cell-cycle arrest signalling. Two of the assays respond to important pathways related to (in)directly acting genotoxins, p53 and Nrf2. The third assay serves as a control for cytotoxicity and luciferase expression in U2OS-based CALUX bioassays in general. Increased p53 levels can be regarded as indicative of direct genotoxicity, as the p53 protein plays a pivotal role in the cellular response to DNA damage by acting as a transcription factor in genes related to cell-cycle arrest, DNA-damage repair and apoptosis [19], and is directly correlated with frequently monitored endpoints such as micronucleus formation [20,21]. Nrf2 is a transcription factor that activates genes containing an anti-oxidant responsive element (ARE) in the promoter region, such as phase-II detoxifying enzymes, transporters (phase-III enzymes) and antioxidant-stress proteins that protect against oxidative stress [22]. Activation of the Nrf2 pathway is indicative of oxidative stress which can indirectly result in genotoxic effects. The third assay does not respond to specific pathways, but serves as a measure of cytotoxicity, and as a control for non-specific activation or inhibition of luciferase expression [23,24].

As all cell lines in the panel utilize the same U2OS cell-line construct combination, the luciferase expression-control assay can serve as a control for the specificity of the response for all current and future cell lines in the panel. These assays supplement our current panel of specific reporter-gene assays that respond to nuclear receptors. Together, they can serve as a powerful tool to gain insight in the mechanisms behind potentially adverse effects of individual compounds as well as of those present in complex (e.g., environmental) mixtures.

## 2. Materials & methods

### 2.1. Chemicals

A total of 61 reference compounds were used in this study: 20 genotoxic carcinogens (positives), 22 non-genotoxic compounds (negatives) and 19 "misleading positives", i.e. compounds that are known to give positive results in vitro at high concentrations, but are regarded as negative in vivo. The selection of compounds was based on the list suggested by Kirkland et al. [25], with the exception of ephedrine sulphate, which could not be obtained due to purchase restrictions. All chemicals were of the highest purity available and were ordered from Sigma–Aldrich except for IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline) and PhIP.HCl (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine), which were obtained from Wako (Germany). Compounds are prepared as $10^{-1}$ M stock solutions (or lower if solubility is limiting), preferably in dimethyl sulfoxide (DMSO) (Acros, Geel, Belgium). Due to limited solubility in DMSO, cadmium chloride, trisodium-EDTA-trihydrate and sodium arsenite were dissolved in high-purity water in order to reach the high concentrations needed.

### 2.2. Cell culture

Human osteoblastic osteosarcoma U2OS cells (American Type Culture Collection [ATCC], Manassas, VA, USA) were cultured as described previously in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF, Invitrogen, Breda, Netherlands) supplemented with 7.5% foetal calf serum (FCS) (Invitrogen), nonessential amino acids (NEAA) (Invitrogen) and 1% penicillin-streptomycin (final concentrations 10 U/ml and 10 μg/ml, respectively). Cultures were maintained in a humidified atmosphere with 5% $CO_2$ at 37 °C and medium was refreshed every 3 or 4 days during sub-culturing. Stably transfected p53, Nrf2 and control cells were cultured in DF medium supplemented with 7.5% FCS, NEAA, penicillin/streptomycin and G418 (or geneticin is an antibiotic that inhibits polypeptide synthase and it is usually lethal to eukaryotic cells. It is used as a selection marker to seperate the successfully transfected cells from the other cells: transfected cells survive and non-transfected cells die) (0.20 mg/ml medium).

### 2.3. Development of a p53, Nrf2 luciferase-reporter and luciferase control vector

The p53 reporter construct was designed as follows: 4 repeats of a blunt HindIII/SPHI 3 × p53 consensus responsive element (p53RE) (GAACATGCCCAACATGTTG) [26] fragment were inserted into a promoter-less luciferase reporter-construct pLuc [12]. For the Nrf2-construct, two oligos were synthesized (GeneArt®/Invitrogen) containing four different EPRE sequences: 1 × consensus EPRE (TCACAGTGACTAAGCAAAAT), 1 × hNQO1 EPRE (TCACAGTGAC TCAGCAGAAT), 1 × hGCLM EPRE (AGACAATGACTAAGCAGAAA) and 1 × hGCLC EPRE (TCACAGTCAGTAAGTGATGG). The two oligos were also ligated into a promoter-less luciferase reporter-construct pLuc [12]. The luciferase control-vector for the Cytotox CALUX consisted of the luciferase reporter inserted into a pSG5-neo [12] expression vector. In all plasmids, a minimal TATA promoter sequence was inserted downstream in the pGL3-basic construct. Because the U2OS cells express both the p53 and the Nrf2 pathways endogenously, a selection construct (pSG5-neo) was used to differentiate between expressing and non-expressing clones by use of G418 [12].

### 2.4. Development of p53 CALUX, Nrf2 CALUX and Cytotox CALUX reporter-gene assays

U2OS cells were transfected with the constructs described above by means of using calcium phosphate precipitation. G418-resistant clones were tested for their response using $10^{-8}$ M actinomycin D (p53), $10^{-5}$ M *t*-butylhydroquinone (*t*-BHQ) (Nrf2) and DMSO (luciferase expression control). Up to 20 clones were selected that showed a promising combination of responsiveness (strongest induction), standard deviation between triplicates (preferably < 15%) and level of light produced (at least 10× the background level of the luminometer, expressed as relative light units (RLU)). Clones were cultured twice a week and their response was monitored over time, with actinomycin D (p53 assay) and *t*-BHQ (Nrf2 assay) as reference compounds. Clones consistently combining high RLU levels with responsiveness and low standard deviations were selected.

### 2.5. Reporter-gene assay, exposure and analysis

The CALUX reporter cells were trypsinized, counted and resuspended in DCC-medium (cell culture medium without phenol red and supplemented with 5% dextran-coated charcoal-stripped FCS (DCC–FCS), instead of 7.5% FCS) to a final concentration of $10^4$ cells/well (100 μl) in a clear 96-well plate. The plates were incubated for 24 h in a humidified atmosphere at 37 °C under 5% $CO_2$. Following the pre-incubation, 9 serial dilutions in the range of $10^{-3}$ M–$3 \times 10^{-7}$ M (log 10 dilution steps) of the compounds to be tested and a positive control were added as 100-μl aliquots to CALUX cells, consisting of DCC-FCS supplemented with the compound to be tested (2% DMSO). In case the compound was dissolved in water, DMSO-supplemented medium was used so that in all cased the final volume in the well was 200 μl, at a DMSO concentration of 1%. Actinomycin D ($10^{-8}$ M) and *t*-BHQ ($10^{-5}$ M) were used as positive controls in the p53 CALUX and Nrf2 CALUX assay, respectively. Benzo[*a*]pyrene ($10^{-4}$ M) was used as a positive control in the p53 assay in combination with S9. Sodium arsenite was used as a cytotoxicity control for the Cytotox CALUX assay. All compound concentrations were tested in triplicate. After the addition of the compounds, the plates were incubated for 24 h in a humidified atmosphere at 37 °C under 5% $CO_2$. In the case of exposure in combination with S9, a mixture containing S9 (MP-Biomedicals) was added at a final concentration of 0.33 mg/ml medium, in

combination with 200 μM NADPH (Applchem), 3 mM glucose-6-phosphate, 0.3 U/ml glucose-6-phosphate dehydrogenase, and 5 mM magnesium chloride. Three hours after addition of the S9 mixture, the medium containing the S9 mixture and compounds was removed. Cells were washed with 200 μl phosphate-buffered saline (PBS, Invitrogen), 200 μl of fresh DCC medium was added to each well, and the cells were incubated for another 16 h. After a total of 24 h of incubation, the medium was removed from the cells and 30 μl Triton lysis buffer was added. Plates were shaken for 15 min and the amount of luminescence was measured with a luminometer (Tristar, Berthold, Germany). The amount of light produced was measured during four seconds, after which the signal was quenched with 0.2 M sodium hydroxide.

### 2.6. Reporter-gene assay validation

The cell-line panel was exposed to a wide range of genotoxic and non-genotoxic compounds, based on the list suggested by Kirkland et al. [25]. All compounds were tested in the range of $10^{-3}$ M to $3 \times 10^{-7}$ M, with 3-fold dilution steps. All compounds were tested at least three times. To validate the cell line for cytotoxicity and to control for changes in luciferase expression or cell proliferation, all compounds were also tested for cytotoxicity by use of the MTT test. In short, cells were seeded and exposed in the same way as for luciferase analysis. After 24 h exposure to the compounds of interest, the medium was replaced by a mixture of PBS and medium (1:5) containing 1 mg/mL 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The cells were incubated for approximately two hours at 37 °C, until formation of formazan crystals became evident under the microscope. After the incubation, the blue crystals were dissolved by addition of 100 μL DMSO and the amount of formazan formed was quantified with a spectrophotometer (595 nm).

### 2.7. Data analysis and calculations

The induction factor (IF) in luciferase activity per well was calculated by dividing the level of relative light units (RLU) in the well by the average RLU level of the solvent control wells (DMSO only). Compounds were considered to be positive in the assays when the response of at least one concentration showed an increase of at least 50% (i.e., a 1.5-fold induction), except for cells treated with S9, for which a cut-off value of a two-fold induction was used, due to the higher standard deviation in the assay. The first concentration to exceed this threshold was reported as the lowest observed effect concentration (LOEC). MTT results were expressed as fold change compared with cells exposed to DMSO only. The highest concentration tested was 1 mM, or lower if that concentration could not be reached due to low solubility. No selection with regard to concentrations to be tested and cytotoxicity was applied a priori. The top concentrations selected are in accordance with the ICH guideline for genotoxicity testing by means of mammalian cell assays [27], and other recent papers [28,29]. Concentrations that showed precipitation in cell-culture medium were not included in the analysis, as precipitates might invoke non-specific stress responses and might therefore lead to false-positive results. Similarly, in accordance with the ICH guidelines [27] only concentrations that showed less than 50% cytotoxicity (i.e. <50% decrease in the Cytotox CALUX assay) were considered, in order to rule out non-specific responses due to general cellular stress. The lowest concentration tested should not be cytotoxic: if the signal in the Cytotox CALUX decreased by more than 50%, the compound is too cytotoxic at all concentrations tested, and the tests should be repeated with lower concentrations, with at least two non-cytotoxic concentrations.

## 3. Results

### 3.1. Maintenance and stability of the cell lines

Three U2OS-based CALUX reporter cell lines were developed, of which two are based on the specific activation of pathways relevant for genotoxic activity: p53 and Nrf2. The third cell line serves as a control for the luciferase expression in all cell lines that are part of the U2OS CALUX cell-line panel. For each cell line, the clone was selected that showed the highest induction, combined with an RLU response higher than 10 times the background of the luminometer and a standard deviation <15%. For all cell lines, the activity of the selected clones was maintained for more than 50 passages, which means that the cells can be used continuously for over five months without losing significant inducibility when the cells were cultured twice a week.

### 3.2. Detection of cytotoxicity and luminescence interference in the Cytotox CALUX assay

To exclude concentrations that exceed 50% cytotoxicity in the analysis (because of non-specific responses) [27], as well as to monitor non-specific changes in luciferase expression, all compounds were tested in the Cytotox CALUX assay. As this assay relies on the constitutive expression of luciferase, a decrease in activity is indicative of cytotoxicity. The cytotoxicity was confirmed qualitatively by observation under a microscope, and quantitatively by means of an assay not based on luciferase, i.e. the MTT test (see Fig. 1). Of the 61 compounds tested in this study, 12 showed signs of cytotoxicity

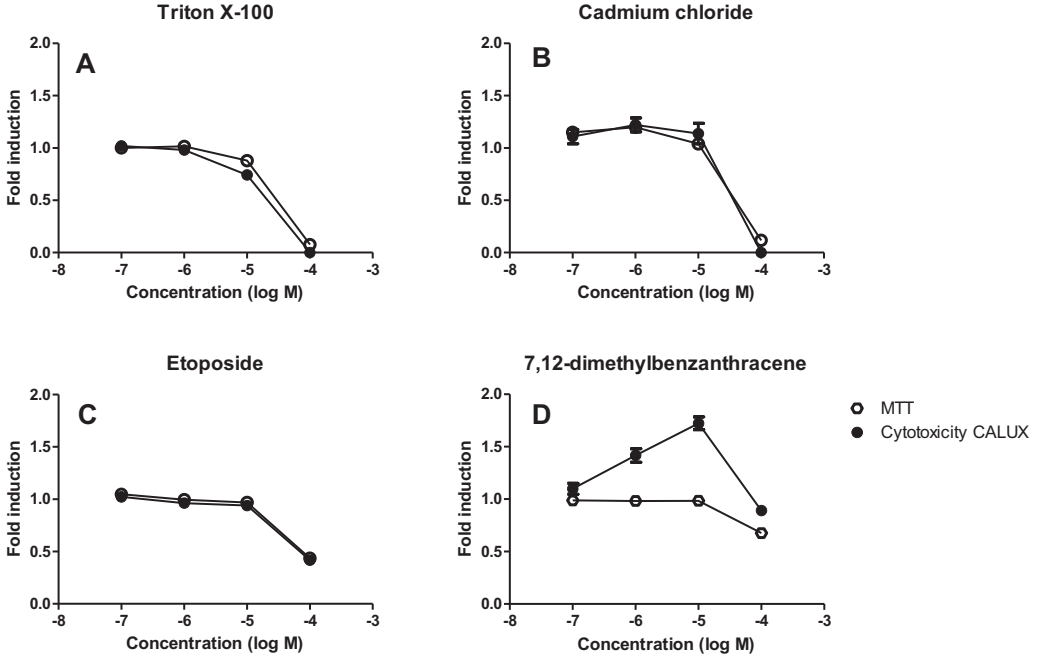


**Fig. 1.** Dose–response curves for three cytotoxic compounds. Cytotoxicity is assessed using the MTT test and the Cytotoxicity CALUX. The Cytotoxicity CALUX performs similarly to the MTT test (a, c and b) but also serves as a control for non-specific luciferase expression (d).

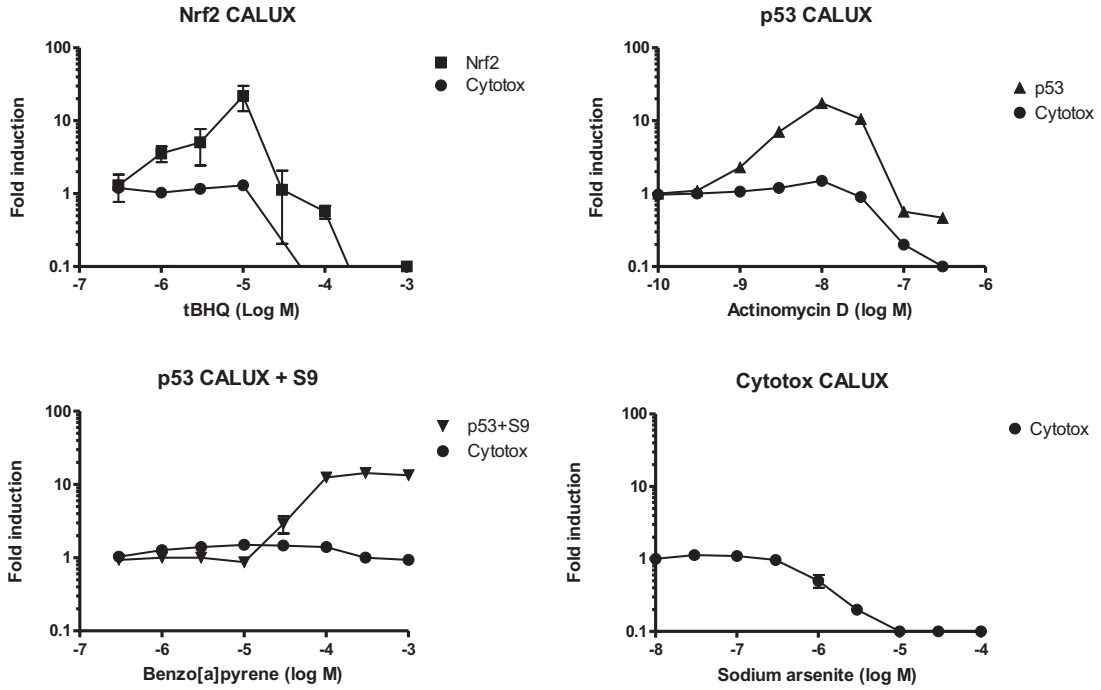


**Fig. 2.** Dose–response examples showing the responses of the p53 assay (with and without S9), Nrf2 assay and control assay for the reference compounds.

when tested up to $10^{-3}$ M. In most cases, cytotoxicity occurred only at relatively high concentrations exceeding $10^{-5}$ M (It was mentioned here because for human cells in vitro, concentrations higher than $10^{-5}$ M can generally be regarded as (unrealistically) high. Many nonspecific effects can start to occur at higher concentrations. However, for genotoxicity screening, even higher concentrations are frequently used, up to $10^{-3}$), although compounds like sodium arsenite and actinomycin D showed cytotoxicity at much lower concentrations. Three compounds consistently showed an increase rather than a decrease in RLU levels, with a response that sometimes exceeded the 1.5-IF threshold in the Cytotox CALUX assay: benzo[*a*]pyrene (Fig. 3c), 7,12-dimethylbenzanthracene (Fig. 1d)

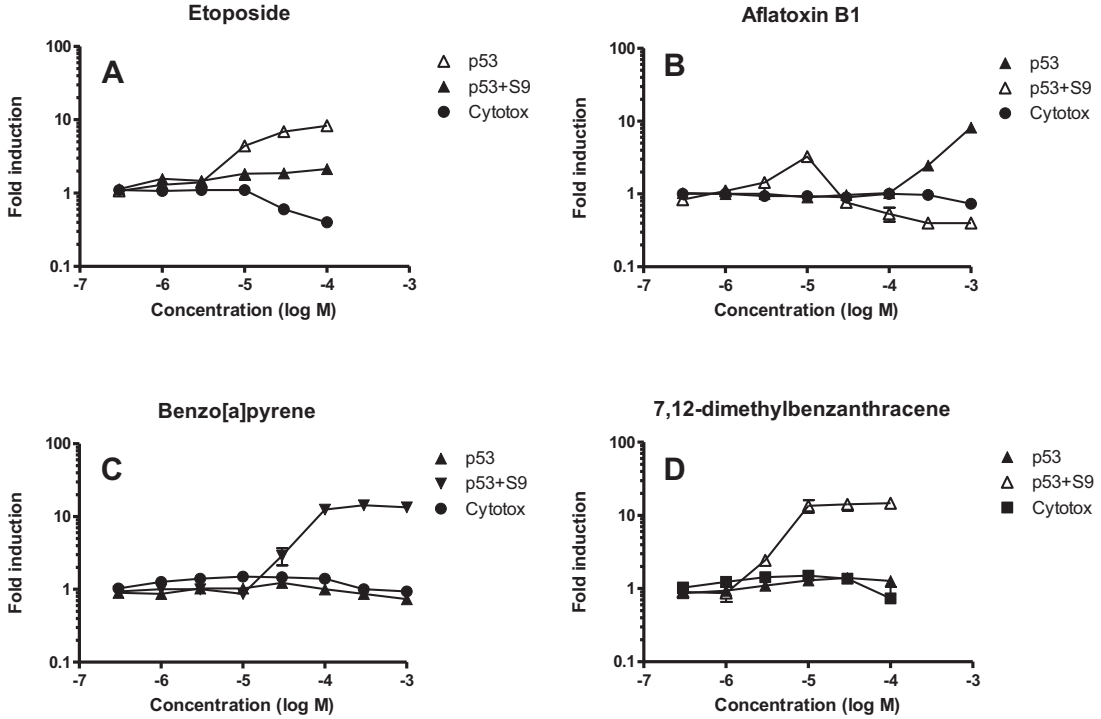


**Fig. 3.** Dose–response examples showing the responses of four compounds in the p53 CALUX assay that are genotoxic without (A and B) and with the addition of S9 (C and D) as a metabolizing system. To be regarded as positive in the p53 CALUX, compounds need to elicit a response above 1.5 times (p53 CALUX) or 2 times (p53 CALUX with S9) fold induction, at concentrations that show less than 50% cytotoxicity (Cytotox CALUX IF > 0.5) as higher concentrations can lead to non-specific responses. Compounds are tested without any knowledge regarding their cytotoxicity a priori, in the range of $3 \times 10^{-7}$ M up to $1 \times 10^{-3}$ M. For clarity, only the concentrations that show less than 50% cytotoxicity are plotted.

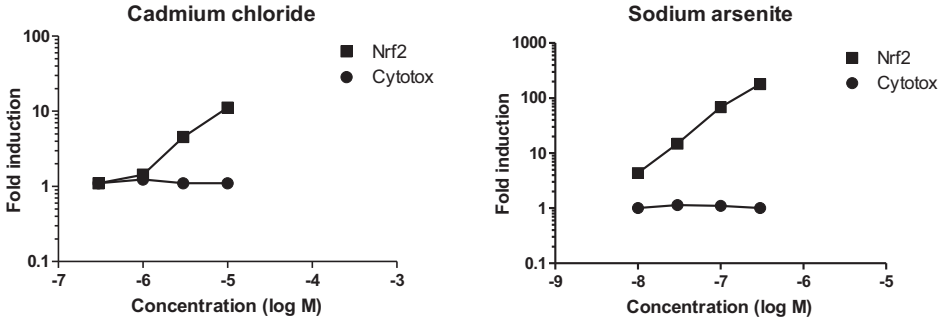


**Fig. 4.** Dose–response examples showing the responses of the Nrf2 assay for two different compounds. To be regarded as positive in the screen, compounds need to elicit a response above 1.5 times fold induction, at concentrations that show less than 50% cytotoxicity (Cytotox CALUX IF > 0.5) as higher concentrations can lead to non-specific responses. Compounds are tested without any knowledge regarding their cytotoxicity a priori, in the range of $3 \times 10^{-7}$ M up to $1 \times 10^{-3}$ M, but due to the cytotoxicity of sodium arsenite this compound was tested at lower concentrations. For clarity, only the concentrations that show less than 50% cytotoxicity are plotted.

and *N*,*N*-dicyclohexyl thiourea (Fig. 5c ). As an increase in RLU levels in the Cytotox CALUX assay indicates a nonspecific induction of luciferase, these compounds might give false-positive responses in other assays that are based on the same cell line-reporter combination.

### 3.3. Detection of genotoxic activity with the p53 CALUX assay

Direct-acting genotoxins are expected to activate the p53 pathway, although this sometimes requires a metabolic activation step [25]. Examples of dose-response curves with and without metabolic activation are given in Fig. 3. Of 20 genotoxic compounds tested, 11 came out positive in the p53 CALUX assay without addition of a metabolizing system (see Table 1). Of the nine chemicals listed to require metabolic activation before becoming genotoxic, four compounds produced a response in the p53 CALUX assay without a metabolic system: PhIP-HCl, 2-acetylaminofluorene, IQ (2-amino-3 methylimidazo[4,5-*f*]quinoline) and Aflatoxin B1. However, in most cases the addition of a metabolizing system (S9) to the assays resulted not only in a much higher response, but also in a decrease of the LOEC (see Table 1). Testing with S9 also resulted in the positive identification of the seven other genotoxic compounds, some known to require metabolic activation. Two compounds, benzo[*a*]pyrene and 7,12-dimethylbenzanthracene, showed a similar response close to the cut-off value of 1.5 IF in the Cytotox CALUX, Nrf2 CALUX and p53 CALUX assays, indicating a possibly false-positive response. However, both compounds were clearly positive in the p53 CALUX assay upon addition of S9, resulting in both cases in a response > 10 IF. Overall, the p53 CALUX assay (with and without S9) detected almost all (18 of the 20) genotoxic compounds as positive. Of the non-genotoxic compounds (Table 2), only one compound (phenanthrene) came out positive in the p53 CALUX assay, without S9 and at non-cytotoxic concentrations. Of the compounds that are known to produce false-positive results (Table 3), propyl gallate showed a positive results in the p53 CALUX assay without S9, while three more compounds showed a positive result after addition S9 to the test system. Overall, the p53 CALUX assay showed a sensitivity of 85% and a specificity of 95% without the

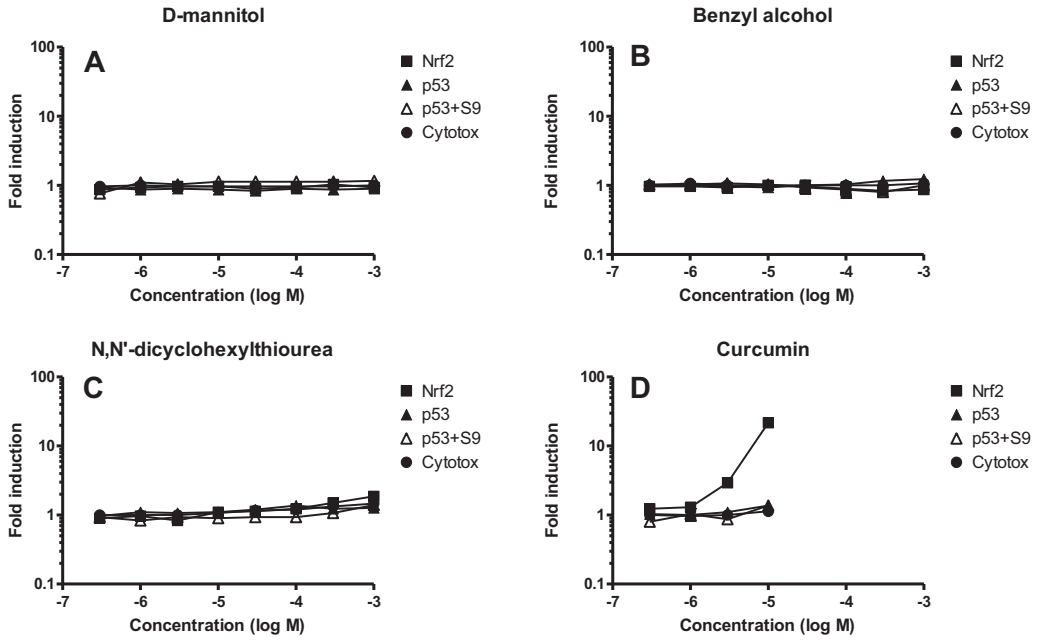


**Fig. 5.** Dose–response examples showing the responses of non-genotoxic compounds in the p53 CALUX (with and without S9), Nrf2 CALUX and Cytotox CALUX. To be regarded as positive in the screen, compounds need to elicit a response above 1.5 times fold induction, at concentrations that show less than 50% cytotoxicity (Cytotox CALUX IF > 0.5) as higher concentrations can lead to non-specific responses. Compounds are tested without any knowledge regarding their cytotoxicity a priori, in the range of $3 \times 10^{-7}$ M up to $1 \times 10^{-3}$ M. The compound N,N′-dicyclohexylthiourea (C) showed a slightly positive response regardless of the type of assay and is therefore regarded as a false positive. The non-genotoxic compound curcumin is a know Nrf2 pathway activator.

**Table 1**
Compounds that are known to be genotoxic in vivo that are expected to be positive in the genotoxicity screen. All concentrations are expressed as mol/l (M) in the assay medium.

| Chemical | CAS | Metabolic activation | Solvent | [Max] (M) | p53 | LOEC-p53 | p53+S9 | LOEC-p53+S9 | Nrf2 | LOEC-Nrf2 |
|---|---|---|---|---|---|---|---|---|---|---|
| 2,4-Diaminotoluene | 95-80-7 | Yes | DMSO | 1.0E−03 | − | N | + | 1.0E−03 | − | N |
| 2-Acetylaminofluorene | 53-96-3 | CYP1A2 | DMSO | 1.0E−03 | + | 1.0E−05 | + | 1.0E−05 | − | N |
| 7,12-Dimethylbenzanthracene | 57-97-6 | CYP1B1 | DMSO | 1.0E−03 | − | N | + | 3.0E−06 | + | 1.0E−05 |
| Aflatoxin B1 | 1162-65-8 | | DMSO | 1.0E−03 | + | 1.0E−04 | + | 3.0E−06 | − | N |
| Azidothymidine | 30516-87-1 | | DMSO | 1.0E−03 | − | N | − | N | − | N |
| Benzo[a]pyrene | 50-32-8 | CYP1A1, 1B1 | DMSO | 1.0E−03 | − | N | + | 1.0E−05 | − | N |
| Cadmium chloride | 10108-64-2 | | Water | 1.0E−03 | − | N | − | N | + | 3.0E−06 |
| Chloramphenicol | 56-75-7 | | DMSO | 1.0E−03 | − | N | + | 1.0E−03 | − | N |
| Cisplatin | 15663-27-1 | | DMSO | 1.0E−03 | + | 3.0E−04 | + | 1.0E−05 | + | 3.0E−05 |
| Cyclophosphamide | 6055-19-2 | CYP2B | DMSO | 1.0E−03 | − | N | + | 1.0E−03 | − | N |
| Dimethylnitrosamine | 62-75-9 | CYP2E1 | DMSO | 1.0E−03 | − | N | + | 1.0E−03 | − | N |
| ENU | 759-73-9 | | DMSO | 1.0E−03 | + | 1.0E−03 | − | N | − | N |
| Etoposide | 33419-42-0 | | DMSO | 1.0E−03 | + | 1.0E−05 | + | 1.0E−04 | − | N |
| Hydroquinone | 123-31-9 | | DMSO | 1.0E−03 | + | 1.0E−04 | − | N | + | 1.0E−06 |
| IQ (2-amino-3methylimidazo[4,5-f]quinoline) | 76180-96-6 | Yes | DMSO | 1.0E−04 | + | 3.0E−05 | + | 3.0E−05 | − | N |
| Methyl methanosulfonate | 66-27-3 | | DMSO | 1.0E−03 | + | 3.0E−04 | − | N | + | 3.0E−05 |
| p-Chloroaniline | 106-47-8 | | DMSO | 1.0E−03 | − | N | + | 1.0E−03 | − | N |
| PhIP.HCl (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) | 105650-23-5 | | DMSO | 1.0E−03 | + | 3.0E−04 | + | 3.0E−05 | − | N |
| Sodium arsenite | 7784-46-5 | | Water | 3.0E−06 | + | 1.0E−7 | + | 3.0E−07 | + | <1.0E−08 |
| Taxol | 33069-62-4 | | DMSO | 1.0E−03 | + | <3.0E−07 | + | <3.0E−07 | − | <3.0E−07 |

[Max] is the maximum concentration (in the medium) tested in the assay. LOEC-p53 is the lowest observed positive concentration in the p53 CALUX bioassay (IF > 1.5). LOEC-p53+S9 is the lowest observed positive concentration in the p53 CALUX bioassay with the addition of the metabolizing system S9 (IF > 2). LOEC-Nrf2 is the lowest observed positive concentration in the Nrf2 CALUX (IF > 1.5). N denotes a negative response in the respective assay.

use of a metabolizing system. By including S9 to generate active metabolites, more genotoxic compounds are detected. However, due to the higher number of false positives, the sensitivity slightly decreases to 82%, and the specificity to 90%.

### 3.4. Detection of oxidative stress with the Nrf2 CALUX assay

To gain insight into the mechanism behind the genotoxic activity of single chemicals, all compounds were also tested in the Nrf2-based luciferase-reporter assay. This assay responds to the activation of the Nrf2 pathway, which activates detoxifying enzymes and antioxidant stress proteins [22]. Two examples of dose-response curves in the Nrf2 CALUX assay are given in Fig. 4. Of the 20 genotoxins, seven were detected as positive in this assay (Table 1) of which two examples are shown in Fig. 4. Of the non-genotoxic compound, only fluometron produced (inconsistently) a positive response (see Table 2), which just exceeded the cut-off value of 1.5-fold induction. Of the compounds that frequently lead to false-positive results, six of the 19 compounds were detected as positive for oxidative stress (see Table 3). Four of these six compounds produced strong responses in the Nrf2 CALUX assay, and were also found positive in the p53 CALUX assay (after addition of S9). The two remaining compounds (2,4-dichlorophenol and *p*-nitrophenol) gave no response in the p53 CALUX assay, but produced a response in the Nrf2 CALUX assay near the cut-off value for that test.

## 4. Discussion

In the present study, two reporter-gene assays and a control assay based on the human U2OS cell line are presented that allow fast screening of compounds for p53 and Nrf2 activation, which are indicators of genotoxicity and oxidative stress, respectively. In all assays a luciferase signal is used as a read-out, which has some clear advantages over the use of fluorescence as it is more sensitive, avoids false-positive signals from autofluorescence of certain chemicals (e.g., curcumin [37]) or from the use of S9 mixture (which contains autofluorescent components [30]). The utilized U2OS cell line is p53-proficient, is easy to maintain in culture and has a short doubling time. Moreover, the assays can be readily incorporated into a more extensive panel of existing U2OS-based reporter-gene assays, providing a reliable way to screen chemicals for their ability to activate multiple pathways of interest and contributing to chemical risk assessment based on toxicity pathways [2,4]. The resulting assay systems can be cultured for several months, making the assays ideal for high-throughput activity-profiling of compounds. Although this study still used the 96-well plate format, the high level of relative light units that they produce makes these systems ideal for automated use in a format with even higher throughput such as 384-well plates (manuscript in preparation).

A separate cell line, the Cytotox CALUX, was developed with the same cell line-construct combination, utilizing luciferase that is expressed constitutively. This assay served as a control for cytotoxicity, which is mainly observed for compounds that are either genotoxic or known to be false (or misleading) positives as classified by Kirkland et al. [25]. Therefore, cytotoxicity probably occurred in most cases as a result of prolonged exposure to high concentrations of genotoxicants, ultimately resulting in apoptosis. Of the non-genotoxic compounds tested, only phenanthrene and hexachloroethane led to cytotoxicity, both showing precipitation at the highest test concentrations. The genotoxic response in the p53 CALUX assay clearly precedes the cytotoxic response in the Cytotox CALUX test, as can be seen, e.g., in Fig. 2.

The Cytotox CALUX assay also served as a control for non-specific expression or inhibition of the luciferase gene. It serves

**Table 2**
Compounds that non-genotoxic (non)-carcinogens that should be negative in the genotoxicity screen. All concentrations are expressed as mol/l (M) in the assay medium.

| Chemical | CAS | Solvent | [Max] | p53 | LEC-p53 | p53+S9 | LEC-p53+S9 | Nrf2 | LEC-Nrf2 |
|---|---|---|---|---|---|---|---|---|---|
| (2-Chloroethyl)trimethyl-ammonium chloride | 999-81-5 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Amitrole | 61-82-5 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Ampicillin trihydrate | 7177-48-2 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Cyclohexanone | 108-94-1 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Di-(2-ethylhexyl)phthalate | 117-81-7 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Diethanolamine | 111-42-2 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| D-Limonene | 5989-27-5 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| D-Mannitol | 69-65-8 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Erythromycin stearate | 643-22-1 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Fluometron | 2164-17-2 | DMSO | 1.0E−03 | – | N | – | N | + | 3.0E−04 |
| Hexachloroethane | 67-72-1 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Melamine | 108-78-1 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Methyl carbamate | 598-55-0 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| *N*,*N*-Dicyclohexyl thiourea | 1212-29-9 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| n-Butyl chloride | 109-69-3 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Phenanthrene | 85-01-8 | DMSO | 1.0E−03 | + | 1.0E−04 | – | N | – | N |
| Phenformin HCl | 834-28-6 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Progesterone | 57-83-0 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Pyridine | 110-86-1 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| *Tert*-Butyl alcohol | 75-65-0 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Tris(2-ethylhexyl)phosphate | 78-42-2 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Trisodium EDTA trihydrate | 150-38-9 | Water | 1.0E−03 | – | N | – | N | – | N |

[Max] is the maximum concentration (in the medium) tested in the assay. LOEC-p53 is the lowest observed positive concentration in the p53 CALUX bioassay (IF > 1.5). LOEC-p53+S9 is the lowest observed positive concentration in the p53 CALUX bioassay with the addition of the metabolizing system S9 (IF > 2). LOEC-Nrf2 is the lowest observed positive concentration in the Nrf2 CALUX (IF > 1.5). N denotes a negative response in the respective assay.

**Table 3**
Compounds that are known to give positive results in *in vitro* genotoxicity screen but not in vivo and/or are believed to act via non-relevant mechanisms and are thus regarded a non-genotoxic compounds. These compounds are therefore expected to score negative in the genotoxicity screen. All concentrations are expressed as mol/l (M) in the assay medium.

| Chemical | CAS | Solvent | [Max] | p53 | LEC-p53 | p53+S9 | LEC-p53+S9 | Nrf2 | LEC-Nrf2 |
|---|---|---|---|---|---|---|---|---|---|
| 1,3-Dihydroxybenzene (resorcinol) | 108-46-3 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| 2,4-Dichlorophenol | 120-83-2 | DMSO | 1.0E−03 | – | N | – | N | + | 3.0E−05 |
| 2-Ethyl-1,3-hexanediol | 94-96-2 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Benzyl alcohol | 100-51-6 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Curcumin | 458-37-7 | DMSO | 1.0E−03 | – | N | – | N | + | 3.0E−06 |
| D,L-Menthol | 15356-70-4 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Ethionamide | 536-33-4 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Ethyl acrylate | 140-88-5 | DMSO | 1.0E−03 | – | N | + | 1.0E−05 | + | 3.0E−04 |
| Eugenol | 97-53-0 | DMSO | 1.0E−03 | – | N | + | 1.0E−04 | – | N |
| Isobutyraldehyde | 78-84-2 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| o-Anthranilic acid | 118-92-3 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Phthalic anhydride | 85-44-9 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| p-Nitrophenol | 100-02-7 | DMSO | 1.0E−03 | – | N | – | N | + | 1.0E−03 |
| Propyl gallate | 121-79-9 | DMSO | 1.0E−03 | + | 3.0E−05 | – | N | + | 3.0E−06 |
| Sodium saccharin | 128-44-9 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Sodium xylene sulfonate | 1300-72-7 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| Sulfisoxazole | 127-69-5 | DMSO | 1.0E−03 | – | N | – | N | – | N |
| *Tert*-butylhydroquinone | 1948-33-0 | DMSO | 1.0E−03 | – | N | + | 3.0E−05 | + | 1.0E−06 |
| Urea | 57-13-6 | DMSO | 1.0E−03 | – | N | – | N | – | N |

[Max] is the maximum concentration (in the medium) tested in the assay. LOEC-p53 is the lowest observed positive concentration in the p53 CALUX bioassay (IF > 1.5). LOEC-p53+S9 is the lowest observed positive concentration in the p53 CALUX bioassay with the addition of the metabolizing system S9 (IF > 2). LOEC-Nrf2 is the lowest observed positive concentration in the Nrf2 CALUX (IF > 1.5). N denotes a negative response in the respective assay.

**Table 4**
The sensitivity, specificity and predictivity of the p53 CALUX (with and without S9) and the Nrf2 CALUX based on the compounds recommended by ECVAM. Compound in category 1 should test positive (positive *in vivo*), compounds in category 2 should test negative (negative *in vivo* or positive due to non-genotoxic mechanism) and compounds in category 3 should test negative (as they are negative *in vivo* but are known to produce positive results in *in vitro* genotoxicity tests).

| | p53 CALUX (−S9) | p53 CALUX(+S9) | p53 CALUX (+/−S9) |
|---|---|---|---|
| Genotoxicant that test positive (cat. 1) | 11 | 15 | 18 |
| Genotoxicant that test negative (cat. 1) | 9 | 5 | 2 |
| Non-genotoxicant that test positive (cat. 2 + 3) | 1 + 1 | 0 + 2 | 1 + 3 |
| Non-genotoxicant that test negative (cat. 2 + 3) | 21 + 18 | 22 + 17 | 21 + 16 |
| Sensitivity (%) | 85 | 88 | 82 |
| Specificity (%) | 95 | 95 | 90 |
| Positive predictive value | 85 | 88 | 82 |
| Negative predictive value | 81 | 89 | 95 |

**Table 5**
The sensitivity, specificity and predictivity of the p53 CALUX (with and without S9) based on the compounds recommended by ECVAM compared to alternative reporter gene assays. Compound in category 1 should test positive (positive *in vivo*), compounds in category 2 should test negative (negative *in vivo* or positive due to non-genotoxic mechanism) and compounds in category 3 should test negative (as they are negative *in vivo* but are known to produce positive results in *in vitro* genotoxicity tests).

| | p53 CALUX (+/−S9) | HepG2-p53[a] (−S9) | GreenScreen-Luc[b] (+/−S9) | GreenScreen-GFP[c] (+/−S9) | p53R2-Luc[d] (+/−S9) |
|---|---|---|---|---|---|
| Genotoxicant that test positive (cat. 1) | 18 | 17 | 16 | 18 | 17 |
| Genotoxicant that test negative (cat. 1) | 2 | 3 | 4 | 2 | 3 |
| Non-genotoxicant that test positive (cat. 2 + 3) | 1 + 3 | 2 + 1 | 0 + 4 | 0 + 5 | 1 + 2 |
| Non-genotoxicant that test negative (cat. 2 + 3) | 21 + 15 | 20 + 18 | 22 + 15 | 22 + 14 | 21 + 17 |
| Sensitivity (%) | 82 | 85 | 76 | 75 | 85 |
| Specificity (%) | 90 | 93 | 88 | 85 | 93 |
| Positive predictive value | 82 | 85 | 76 | 75 | 85 |
| Negative predictive value | 95 | 93 | 90 | 95 | 93 |

[a] Westerink et al. [21].
[b] Hughes et al. [32].
[c] Birrell et al. [37].
[d] Mizota et al. [7].

as a control for the entire existing panel of CALUX assays based on the use of the U2OS cell line. Several compounds are known to non-specifically influence the activity of luciferase [23,31], and the novel Cytotox CALUX reporter assay helps avoiding misinterpretation of such results. Within the set of 61 compounds tested in this study, non-specific inhibition of luciferase was not observed. However, some compounds, e.g., 7,12-dimethylbenzanthracene (Fig. 1d) and *N*,*N*-dicyclohexylthiourea (Fig. 5c), did appear to elicit a slight, non-specific increase in luciferase activity. Although chemicals might enhance the luciferase response due to increased cell proliferation, the increase in luciferase activity could not be contributed to this, as was assessed by means of the MTT test (see Fig. 1d). Thus, while exceeding the threshold limit, all these compounds should be regarded as giving no response in the p53 and Nrf2 CALUX assays, which corresponds with their known mode of action. Benzo[*a*]pyrene and 7,12-dimethylbenzanthracene are known genotoxins that require metabolic activation [25] and indeed, both agents show a clear increase in response in the p53 CALUX assay after addition of S9 (fold induction > 10 IF), thus classifying both compounds as genotoxins that require metabolic activation. The third compound, *N*,*N*-dicyclohexyl thiourea, displayed the same activity (up to 1.7 IF) under all conditions. Therefore, it is considered as negative in all assays, which is consistent with the results from tests utilizing HepG2 [21] and TK6 cells [32].

The p53 CALUX assay detected 11 of 20 (55%) genotoxic compounds as positive without the addition of S9, including most of the Ames-positive compounds that do require metabolic activation. Of the Ames-negative compounds, only azidothymidine is not detected as being genotoxic in p53 CALUX assay, neither with nor without the addition of S9. To exert its genotoxic effect, azidothymidine needs to be incorporated in the DNA, which is dependent on both time and concentration [33] and does not require metabolic activation. However, longer exposure times and higher concentrations of azidothymidine do not elicit a positive response in the p53 CALUX test (results not shown).

Metabolic activation is of importance for the bioactivation of genotoxic compounds [25]. As U2OS cells do not show detectable levels of cytochrome P450 enzymes that are important for the bioactivation of genotoxins, e.g., CYP1A1 and CYP1B1 ([34,35]; our unpublished data), addition of S9 can be used as a surrogate. Although the use of S9 is not a perfect representation of in vivo metabolic activity, is does provide the most important metabolic steps. The p53 CALUX assay in combination with a metabolizing system (in the form of S9) correctly detected 18 of the 20 (90%) genotoxic compounds, including most agents that are known to require metabolic activation, e.g., cyclophosphamide. Some of these compounds are also active in the assay without S9, e.g., PhIP.HCl, but then higher concentrations are needed to obtain a response. Although mammalian (reporter) cell lines exist that allow some of the relevant metabolic activation steps [21], no cell line or other methods are currently available that can provide a quantitatively and qualitatively accurate representation of human in vivo enzyme levels.

Although the addition of S9 enables the detection of genotoxic compounds that require metabolic activation, the addition of S9 to the p53 CALUX assay results in more false or misleading positives, all coming from the set of 19 compounds that are frequently identified "incorrectly" as genotoxic compounds (Table 3). According to the classification recommended by Kirkland et al. [25], which is based on in vivo rodent data, these compounds should not be detected as being genotoxic, although there is still some debate about the correct classification of some of these compounds [21,36]. Regardless of the classification, there is extensive overlap between results from similar assays based on the use of human cell lines, such as the GreenScreen-GFP [37], BlueScreen HC [32], and HepG2-p53 [21], with compounds like *t*-butylhydroquinone and propyl gallate that produce consistently positive results in the different assays. Apparently, these compounds do induce different steps in the DNA damage-signalling pathways and induce chromosomal aberrations at the cellular level at the concentrations tested. However, the concentrations tested in vitro might not reflect the concentrations that are achieved in vivo and the metabolic activation steps might be under- or over-estimated by the S9 used. The positive response of, e.g., *t*-butylhydroquinone seems to be due to the high level of oxidative stress, which can be seen from the strong Nrf2 response. Indeed, *t*-butylhydroquinone is a known anti-oxidant, and many anti-oxidants can promote the generation of reactive oxygen species at elevated concentrations [36]. Of the remaining chemicals with misleading positive results, 2,4-dichlorphenol came out positive after the addition of S9. As this compound is also positive in the recently introduced BlueScreen HC assay [32] but not in the HepG2-p53 test [21], possibly the key metabolite for the response is not produced in HepG2 cells.

While the activity in the p53 CALUX reporter assay is indicative of genotoxicity of a compound, activity in the Nrf2 reporter assay provides insight into the mechanism behind the genotoxic activity. Compounds that show a strong activity in both the Nrf2 CALUX assay and the p53 CALUX test are known to act via a mechanism that involves oxidative stress (Table 4). Well-known examples are sodium arsenite and cadmium chloride, which would be classified as genotoxic compounds that exert their effect via an

oxidative-stress mechanism. Other compounds like MMS would also be classified as compounds that cause oxidative stress, which indeed may contribute in part to their genotoxic stress [38]. Test compounds that are known to cause oxidative stress all elicit a response much higher than just the statistical threshold of 1.5-fold induction: all reach an IF of 4 or higher. The lowest concentrations at which these compounds elicit there effect in the Nrf2 CALUX assay is generally lower than in the p53 CALUX test, indicating that indeed part of the positive response in the p53 CALUX assay might be explained as an indirect effect via oxidative stress.

Recently, other reporter-gene assays have been described that focus on pathways relevant to genotoxic stress. These all utilize different mammalian cell lines and are aimed at determining the transcriptional activity of specific genes associated with genotoxic stress [6,7,21,39]. Various studies have shown that in principle all these tests are sensitive and selective, and can identify direct-acting genotoxic compounds as well as chemicals that elicit oxidative stress (see Table 5). Several of these assays utilize relatively large promoter regions from genes that are responsive at least (but not only) to p53 and Nrf2 activation, like Gadd45a [6] and p53R2 [7]. As such, most assays do not allow differentiating between these two classes of compounds. Also the HepG2-reporter assays do not allow differentiating between both classes of compounds, which is possibly due to the continuous metabolic activity of the cell. The assays presented by Hendriks et al. [39] with mouse ES cells appear to be able to differentiate between genotoxic and oxidative stress. However, these assays still rely on large gene promoters, which makes it uncertain whether these genes respond to genotoxic processes only. Additionally, the choice of DsRed as a read-out makes it more difficult to use these assays in a high-throughput format, and it may limit its use when testing auto-fluorescent compounds.

All the alternative assays described above are solely aimed at detecting genotoxic compounds. To obtain more information regarding the mode(s) of action of chemicals, more than one assay is needed. When several tests are used in parallel, each covering a different pathway or endpoint, assays can discriminate between compounds that are genotoxic, directly or via oxidative stress. Including additional tests may allow identification of compounds that are genotoxic, and those that trigger other pathways relevant for carcinogenicity. For example, several compounds on the ECVAM list are described to elicit carcinogenic effects via nuclear-receptor pathways [25] and many estrogens are carcinogens in humans and rodents, which might be related to both their genotoxic potential as well as their estrogenicity [40,41]. However, additional assays will generally rely on different combinations of cell lines, reporter genes, constructs and readout. Therefore, it is important to realize that combining the results of assays that all rely on different constructs, cell lines and read-outs will not only provide extra information regarding the mode(s) of action of compounds, but will also add extra uncertainty. Small changes, like changing the read-out from fluorescence to luciferase while keeping the same construct and cell line [32], or changing the cell type while keeping the same construct [7], can already significantly change some of the outcomes of a test, leading to different classifications regarding the genotoxic potential of test chemicals. By keeping the number of cell lines and constructs to a minimum, we believe that false-negative and false-positive responses can be defined more clearly, avoiding incorrect classification of the mode of action(s) of a compound.

The cell lines described in this paper belong to a larger panel of bioassays that respond to specific modes of action, including nuclear hormone receptors and other signalling pathways that are related to adverse effects. Some of these assays focus on pathways that are linked to the mode of action of non-genotoxic carcinogens like those induced by e.g., the oestrogen receptor, progesterone receptor [12] and PPAR receptors [42]. All these assays are based on the same cell line (U2OS), with a reporter containing a minimal promoter linked to luciferase, containing a minimal promoter (TATA box). By using only defined responsive elements, the cell lines specifically respond only to the pathway of interest, and interpretation of results becomes much more straightforward than with tests that utilize complex promoters. In addition, by building a panel with the same cell line-construct combination the risk of finding false-positives through non-specific effects are minimized, even if those effects appear to behave in a dose-responsive manner.

In conclusion, we have extended our panel of U2OS cell lines with three new assays. Two of these are aimed at detecting activation of pathways relevant for genotoxic activity: the p53 CALUX assay (with and without metabolic activation) is aimed at detecting direct-acting genotoxic compounds, while the Nrf2 CALUX test detects compounds that elicit oxidative stress (which can lead indirectly to genotoxic stress). The third cell line, the Cytotox CALUX, is used as a control for cytotoxicity and for luciferase interference. All three cell lines currently undergo validation in different laboratories and can be used in a high-throughput screening setting with 96-well or higher plate formats. As the constructs used in these cell lines consist of a minimal promoter combined with specific responsive elements only, these cell lines are highly sensitive and specific in their response.

This panel of human reporter cell lines can be used for high-throughput screening to determine genotoxicity of compounds along with providing valuable insight into their toxic mechanism(s).

## Conflict of interest statement

Sander C. van der Linden, Barbara M.A. van Vught-Lussenburg, and Lydia R.A. Jonker are employed by Biodetection Systems BV, the company which develops and sells CALUX© bioassays. Bart van der Burg is scientific director of Biodetection Systems BV, the company which develops and sells CALUX© bioassays. Anne R.M. von Bergh, Marc Teunis, Cyrille A.M. Krul have no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mrgentox.2013.09.009.

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