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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–3×10^{-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