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Upgrading cytochrome P450 activity in HepG2 cells co-transfected with adenoviral vectors for drug hepatotoxicity assessment

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ABSTRACT

In a number of adverse drug reactions leading to hepatotoxicity, drug metabolism is thought to be involved by the generation of reactive metabolites from non-toxic drugs. The use of hepatoma cell lines, such as HepG2 cell line, for the evaluation of drug-induced hepatotoxicity is hampered by their low cytochrome P450 expression which makes impossible the study of the toxicity produced by bioactivable compounds. Genetically manipulated cells constitute promising tools for hepatotoxicity applications. HepG2 cells were simultaneously transfected with recombinant adenoviruses encoding CYP1A2, CYP2C9 and CYP3A4 to confer them drug-metabolic competence. Upgraded cells (Adv-HepG2) were highly able to metabolize the toxin studied in contrast to the reduced metabolic capacity of HepG2 cells. Aflatoxin B1-induced hepatotoxicity was studied as a proof of concept in metabolically competent and non-competent HepG2 cells by using high content screening technology. Significant differences in mitochondrial membrane potential, intracellular calcium concentration, nuclear morphology and cell viability after treatment with aflatoxin B1 were observed in Adv-HepG2 when compared to HepG2 cells. Rotenone (non bioactivable) and citrate (non hepatotoxic) were analysed as negative controls. This cell model showed to be a suitable hepatic model to test hepatotoxicity of bioactivable drugs and constitutes a valuable alternative for hepatotoxicity testing.

1. Introduction

Biotransformation reactions are aimed at facilitating the elimination of lipophilic xenobiotics from cells. However, drug-induced hepatotoxicity is often caused by cytochrome P450-dependent activation of the drugs into reactive metabolites in the course of Phase I reactions (Gomez-Lechon et al., 2010a; Tang and Lu, 2010; Zhou et al., 2005). Therefore, the toxicity of a compound may be caused not only by the parent molecule but also by its metabolites. The occurrence of this phenomenon cannot be assessed in the cell lines currently used for basal cytotoxicity screening, as they lack biotransforming enzymes (Donato et al., 2008). This explains the differences in the intrinsic *in vitro* toxicity observed for certain compounds when assessed with metabolically competent vs. non-competent cells and evidences the need of appropriate *in vitro* models to detect bioactivable hepatotoxin.

Recombinant adenoviral vectors can be used to deliver specific genes into cultured cells with very high efficiencies (Aoyama et al., 2009; Castell et al., 1997) and provide a versatile system for gene transfer to a broad spectrum of cell types, independently of cell division activity. This technology has facilitated the generation of transient metabolically competent hepatic cells after transduction with recombinant-defective adenoviral vectors encoding for cytochrome P450 (CYP) genes involved in foreign compound metabolism (Bai and Cederbaum, 2004; Castell et al., 1997; Donato et al., 2010; Hosomi et al., 2010).

In this study, we developed upgraded HepG2 cells transiently expressing functional levels of three major enzymes (CYP1A2, CYP2C9 and CYP3A4) responsible for oxidative metabolism of drugs in human liver. To this end, HepG2 cells were simultaneously transfected with a mixture of recombinant CYP adenoviruses (Adv-HepG2). HepG2 cells were selected for this purpose because of their human liver origin, wide use for hepatotoxicity studies and

Abbreviations: BSO, L-buthionine *S,R*-sulphoximine; CYP, cytochrome P450; EDTA, ethylenediaminetetraacetic acid; HCS, high content screening; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Adv-HepG2, upgraded HepG2 cells co-transfected with adenoviral vectors; TMRM, tetramethyl rhodamine methyl ester.

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appropriate levels of elements required to support CYP activities (i.e. NADPH-cytochrome P-450 reductase, cytochrome b5) (Gonzalez and Korzekwa, 1995; Rodriguez-Antona et al., 2002). Cotransfected Adv-HepG2 cells expressed high levels of CYP1A2, CYP2C9 and CYP3A4 activities and were proposed as a new *in vitro* model able to identify bioactivable hepatotoxins.

Our screening strategy is based on the analysis of multiple markers of toxicity in a 96-well plate format using high content screening (HCS) technology. HCS is a recent advance in the automation of quantitative epifluorescence microscopy and image analysis, and in the application of multiprobe technology. By combining the use of sensitive, scarcely invasive, fluorescence-based multiparametric methods and the event-integrating concept of individual cells, HCS has been demonstrated to be a promising and valuable tool for the mechanistic understanding of drug-induced hepatotoxicity (Noor et al., 2009; O'Brien et al., 2006). In contrast to conventional *in vitro* toxicity assays that measure a single parameter indicative of cell injury, multiparametric approaches cover simultaneously a wide spectrum of mechanistic effects, which can notably increase its sensitivity.

Aflatoxin B1 was selected as a model compound to be used as a proof of concept of metabolism-induced hepatotoxicity in metabolically competent (Adv-HepG2) vs. non-competent (HepG2) cells. Aflatoxin B1 is a potent hepatotoxic and genotoxic compound, produced by the genus Aspergillus, which is known to contaminate agricultural products, such as cereal grains (Bedard and Massey, 2006). Biotransformation by P450 enzymes is crucial for its toxicity (Bedard and Massey, 2006; Kirby et al., 1996; Mace et al., 1997) and particularly, CYP1A2 and CYP3A4 appear to be the most important isoforms for aflatoxin B1 activation in human liver (Forrester et al., 1990; Mace et al., 1997). In addition, rotenone (a non-bioactivable toxic chemical) and citrate (a non-cytotoxic compound) were included in the study. Our results showed Adv-HepG2 cells as a suitable hepatic model to test hepatotoxicity by bioactivable drugs which could constitute a valuable approach for hepatotoxicity testing.

2. Materials and methods

2.1. Construction of recombinant adenoviruses

CYP1A2, CYP2C9 and CYP3A4 cDNA were obtained and purified as described (Donato et al., 2010). Purified CYP cDNAs were double digested with the appropriate restriction enzymes and ligated into the adenoviral pAC/CMVpLpA plasmid (Gomez-Foix et al., 1992). Each recombinant plasmid pAC/CMVpLpA containing CYP cDNA (transfer vector) was cotransfected with pJM17 into 293 cells by calcium phosphate/DNA co-precipitation to obtain a recombined adenovirus expressing each CYP (Becker et al., 1994). The resulting viruses were plaque-purified, expanded into a high-concentration stock and titrated by plaque assay as previously described in detail (Donato et al., 2010).

2.2. Culture of HepG2 cells and adenovirus infection

HepG2 cells were cultured in Ham's F-12/Leibovitz L-15 (1:1 v/v) supplemented with 7% newborn calf serum, 50 U penicillin/ml and 50 μg streptomycin/ml. For subculturing purposes, cells were detached by treatment with 0.25% trypsin/0.02% EDTA at 37 °C. For adenovirus infection studies, cells were seeded in 96-well plates (8000 cells/well) and 48 h later adenovirus-containing medium was added. To optimize the expression of functional CYP enzymes in Adv-HepG2 cells, different doses (MOI, multiplicity of infection defined as plaque formation units per cell) of each adenovirus were tested. After 24 h, cells were shifted to adenovirus-free medium and cultured for additional 24 h prior to incubations with test chemicals.

2.3. Drug-metabolism assessment

CYP activities were evaluated after 48 h of cell infection with adenoviral vectors by the incubation with a cocktail of selective substrates for each CYP enzyme: 10 μM phenacetin (CYP1A2), 10 μM diclofenac (CYP2C9), and 5 μM midazolam (CYP3A4). After the hydrolysis of the potential conjugates by incubation with β-glucuronidase and arylsulfatase (Donato et al., 1993), acetaminophen (CYP1A2), 4′-hydroxydiclofenac (CYP2C9) and 1′-hydroxymidazolam (CYP3A4) formed and released into culture medium were quantified by high-performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS), as previously described in detail (Lahoz et al., 2007, 2008).

2.4. MTT assay

Cytotoxicity induced by the adenoviruses was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. After the treatments with a mixture of the recombinant adenoviruses CYP1A2, CYP2C9 and CYP3A4, cell monolayers were washed with PBS and 100 μL/well of MTT reagent (5 mg/mL in medium) were added. After 2 h in a cell incubator at 37 °C, culture supernatants were discarded, the dye was extracted with DMSO and optical density was read at 540 nm on a microplate reader (Versamax, Molecular Devices). The percentage of inhibition of the succinic dehydrogenase reduction of MTT was calculated in relation to control cells in each experimental series (control cells were assumed to have 100% viability).

2.5. Incubation of cells with model hepatotoxins

In order to minimize the effect of glutathione (GSH), HepG2 cells were treated with 100 μM L-buthionine *S,R*-sulphoximine (BSO, Sigma–Aldrich), a potent GSH depletion agent, 24 h before the incubation with the test chemicals. Then, cells were exposed for the following 24 h to eight concentrations of the bioactivable hepatotoxin aflatoxin B1 (positive control). Rotenone and citrate were chosen as non-bioactivable and non-hepatotoxic compounds, respectively (negative controls).

2.6. High content screening assay: incubation of fluorescent probes, imaging and analysis

Following treatment, cells were simultaneously loaded with 1.5 μg/ml Hoechst 33342 (Sigma Aldrich), 1.5 μg/ml of propidium iodide (Sigma Aldrich), 75 ng/ml tetramethyl rhodamine methyl ester (TMRM, Molecular Probes) and 0.27 μg/ml Fluo-4 AM (Molecular Probes). After 30 min of incubation at 37 °C with culture media containing fluorescent probes, cells were imaged using Scan^R system (Olympus, Germany).

In order to acquire enough cells (>1000) for analysis, nine fields per well were imaged. The 10× objective was used to collect images for the distinct fluorescence channels. Dyes were excited and their fluorescence monitored at excitation and emission wavelengths with an appropriate filter set and then analyzed by using Scan^R analysis module which allows simultaneous quantification of subcellular inclusions that are stained by different fluorescent probes and measures fluorescence intensity associated with predefined nuclear and cytoplasmic compartments. Cell count was generated from the number of Hoechst 33342 stained nuclei. This probe also allowed the detection of condensed pycnotic nuclei in apoptotic cells. Cell viability was determined by propidium iodide exclusion. Since propidium iodide is not permeant to live cells, it is