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Assessment of an automated *in vitro* basal cytotoxicity test system based on metabolically-competent cells

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ABSTRACT

When *in vitro* test systems are evaluated for assessment of the toxicity of chemical compounds, particular efforts are made to mimic the *in vivo* reality as close as possible. Cellular models with appropriate metabolic competence, i.e. with the potency to biotransform chemical compounds, are considered crucial since some metabolites have a different toxicity than their parent compounds. In this study a cell based *in vitro* test system is proposed to investigate the basal cytotoxicity of several reference chemicals. Both metabolic competent HepaRG cells and cells with no or low hepatic enzyme activity (undifferentiated HepaRG and proliferating HepG2) were used. The classic Neutral Red Uptake (NRU) assay proved to be robust and reliable to be applied as viability assay. The test was performed on a robotic platform, which enabled fully automated and simultaneous screening of the compounds. The outcome of these tests grouped the tested compounds in three categories following their detoxification effect (benzo(a)pyrene, valproic acid), their bio-activation effect (aflatoxin B1) and their specific effect on inhibition of cell proliferation (cycloheximide, sodium lauryl sulphate, atropine sulphate monohydrate, acetylsalicylic acid).

1. Introduction

Recent European legislation calls for toxicity assessment of chemicals (REACH) as well as the development and implementation of alternative methods to animal testing (EU, 2003). *In vitro* test systems based on cellular models are good alternatives for animal based tests and can contribute to the hazard assessment of substances that are produced by cosmetic, pharmaceutical, chemical or food industry (IEH, 2001; Hofer et al., 2004). In recent years, several cell-based *in vitro* assays have been developed for application to toxicity testing studies (Hewitt et al., 2001; ICCVAM, 2006a,b). A major drawback of most of these assays is that they are based on cellular models that do not express the active metabolizing enzymes that play an important role in the toxicity of chemicals (Coon et al., 1992; Coecke et al., 2006). To improve the toxicity determination of compounds that are bio-transformed, assays that lack metabolic competence should be adapted. The assessment of the intrinsic cytotoxicity of chemical entities that do not form the substrate of metabolism should however be independent from the metabolic capacity of the cell model used.

Since the liver is the main target organ in systemic toxicity (Davila et al., 1998) and since it plays a major role in the metabolism of many compounds, the hepatic cell models are among the most used in *in vitro* toxicity experiments (Maurel et al., 2010). Primary human hepatocytes are considered the gold-standard when presence of metabolic activity is required (Guillouzo et al., 1993; LeCluyse, 2001). Beside the use of fresh isolated human hepatocytes, use of primary cryo-preserved hepatocytes is gaining increasingly attention (Hengstler et al., 2000; McGinnity et al., 2004). Nevertheless, the use of primary hepatocytes remains limited, since these cells are scarce, have a low proliferative capacity and their metabolic activity is limited to a very short time periods (Abdelrazzak et al., 1993).

Currently one of the most used human hepatic cell model is the HepG2 cell line that was isolated from a human hepatocellular carcinoma (Chiu et al., 1990). Although some studies attribute sufficient metabolic activities to these cells (Schoonen et al., 2005a,b), they are in general considered to lack substantial liver-specific functions and hepatic enzyme activity (Castell et al., 2006). The human origin of this hepatic cell line and its high rate of cellular proliferation, which represents a sensitive end-point for cytotoxicity potential, still make the HepG2 cells a relevant cell line in basal toxicity testing.

Abbreviations: NRU, neutral red uptake; HTS, high throughput screening; CYP, cytochrome P450; OD, optical density.

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Another cell line isolated from a patient suffering from hepatocarcinoma is the HepaRG. In their undifferentiated stage these cells are highly proliferative and do not express a hepatic phenotype (Gripon et al., 2002). However, after differentiation HepaRG cells express most of the hepatic cell markers as well as the enzymatic machinery necessary for biotransformation at levels comparable to fresh isolated human hepatocytes, which is maintained for several weeks (Aninat et al., 2006). Due to these characteristics, HepaRG cells are gaining increasing attention in drug metabolism, hepatotoxicity, genotoxicity and enzyme induction studies (Guillouzo et al., 2007; Kanebratt and Andersson, 2008). On the contrary, undifferentiated HepaRG cells are not widely used. In this study we also use undifferentiated HepaRG at a differentiation stage at which the cells are confluent, but have no, or very limited, proliferative capabilities. This cell model is very similar to differentiated HepaRG, but does not express active cytochrom P450 enzymes. Besides their genomic equality, both undifferentiated and differentiated HepaRG cells should have equivalent sensitivity to toxicants and to the endpoint detection method. The comparison between differentiated and undifferentiated HepaRG has been documented before (Hart et al., 2010). However in these studies the undifferentiated HepaRG were highly proliferative growing at sub-confluence levels, thus a stage previous to the stage we use in our study.

In this study an *in vitro* test system that can be applied as a template for the assessment of basal cytoxicity is presented. The approach is based on the determination of the cytotoxicity of a set of reference compounds in three different hepatic cell lines: differentiated HepaRG cells, undifferentiated HepaRG cells and HepG2 cells. The specific metabolic competences of each cell line permit to evaluate the influence of hepatic metabolism on basal cytotoxicity. The different proliferative properties of the different cell types provide information on acute cell death versus inhibition of cell proliferation.

The assay chosen to assess basal cytotoxicity is the classic neutral red uptake (NRU), which is a cell survival/viability test based on the ability of living cells to incorporate and bind neutral red (NR). Cytotoxicity is expressed as a concentration dependent reduction of the uptake of NR after chemical exposure, thus providing a sensitive, integrated signal of both cell integrity and growth inhibition (Borenfreund and Puerner, 1985).

The NRU assay was performed on a automated platform composed of modules for liquid handling, transportation, incubation and spectrophotometric reading, following the implementation procedure described by Bouhifd et al. (2012). Although a relatively small number of chemicals were tested, the system can cope with much higher number of testing samples. The use of the automated platform in this study provided high quality data due to the accuracy of the automated mode when compared to manual assays.

2. Material and methods

2.1. Cell culture

2.1.1. HepG2

HepG2 cells were purchased from ATCC (HB8065 #58210525, LGC standards, UK). A working cell bank was prepared and stored in liquid nitrogen so that cells from the same cell batch could be used in all the experiments. The cells were cultured in 75 cm^2 culture flasks (BD-Biosciences, Italy) in a humidified incubator (37 °C, 5% CO_2). The culture medium used was composed of EMEM (30-2003#3000715, LGC standards, UK) containing 10% (v/v) fetal calf serum (A15-151#A15108-0088, PAA, UK). After recovery from liquid nitrogen, cells were split three times before any experiment. HepG2 cells were seeded in 96-well culture plates at a density between 7500 and 10,000 cells/well. Subsequently the cells were incubated at 37 C in a humidified 5% CO_2 incubator for a period of 24 h before testing. The working passage number of the cells was 81.

2.1.2. Differentiated HepaRG

A vial of cryo-preserved Human Cell Line HepaRG was obtained from INSERM's laboratory U522 (Institut National de la Santé et de la Recherche Médicale, U522, Hôpital de Pontchaillou, 35033 Rennes, France) and a cell culture bank was established in house. Both a small master cell bank (passage number 14) and a working cell bank (passage number 15) were set up to assure that the cells used in different tests had equal passage numbers. For each biological replicate, defined as an independent cell batch, a new cryopreserved vial was used.

The frozen undifferentiated cells were thawed and seeded in a 75 cm^2 culture flask (BD-Biosciences, Italy) in culture medium (CM) composed of Williams' E medium (#22551-089, Invitrogen, Italy) supplemented with 10% Serum (FetalClone III#SH30109.03 Lot ASH30211, Hyclone, UK), 5000 IU/mL, 5000 μg/mL Pen/Strep (#15140-122, Gibco, Italy), 5 g/ml insulin (#16634, Sigma, Italy), 2 mM L-glutamine (#25030024, Gibco, Italy) and 0.5 μM hydrocortisone (#H0888, Sigma, Italy). After approximately one week at confluence the cells were split (dilution 1:5) into new 75 cm^2 culture flasks in which they were kept for differentiation.

Differentiation was obtained by firstly growing the cells for 2 weeks at confluence with CM and then further culturing for two more weeks with CM supplemented with 2% DMSO. The medium was renewed every 2–3 days.

After differentiation HepaRG cells were detached by gentle trypsinization, seeded at a density of 4–5 $\times$ 10^4/well in 96 well microtiter plate (BD Biosciences, Italy) to allow the selection of hepatocyte-like populations. This procedure resulted in a higher percentage of hepatic-like cells versus biliary like epithelial cells of the HepaRG cell culture. The cells were used for testing within one week after plate seeding.

2.1.3. Undifferentiated HepaRG

Undifferentiated HepaRG cells were obtained by reducing the culture time of these cells. As with differentiated HepaRG, the cells were thawed in a 75 cm^2 culture flask, were grown in culture medium for one week at confluence as described previously before they were split and seeded into new culture flasks. The differentiation process was interrupted after one week at confluence. At this moment undifferentiated cells were seeded at a density of 4–5 $\times$ 10^4/well in 96 well microtiter plates that were used in tests within one week.

2.2. Reference chemicals

A total of 10 compounds were selected for testing, based on their toxicological profile (Table 1). The selection was based on the toxicological profiles formerly assessed in previous studies (http://www.acuteto.org; http://www.liintop.cnr.it; (ICCVAM, 2006b). The choice of test substances envisaged to cover different toxicity classes and different specific toxicities such as hepatic toxicity, non-hepatic toxicity, and cytotoxicity. Also attention was given to compounds that were known to be bio-transformed. All compounds were purchased from Sigma–Aldrich Srl Italy.

2.3. Cytotoxicity assay

To assess the basal cytotoxicity, the cells were exposed to eight chemical concentrations for a period of 48 h. After chemical exposure the cells were washed with PBS (D8662 Sigma, Italy) and incubated further for 3 h in a neutral red solution in culture medium (25 μg/mL; N2889 Sigma, Italy). The cells were then rinsed with