Chapter 2: *In Vitro* Procedure

2.1. Introduction

2.1.1. Background

The use of *in vitro* methods/models has increased greatly in order to reduce the use of animal testing models (Davila *et al.*, 1998). Many hepatic *in vitro* test systems have been developed in the past including isolated perfused organs, tissue slices, cell culture/suspensions, isolated organelles/membranes/enzymes, invertebrates and non-living systems (Silber *et al.*, 1994).

Primary human hepatocytes are considered the ‘‘gold standard” in the pharmaceutical industry for rapid evaluation of new chemical entities for both liver-related pharmacokinetic and toxicity liabilities (Hewitt *et al.*, 2003). However, there are some drawbacks in using primary cultures. Most cryopreserved human hepatocyte preparations do not attach to the culture plate surface, thereby limiting their survival time (usually less than 24 h) (Flynn and Ferguson, 2008). Also, primary hepatocytes lose normal hepatocyte characteristics and functions, a few days after explantation, making them unsuitable for longer term studies (Katsura *et al.*, 2002). Furthermore, the availability of freshly plated human hepatocytes can be sporadic and different donors need to be used between temporarily separated batches of experiments (Flynn and Ferguson, 2008), leading to large donor-to-donor variability (Reid *et al.*, 2009).

The HepG2 hepatocarcinoma perpetual cell line has been widely used by many researchers for more than a decade in various *in vitro* models focusing on hepatotoxicity (Castell *et al.*, 1997; Sahu, 2003; Dambach *et al.*, 2005; Farkas and Tannenbaum, 2005; Baudoin *et al.*, 2007; Gomez-Lechon *et al.*, 2010b; Swift et al., 2010). Amongst others, the HepG2 cell line was chosen as one of the cell lines used in the LIINTOP project. This project is aimed at

establishing experimental *in vitro* models and protocols for testing hepatic metabolism and toxicity of molecules of pharmacological interest. This model also strives to replace animal experimentation with alternative models in the drug development process (Gomez-Lechon *et al.*, 2010b).

HepG2 cells display most of the genotypic and phenotypic features of normal liver cells (Sassa *et al.*, 1987) preserving many of the normal functions of human liver cells. These include aldehyde dehydrogenase regulation (Marselos *et al.*, 1987), high drug metabolising capacity (Dashti *et al.*, 1984; Knowles *et al.*, 1980), a functional glutathione system (Dierickx, 1987) and CYP subfamilies 1, 2 and 3, which are the major classes involved in drug and xenobiotic metabolism (Alexandre *et al.*, 1999; Westerink and Schoonen, 2007).

2.1.2. Hepatotoxic endpoints

There are a substantial number of endpoints that can be considered in hepatotoxicity studies. Scrutiny of relevant literature indicated that the toxicity endpoints suggested by "*The non-clinical guideline on drug-induced hepatotoxicity"* assembled by the European Medicines Evaluation Agency (EMEA) (Committee for medicinal products for human use, 2008) were the most appropriate. The parameters suggested by the EMEA together with the parameters that were tested in the present study are listed in Table 2.1.

In the present study one major substitution was made to the list of parameters suggested by the EMEA. "*Loss of critical macromolecules/small molecular scavengers*" was substituted with "*CYP1A1 induction*" (Table 2.1). ROS generation, which was already part of the panel of tests suggested by the EMEA, and cellular glutathione concentration, which would be a measure of "*Loss of critical macromolecules/small molecular scavengers*", evaluate the oxidative state of cells. To avoid duplication (two assays that assess oxidative status; ROS generation and GSH depletion), CYP1A1 was included in the panel of tests used in the present study. The broad xenobiotic selectivity of CYPs (Park *et al.*, 2005) increases the possibility to detect toxicity that may induce subtle adverse effects. Also, in HepG2 cells the

levels of CYP1A1, 1A2, 2B6 and 3A4 correlate well with primary cultures (Westerwink and Schoonen, 2007).

Table 2.1. **Parameters of cellular physiology that are suggested by the EMEA and those that were examined in the present study.**

Suggested by the EMEA	Examined in present study
Loss of membrane integrity	Loss of membrane integrity
Apoptosis induction	Apoptosis induction
Loss of critical macromolecules/small molecular scavengers	CYP1A1 induction
Increased ROS generation	Increased ROS generation
Mitochondrial effects	Mitochondrial effects
Anti-proliferative effects	Cell viability

2.1.3. Scope of toxicity

The toxicological parameters evaluated in the present study are graphically represented in Figure 2.1. Examining these parameters provides a good overview of the toxic response of the cells to the test compound. In an initial toxic response, cells (especially liver-derived cells) attempt to eliminate a xenobiotic through metabolic inactivation by especially CYPs, as these enzymes allow cells to adapt to changes in their chemical environment (Denison and Whitlock, 1995; Delescluse *et al.*, 2000). The focus in this study was on CYP1A1 as it is rapidly and highly induced by a wide range of substances (Whitlock, 1999; Ma, 2001), thereby increasing the chance of detecting an initial toxic response.

Another toxic response is the generation of ROS, which may originate either from the mitochondria (Liu *et al.*, 2002) or from CYP activity (Schlezinger *et al.*, 2006). ROS generation may induce a state of oxidative stress within the cell, which can disrupt homeostasis and in this way lead to cell death. Mitochondria are not only a source of ROS but may also be a

target for ROS under certain conditions, during which ROS mediates the release of cytochrome c from the mitochondrial intermembrane space. In turn, cytochrome c triggers caspase activation, leading to apoptosis (Simon *et al.*, 2000). However, if ROS generation produces excessive levels of radicals, these radicals may inactivate caspase activity, which could then favour necrosis as the mode of cell death (Samali *et al.*, 1999; Prabhakaran *et al.*, 2004).

Quantifying apoptotic and necrotic death provides valuable information regarding the mechanism of cell death. Furthermore, cell viability provides an indication of the concentration at which the test compound induces cell death.

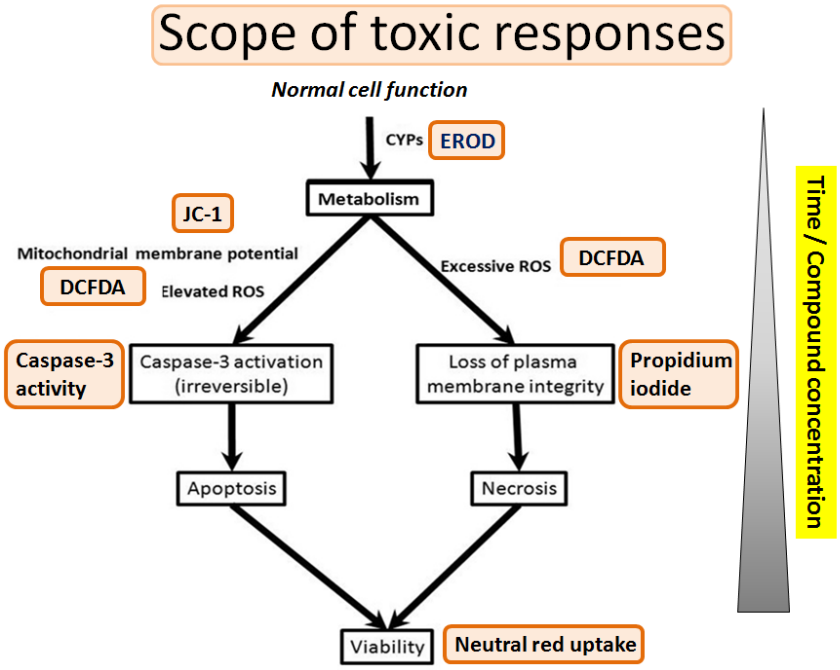

Figure 2.1. Diagram illustrating the scope of toxicity that the chosen parameters are expected to evaluate. As the concentration of test compound increases one would expect to see different cellular responses in an attempt to restore homeostasis. Initially, cells should respond by trying to eliminate xenobiotics through metabolic inactivation (Phase I metabolism). At higher concentrations ROS generation may occur, originating from either mitochondria or from CYP activity. If the mitochondria are affected, they may release factors that will initiate apoptotic death via caspase-3. On the other hand, excessive ROS may result in deactivating caspase activity and lipid peroxidation, which leads to cell death by necrosis. The degree of toxicity can then be assessed utilising an assay that enumerates viable cells. EROD - ethoxyresorufin-*O*-deethylase, JC-1 - 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, DCFDA - 2'7'-dichlorofluorescein diacetate.

2.2. Methods

2.2.1. Propagation of Cells

Ethical approval for performing the work on a commercially available cell line was obtained from the Faculty of Health Sciences Research Ethics Committee (Annexure A). Cells were propagated in a CO_2 tissue culture incubator (Binder C150, USA) in EMEM containing non-essential amino acids and fortified with 10% FCS. Cells were grown in 75 cm^2 culture flasks and medium changed every 2-3 days, as required, until confluent before being harvested with trypsin-/versine and split 1:6. All experiments were performed on cells between passages 20 - 26.

2.2.2. Cell harvest

Propagation medium was discarded and the cell culture flask rinsed with sterile PBS to wash off excess FCS. This was followed by the addition of 2 ml of trypsin/versene and incubated for 5 - 7 min at 37°C. The suspension of detached cells was transferred to a sterile 15 ml tube, filled with EMEM containing 10% FCS to inactivate the trypsin/versene and then centrifuged at 200 *g* for 5 min. The supernatant was discarded and the cell pellet resuspended in 1 ml cell culture medium supplemented with 10% FCS. The suspension was mixed thoroughly with an autopipette to obtain a homogenous single-cell suspension.

2.2.3. Cell counting

A homogenous single-cell suspension was diluted 1:9 into Trypan blue counting solution. The mixture was immediately loaded onto a haemocytometer and a minimum of 200 cells were counted using a Reichert Jung MicroStar 110 microscope at 400x magnification. Trypan blue, a membrane impermeable dye, was used to improve assay consistency by eliminating

non-viable cells from the cell count by staining their cytoplasm blue. Dilutions of the cell suspension in EMEM (10% FCS) were then made to achieve the correct viable cell density required for the experiments.

2.2.4. Microplate setup

The microplate was set up as illustrated in Figure 2.2. The columns of each plate were divided to give 6 duplicate columns of 8 wells each. Each set of 16 wells was used to perform one of six *in vitro* assays. Additionally, each plate was divided into 8 rows spanning all 12 columns. Blanks, positive and negative controls and 5 different concentrations of each test compound (5, 10, 50, 100 and 150 µM) were allocated to each row. This setup allowed each assay to be performed in duplicate on each plate for each of the controls/test compound concentrations/blanks. Nine individual experiments were conducted, 6 for each of the test compounds (18 plates; 12 observations per parameter per compound) and 3 for each of the compounds in combination with NAC pre-treatment (9 plates; 6 observations per parameter per compound). Negative controls were exposed to vehicle solvent (0.5% DMSO) alone. Positive controls used were compounds different from the test compounds that demonstrate a predictable effect on each specific assay.

2.2.5. Test staggering

Depending on the plate row number, cells were exposed to either negative control (0.5% DMSO v/v), positive control (150 µM) or test compound at concentrations of 5, 10, 50, 100 and 150 µM (Figure 2.2). All procedures were carried out under sterile conditions and all incubations took place in a 5% CO_2 incubator at 37°C.

The times of treatment (test staggering) are provided in Table 2.2. The starting time of a specific assay (described individually in each relevant chapter) were different since the time to complete assays (exposure + incubation times), varied. The time lines were as follows:

Table 2.2. **A summary of the timing followed to perform the six *in vitro* toxicity assays on a single microplate. Columns refer to those shown in Figure 2.2.**

Time	Procedure	*Columns
-24 h	Expose for Viability + CYP + Necrosis determination	A-F
-6 h	Expose for Apoptosis determination	K-L
-4 h	ROS assay dye loading	G-H
-3 h	Wash + Expose for ROS determination	G-H
-2 h	Add Viability + CYP assay dyes	A-B; C-D
-1.5 h	Mitochondria assay dye loading	I-J
-1 h	Wash + Expose for Mitochondria determination	I-J
-15 min	Add Necrosis assay dye	E-F
-5 min	Wash Viability + Necrosis assays	A-B; E-F
0	Determine values for CYP + Necrosis + ROS + Mitochondria assays	C-J
+5 min	Lyse cells for Apoptosis assay	K-L
+20 min	Add Apoptosis assay dye	K-L
+ overnight	Determine values for Viability +Apoptosis assays	A-B; K-L

*Columns refer to those shown in Figure 2.2.

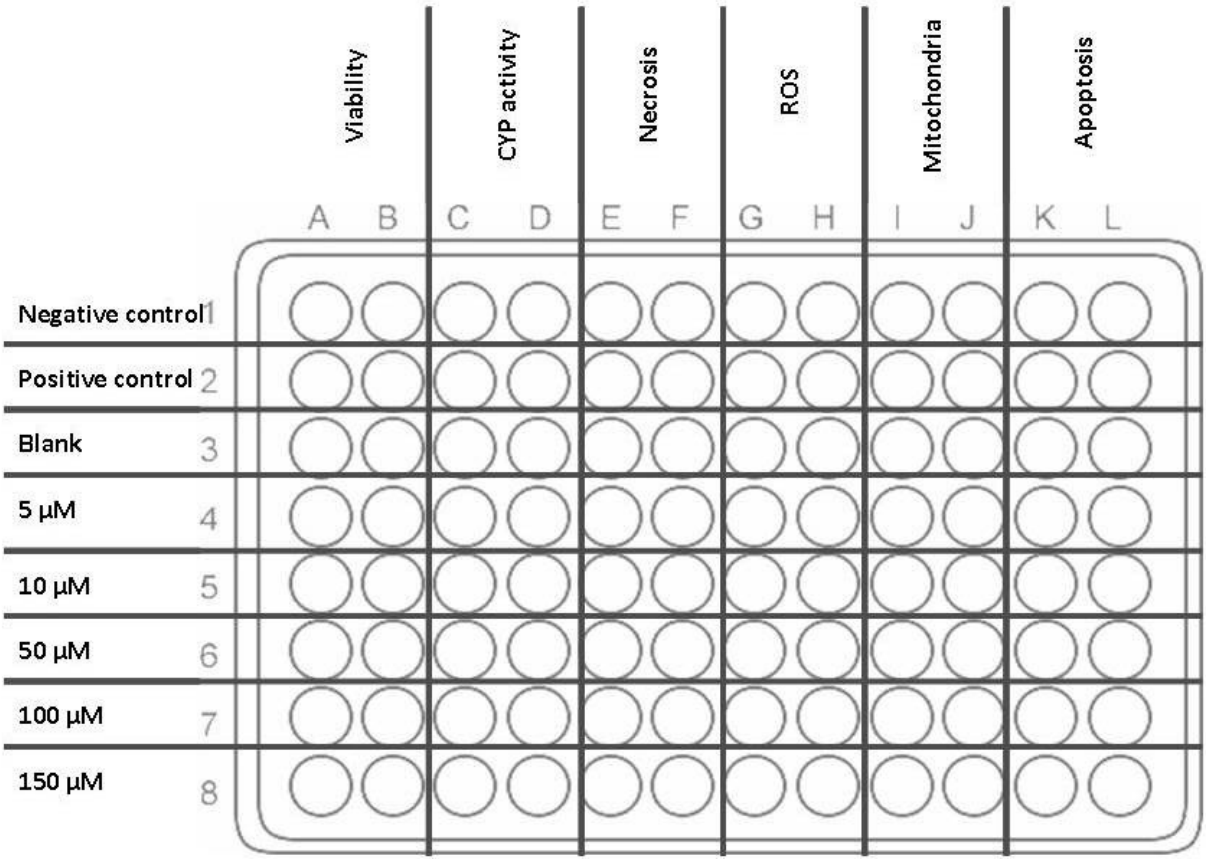

Figure 2.2. Diagram illustrating the plate setup. Plates were divided into six duplicate column sets, one for each of the six different assays to be performed and into eight rows, for the blanks, controls and various concentrations of test compound. Only one compound was tested on a single plate.

Initially cells (2×10^4 cells/well) were seeded into all wells (except for blank wells) and incubated for 48 h, to allow for proper cell adhesion.

-24 h: Cells in wells subjected to viability, CYP activity, and necrosis assays were exposed to control or test compounds.

-6h: After further 18 h of incubation, which was 6 h prior to the endpoints of the viability, CYP activity and necrosis determination, wells for determination of apoptosis were exposed to control or test compounds.

-4 h: After further 2 h incubation, cells in wells for ROS determination were loaded with 2', 7'-dichlorofluoresceindiacetate for 1 h.

-3 h: Wells for ROS determination were washed to remove excess dye and the control or test compounds added.

-2 h: Following a further one hour incubation, the dyes for only the cell viability and CYP determinations were added to the wells and incubation continued.

-1.5 h: After further 30 min incubation, wells used to determine possible mitochondrial toxicity were loaded with the relevant fluorescent dye.

-**1 h:** Following further 30 min incubation, cells in wells loaded for mitochondrial toxicity determination were washed to remove the excess dye and the control or test compounds added.

-15 min: After further 45 min incubation, propidium iodide was added to the wells, in which necrosis was to be determined, and incubated for 10 min.

-5 min: Wells used for necrosis and viability determination were rapidly washed.

Time 0: At 24 h after the initial control or test compound exposure, the following parameters were determined fluorometrically: CYP activity, necrosis, ROS and mitochondrial toxicity effects.

+5 min→ +20 min: Cells in wells used for determination of apoptosis were lysed on ice for 15 min followed by the addition of apoptosis assay substrate and incubation overnight.

+ 18 h: Fluorescence for the apoptosis assay was determined and the dye used for the viability assay was then eluted and the absorbance measured.

All wash steps were performed as follows:

- Aspirate well contents and discard
- Slowly add 200 µl of sterile PBS so as not to disturb the cell monolayer
- Aspirate well contents and discard

The entire procedure i.e. from seeding of the cells to obtaining the results took approximately 90 h (≈4 days).

When evaluating the possible hepatoprotective effects of NAC against test compound toxicity, the same procedure as described above was followed, except that the cells were pre-treated with NAC (100 µg/ml or approximately 620 nM) for 1 h (Souza *et al.*, 2004), prior to addition of the test compound for each assay.