Chapter 8: Concluding discussion

8.1. Procedure summary

The procedure developed during the present study aimed at miniaturising six different *in vitro* assays to be run simultaneously on a single 96 well microplate. Aspects of cellular physiology that were measured include:

- Cell viability
- CYP1A1 activity
- ROS generation
- Mitochondrial homeostasis
- Mode of cell death (apoptosis *vs*. necrosis)

Test compound exposure times for the different assays differed according to the response being measured. Cell viability, CYP1A1 activity and necrotic cell death was measured after 24 h exposure, whereas apoptotic cell death, ROS generation and mitochondrial effects were measured after exposure periods of 6 h, 3 h and 1 h, respectively. In order to perform all six assays on a single microplate, assay initiation had to be staggered, which added to the complexity of the procedure. The entire procedure, from when the first exposure starts, to obtaining the final raw data, took approximately 3 days to complete. This is considerably faster than performing each assay separately. In addition, this method yields results that are truly comparable as the experimental conditions are exactly the same for assays that are conducted on the same plate.

8.2. Test compound mechanism of toxicity

All three test compounds, DDT, DDE and DDD, proved to be cytotoxic. Following 24 h exposure DDT, DDE and DDD yielded IC_{50} values of 54 µM, 64 µM and 44 µM, respectively. Although limited literature is available on the *in vitro* hepatotoxicity, specifically for HepG2 cells, using DDT and none for the known metabolites, DDE and DDD the results from the present study correlates well with two previous studies in that the same threshold value for the onset of DDT-induced toxicity was observed; one on the same cell line (Medina-Diaz & Elizondo, 2005) and the other in a breast carcinoma cell line (Diel *et al.,* 2002).

Cyp1a1 is one of the gene batteries, whose induction is mediated by AhR through a cascade of events known as the AhR signalling pathway (Nebert *et al.*, 2000). In this classic signalling model, inactive AhR is bound to a heat shock protein (HSP90) chaperone within the cytosol. Upon ligand binding, dissociation from HSP90 takes place and activated AhR translocates into the nucleus, where the AhR can dimerise with its transcriptional partner, the Arnt. Within the nucleus, the AhR/Arnt heterodimer binds to the xenobiotic response element (XRE), leading to the transcriptional up-regulation of genes such as *cyp1a1*, which encodes the xenobiotic metabolising enzyme CYP1A1 (Nukaya *et al.*, 2010). Results from the present, and other studies (Dehn *et al.*, 2005), suggest that DDT is capable of up-regulating CYP1A1 activity. Both DDE and DDD were also capable of inducing CYP1A1 activity, which is expected as all three compounds are similar in structure. From a structural point of view CYP1A1 inducers have been classified into three groups (Navas *et al.*, 2004):

(1) Compounds having at least two aromatic rings in a plane, with a very low conformational mobility. This is the case of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Safe, 1995). This compound is considered a typical AhR ligand and CYP1A1 inducer (Navas *et al.*, 2003).

(2) Molecules with conformational mobility i.e. they exist in a variety of conformers that are separated by small energy differences so that the inter-conversion between them can be readily achieved. These compounds have been described as low affinity AhR ligands and CYP1A1 inducers (Anderson *et al.*, 1996).

(3) Compounds having at least two aromatic rings linked by a sp3 carbon in their structure. Due to steric requirements of the tetrahedral carbon atom, the conformations having two aromatic rings in the same plane are energetically unfavourable.

From this classification, CYP1A1-inducing compounds are expected to have defined structures capable of binding the AhR. DDT has two aromatic rings, linked by a sp3 carbon, which would place it in the third group of Navas' classification. However, high affinity of the AhR for DDT is unlikely as the two aromatic rings in the structure of DDT are not in the same plane (Figure 8.1). DDT may also fall into the second classification as each of the two chlorobenzene rings are able to rotate around their respective single bonds with the C2 carbon allowing some degree of conformational mobility.

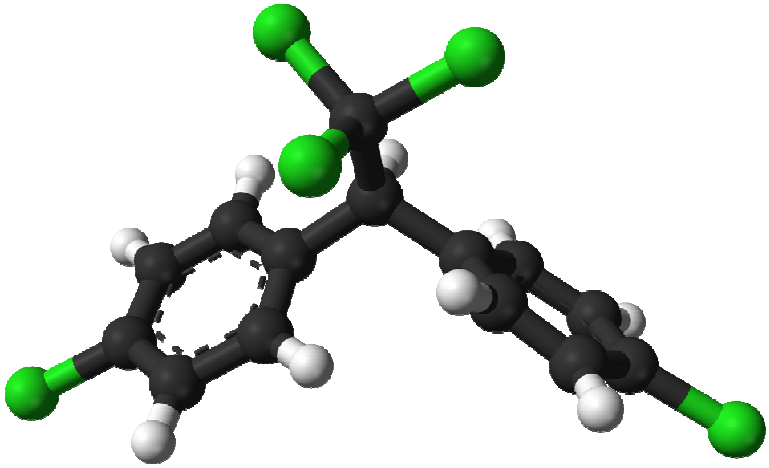

Figure 8.1. Illustration of the three dimensional structure of DDT demonstrating the two aromatic rings, which are not within the same plane (Wikipedia, 2011).

Another unlikely, but possible, explanation for how CYP1A1 was activated by the three test compounds without them binding to AhR may be that the three test compounds may cause weakening of the interactions between AhR and HSP90, facilitating dissociation of cytosolic AhR from its HSP90 chaperones thus activating AhR, which allows it to dimerise with Arnt leading to transcriptional up-regulation (Ledirac *et al.,* 1997).

Exactly how the test compounds may have up-regulated CYP1A1 activity in not known. Nonetheless, up-regulation of this enzyme has been associated with ROS generation (Schlezinger *et al.*, 2000) and DNA damage (Lin *et al.*, 2008). However, ROS evaluation revealed that no ROS generation occurred due to exposure to any of the test compounds under the test conditions (Figure 5.4.). This suggests that if toxicity was due to CYP1A1 up-regulation, it could result in subsequent DNA damage. DDT-induced DNA damage has been reported in peripheral blood mononuclear cells (Yanez *et al.*, 2004; Gajski *et al.*, 2007) and it is well established that DNA damage can result in cell death through apoptosis due to the action of p53 (Bhana and Lloyd, 2008). Activation of p53 leads to the up-regulation or post-translational modification of BH 3-only proteins such as Puma (*p53* up-regulated modulator of apoptosis), which results in apoptosis via the intrinsic apoptotic pathway (Coultas and Strasser, 2003). However, BH 3-only mediated apoptosis is reported to be caspase independent (Coultas and Strasser, 2003) and from the *in vitro* model it was evident that apoptosis was induced via Cas-3 activation, implicating a mechanism of toxicity that is independent of p53 activation and therefore not initiated by DNA damage. These findings indicate that although CYP1A1 was up-regulated by all three test compounds, CYP1A1 was not directly responsible for cell death resulting from to excessive ROS generation, as no ROS were detected. Cell death was also unlikely to have been initiated by DNA damage because Cas-3 was activated, reducing the possibility of apoptosis induction by the caspase-independent BH 3-only proteins.

None of the tested compounds elevated intracellular ROS levels at any of the tested concentrations over a 14 h exposure period. The lack of detectable concentrations of ROS makes this route of cell death unlikely.

Results from the mitochondrial assays showed that all three test compounds were potent inducers of mitochondrial hyperpolarisation, which has been described as an early apoptotic event (Matsuyama *et al.*, 2000). Observed elevations in $\Delta\psi_m$ may be due to inhibition of ATP-synthase (Younis *et al.*, 2002), intracellular GSH depletion (Nagy *et al.*, 2007) or a combination of both. Very recently, it was demonstrated that activated AhR can modulate

mitochondrial function by interacting with the ATP5α1 subunit of the ATP-synthase enzyme, which plays a major role in oxidative phosphorylation (Tappenden *et al.*, 2011). The authors demonstrated that the degree of TCDD-induced mitochondrial hyperpolarisation was not only dependent on the concentration of TCDD but also on the degree of AhR expression in different perpetual cell lines. It is therefore possible that DDT and its metabolites may have induced mitochondrial hyperpolarisation by activating AhR. However, this is unlikely for the following reasons:

(1) Structurally, the test compounds are unlikely to bind to the AhR.

(2) Although NAC pre-treatment counteracted pesticide-induced changes in $\Delta\psi_m$, it did not result in any major changes in test compound-induced CYP1A1 induction.

All three test compounds significantly induced Cas-3 activity in a dose-dependent manner, indicative of apoptotic death. In terms of potency of inducing apoptosis, the test compounds can be ranked as follows: DDD > DDT > DDE. These results correlate very well with the IC_{50} values determined, where, in terms of cytotoxicity, the same ranking was observed: DDD (44 µM) > DDT (54 µM) > DDE (64 µM). Results from NAC pre-treated cells, suggest a switch in mode of cell death from apoptosis to necrosis as Cas-3 activity was significantly decreased with a corresponding increase in PI staining, indicative of necrotic cell death pathway. These findings, coupled with those from $\Delta\psi_m$ experiments, suggest that the Cas-3 activity was possibly the result of mitochondrial hyperpolarisation as NAC was able to decrease test compound-induced changes in both $\Delta\psi_m$ and Cas-3 activation.

In conclusion, results from the developed *in vitro* assay method suggest that all three toxic test compounds, DDT, DDE and DDD, induced cell death *in vitro* in HepG2 cells, after 24 h exposure, by inducing mitochondrial hyperpolarisation, which in turn resulted in Cas-3 activation, with subsequent apoptotic death (Figure 8.2).

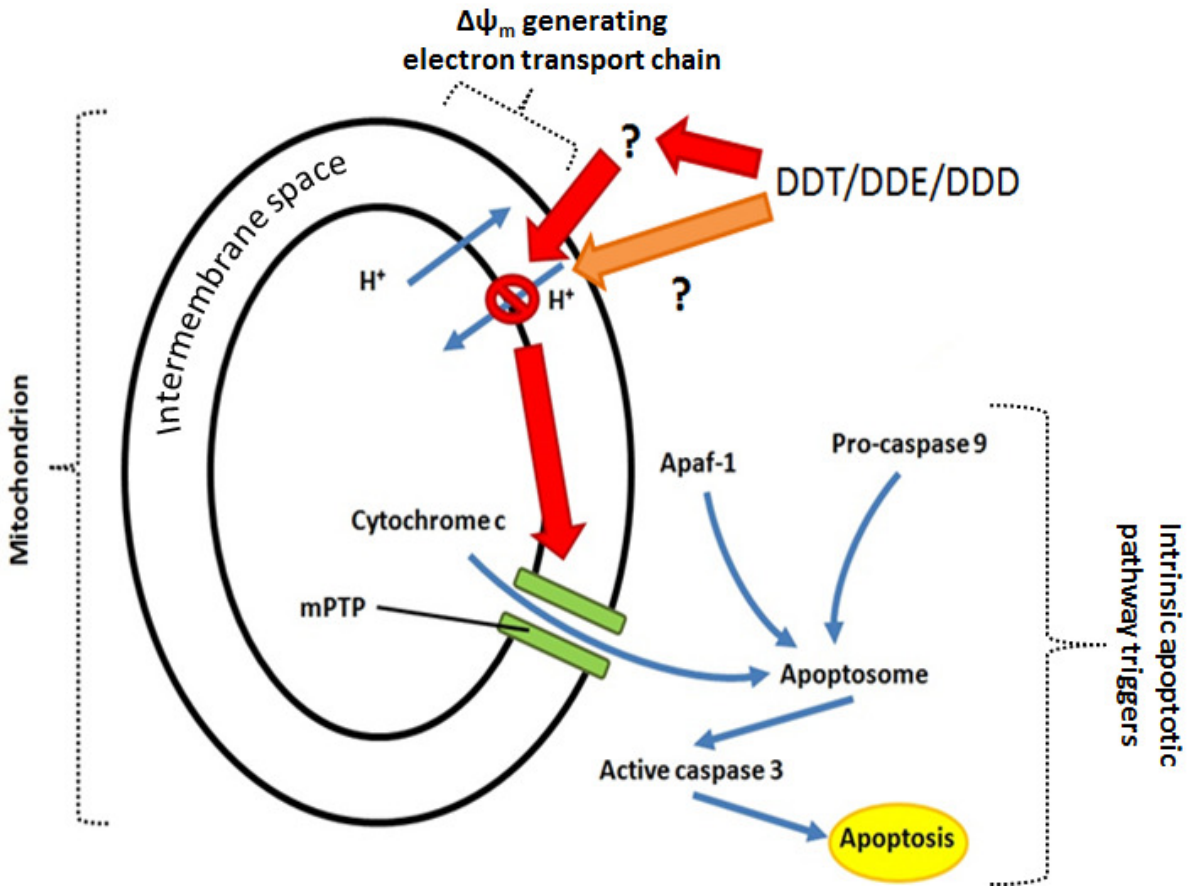

Figure 8.2. **Hypothetical mechanism of the acute toxicity of DDT, DDE and DDD in HepG2 cells after 24 h exposure to 5 - 150 μM of test compound. At high toxin concentrations (> 50 μM), the test compounds inhibit ATP synthase either directly or through some unknown mechanism(s). In turn, this elevates $\Delta\psi_m$, resulting in opening of the mPTP and cyt C release. In the cytosol, cyt C associates with Apaf-1 and pro-caspase 9 to form the apoptosome, which in turn activates Cas-3, leading to cell death by apoptosis. mPTP = mitochondrial permeability transition pore; Apaf-1 = apoptotic protease activating factor-1; $\Delta\psi_m$ = mitochondrial membrane potential.**

8.3. Advantages of the *in vitro* model

Human *in vitro* cellular models constitute valuable tools to understand the molecular and cellular processes of drug-induced liver injury (Gomez-Lechon *et al.*, 2010a). The present study aimed at developing an *in vitro*-based multiparametric screening assay, in an attempt to detect hepatotoxicity manifesting in various ways. Apart from the role this model can play in compound prioritisation and optimisation during drug development, it could enable researchers to gain some insight into the possible mechanism(s) of toxicity of a particular

compound and provide direction to further in-depth toxicity studies, demonstrating a possible role in drug development, especially in light of the three R's of animal testing: replacement, reduction and refinement.

Although this *in vitro* model was not able to pinpoint the mechanism of toxicity of the test compounds, it did identify mitochondrial hyperpolarisation as a key event that could lead to apoptotic cell death. No direct evidence was produced, but the possibility exists that this hyperpolarisation may be the result of GSH depletion without ROS generation. Further studies are needed to confirm this and, if true, determine the mechanism by which the test compounds deplete GSH.

It should be noted that the results presented in this study could be obtained within three weeks, highlighting the efficiency of the model in rapidly accumulating large quantities of cytotoxic and mechanistic data. Apart from being fast the model has a number of advantages over conventional toxicity testing:

(1) Time and infrastructure efficient
(2) Requires small quantities of test material
(3) Requires relatively few HepG2 cells, all from the same batch
(4) Assesses a broad scope of toxicity, under the same conditions
(5) It produced truly comparable data

Probably the greatest advantage of this model is listed as the 5th advantage above: the fact that results obtained from the different assays are truly comparable in that they originate from the same microplate, implying that all the assays are performed on the same passage of cells, with the same plating conditions, monitored on the same day using the same instrument. This would eliminate a considerable amount of variability that may otherwise be unavoidable.

8.4. Further development of the *in vitro* model

This proof-of-concept was successful in establishing a foundation from which further development of this model can be conducted. One of the aspects of the model that may be improved upon would be the ROS generation assay. It may be advantageous to replace the DCFDA assay with one that evaluates intracellular GSH levels rather than looking at ROS generation for two reasons: First, DCFDA only detects H_2O_2 (Gomes *et al.*, 2005), limiting the degree to which ROS generation can be assessed. Secondly, if the levels of ROS generated by a particular compound are sufficient to induce an intracellular oxidative state, it may also present as decreased levels of GSH, further increasing the chances of detecting hepatotoxic insult. Therefore, introducing a GSH assay should improve the scope of the model.

The second aspect that may be improved upon is the membrane integrity assay used to measure necrotic cell death. The method used in the present study did not produce the desired dose-dependent trends for all of the test compounds. There are other assays that rely on the same principle to evaluate a different endpoint. The LDH assay is used to determine cytotoxicity. Although this assay is not classically used to quantify necrosis, it indirectly assesses membrane integrity through measuring the leakage of LDH from cells (McKarns *et al.*, 1997). For this reason, it may be beneficial to evaluate whether or not the LDH assay can be used to quantify necrotic cell death in a microplate setup. If it proves to be successful, it can be compared to the assay used in the present study in the hope of obtaining a more accurate 'necrosis assay', which can then replace the present one.

In terms of performing the work, something that stood out was the complexity of performing this procedure. The procedure will benefit from simplification i.e. assays with fewer steps and more assays with similar exposure times. As this procedure comprises a number of individual assays, the ideal individual assay that would be conducive to this type of procedure would be an assay that:

(1) Utilises fluorescence detection to measure the endpoint

(2) Is started after exposure to the test compound

(3) Does not include any wash steps

(4) Is inert to the test compound

It may be difficult to find an assay for each parameter that satisfies all of the above-mentioned prerequisites and some compromises will have to be made.

Once these issues have been addressed, the predictive power of the model can be evaluated by accumulating data on various known hepatotoxins with different mechanisms of toxicity. A number of mathematical prediction models will then be able to be applied to the acquired data and the ability of each to accurately predict, for instance, known *in vivo* LD_{50}s or human lethal doses, can be determined.

To improve the predictive power of the model, it would probably be best to avoid the loss of assay signal, which occurred with CYP1A1 activity, where toxic concentrations of test compound induced cell death to such an extent that the CYP1A1 induction of the test compounds could not be detected any longer. This may be accomplished by lowering the tested concentration range to one that would provide more 'linear' data. In this regard 'linear' refers to data that follows a linear dose-response i.e. increasing concentration would be associated with linear dose-response, implying the use of sub-toxic concentrations (at least sub-IC_{50}) of test compound. It might also be advantageous to lengthen the toxin exposure period, which may improve assay signal/background ratios.

8.5. In conclusion

The present study set out to develop a method that can simultaneously assess a wide range of toxicity on a single 96 well microplate by measuring the effects of the known hepatotoxin DDT (and its metabolites DDE and DDD) on different parameters of cellular physiology. Furthermore, this type of cell-based procedure provides valuable mechanistic data that could direct future research into the exact mechanism(s) of toxicity of each of the test compounds. The procedure included, fundamentally, parameters suggested by the EMEA for assessing hepatotoxicity: cell viability, phase I metabolism, oxidative stress, mitochondrial homeostasis and mode of cell death (apoptosis *vs.* necrosis). Established *in vitro* assays were used to evaluate the different parameters. In order to miniaturise the relevant assays onto a single microplate required staggering of the assays in such a way that most of the endpoints can be measured at the same time. Some assay optimisation was performed to improve assay sensitivity and data quality. In addition to the test compounds, the method was tested with a known hepatoprotective agent (NAC) to evaluate the ability of the method to detect hepatoprotection. The complexity of the method was more than desired. However, this was superseded by the amount of results that were obtained in a short period of time i.e. the method was found to be highly time and infrastructure efficient. Other advantages of the method were the small amounts of test material required and relatively few cells, which makes it conducive to scalability for the purposes of higher throughput screening platforms. Although the complexity of the method does not yet allow up-scaling, it lays a solid foundation from which further method optimisation can be conducted that is directed at scalability. The quality of the obtained data was of a good standard, with small error margins and predictable dose-dependent trends in most cases. Probably the greatest advantage of this method is that data obtained from this procedure is truly comparable in that all of the assays were subjected to the same conditions and were performed on the same passage of cells, in the same plate. In conclusion, the study aims and objectives were achieved and opened the door to exciting new opportunities in further development of this method.