Chapter 4: Phase I metabolism

4.1. Background

Humans are constantly exposed to many different chemicals. A large percentage of these are hydrophobic in nature and would accumulate in the body if not converted to water-soluble derivatives that can be excreted via the kidneys (Delescluse *et al.*, 2000). The CYPs are a superfamily of hemethiolate enzymes, of which over 2000 individual members are currently known. It has been established that many of these CYPs play major roles in the metabolism of drugs and other xenobiotics. In humans and in other mammalian species, CYPs of the families 1, 2 and 3 are primarily associated with Phase I metabolism of exogenous compounds (Lewis, 2003). Phase I metabolism is responsible for the functionalisation of xenobiotic compounds (Iyer and Sinz, 1999). This is accomplished either by the introduction of polar and reactive groups through processes such as hydroxylation, oxygenation and epoxydation, or through the modification of existing groups on the specific compound through processes such as dealkylation and dechlorination (Guengerich, 2001; Westerink and Schoonen, 2007). Phase I metabolism is aimed at preparing the compound for and facilitating Phase II metabolism by making the molecule more chemically reactive. Whilst Phase I reactions generally result in a more polar metabolite, it is the conjugation reactions (e.g. glucuronidation and sulphonation) that result in marked increases in water solubility, which facilitate renal excretion (Westerink and Schoonen, 2007).

Some of the CYP enzymes are substrate inducible, which allows the cell to adapt to changes in its chemical environment (Denison and Whitlock, 1995; Delescluse *et al.*, 2000). The CYP1A subfamily of enzymes consists of only two members, CYP1A1 and CYP1A2, both of which are involved in oxidative metabolism of exogenous chemicals such as polycyclic aromatic hydrocarbons (PAHs), aromatic amines and heterocyclic amines, to name a few (Fujii-Kuriyama *et al.*, 1992). CYP1A2 accounts for approximately 13% of the total cytochrome P450 content in the liver (Shimada *et al.*, 1994) whereas CYP1A1 is expressed at low levels in the liver (Nakamura *et al.*, 2005) but is highly inducible (Morel *et al.*, 1999; Vrzal *et al.*, 2009). Although CYP1A1 and CYP1A2 have many inducers in common, the

difference between them lies in their tissue expression. When host animals are treated with the relevant inducers, CYP1A1 is not only expressed in liver tissue, but also in many extra-hepatic tissues such as lung, skin and kidney, whereas the expression of CYP1A2 is essentially limited to liver tissue (Fujii-Kuriyama *et al.*, 1992).

The ubiquitous form of CYP1A, one of the most studied CYP enzymes due to its involvement in generating reactive metabolites as a result of xenobiotic metabolism, has been implicated in both cytotoxicity and cancer induction. CYP1A1 can be induced by various environmental factors, such as smoking, dioxin exposure as well as the ingestion of charred meat (Nakamura *et al.*, 2005). A good example demonstrating CYP1A's role in toxicity would be the oxygenation of polycyclic aromatic hydrocarbons, originating from combustion, including cigarette smoke, to generate arene oxides. These are chemically reactive electrophiles capable of covalently binding different cellular components, such as DNA, which gives rise to their tumorigenic effect (Whitlock, 1999). The cancer-promoting role of CYP1A1 is probably best illustrated in the review of bezo(a)pyrene carcinogenicity (Androutsopoulis *et al.*,2009). Apart from its apparent role in cancer, an increase in CYP1A1 activity (similar to CYP2E1 (Chen and Cederbaum, 1998) and CYP3A4 (Perret and Pompon, 1998)), has been shown to release H_2O_2 during its catalytic cycles (Morel *et al.*, 1999), which may lead to oxidative stress and result in cell death. Due to its possible implication in both short and long-term toxicity, assessing CYP1A1 activity would be beneficial to any study evaluating potential hepatotoxicity as the liver is the major detoxifying organ of the body, implicating a major role in Phase I metabolism and, as such, it would be prone to toxic insult from a CYP1A1 inducing agent, which may generate reactive radicals in more than one way.

The best cell type for studying the detoxification and activation processes of compounds are hepatocytes (Westerink and Schoonen, 2007). There are many different techniques for performing the CYP1A1-specific ethoxyresorufin-*O*-deethylase (EROD) assay described in literature (Kennedy and Jones, 1994; Alexandre *et al.*, 1999; Jonsson *et al.*, 2006; Shiizaki *et al.*, 2008; Rudzok *et al.*, 2009). EROD activity is indicative of the rate of the CYP1A-mediated *O*-deethylation of the substrate 7-ethoxyresorufin (7-ER) to form the fluorescent product resorufin. The catalytic activity is an indication of the amount of enzyme present (Kennedy and Jones, 1994). Described methods include techniques for use with microsomal fractions,

fish gills (Jonsson *et al.*, 2006) and HepG2 cells (Alexandre *et al.*, 1999; Shiizaki *et al.*, 2008; Rudzok *et al.*, 2009). However, consistency among the techniques used by various researchers (specifically on the HepG2 cell line) is lacking.

4.2. Methods

4.2.1. Experimental design

Exposure conditions were similar to those previously described in Chapter 3; with the exception that omeprazole (150 µM) was used as positive control due to reports of CYP1A1 induction. Omeprazole produces its inductive effect by activating the aryl hydrocarbon receptor (AhR) pathway (Shiizaki et al., 2008).

Techniques for performing the EROD assay differ mainly in the amount of substrate used and the cofactors used in the final assay solution. For the purposes of this study, three different techniques (Alexandre *et al.*, 1999; Dubois *et al.*, 2006; Jonsson *et al.*, 2006) were tested but failed to detect increased CYP1A1 activity after treatment with the known modulator, omeprazole (Linden *et al.*, 2010; Fujii-Kuriyama *et al.*, 2010). An excessive 7-ER concentration (10 µM) was assumed to be the cause due to the fact that the background fluorescence intensities from blank wells were too high compared to the fluorescence intensities from wells seeded with cells. Consequently, Step 1 of the optimisation aimed at determining the role that 7-ER concentrations play in the EROD assay. HepG2 cells were seeded at a density of 2×10^4 cells/well and allowed to acclimatise for 24 h. White microplates were setup to include blank wells (no cells) along with the ones in which cells were seeded as before. Medium was then aspirated and replaced with 100 µl of fresh EMEM (no FCS) containing various concentrations (0.5 nM - 1000 nM) of 7-ER and incubated for 2 h at 37°C. Resorufin fluorescence was detected using a FluoStar Optima at λ_{ex} = 520 nm and λ_{em} = 595 nm.

Step 2 of optimisation focused on cofactors included in the final assay solution, specifically the use of NADPH in the EROD assay as an electron donor (Nakamura *et al.*, 2005; Rudzok *et*

al., 2009; Lemaire *et al.*, 2011). The use of NADPH in EROD assays performed on microsomal fractions may be a prerequisite because the necessary biochemical co-factors, co-enzymes etc. may not be present because only a sub-fraction of the cell is used. On the other hand, when performing the EROD assay on whole cell cultures, the use of NADPH as a cofactor may not be required as a complete, whole cell is already present with all of its biochemical functions intact. To test this hypothesis, experiments were setup to include both blanks (no cells) and untreated cells, as in Step 1 of optimisation. After the acclimatisation period, cells were then treated with 150 µM Omeprazole in EMEM for 24 h. Medium was then aspirated and replaced with 100 µl EMEM containing 150 nM 7-ER, either with or without 100 nM NADPH (Rudzok *et al.*, 2009). Thereafter, cells were incubated at 37°C for 2 h and fluorescence was monitored using a FluoStar Optima at λ_{ex} = 520 nm and λ_{em} = 595 nm.

4.2.2. Statistical analyses

For optimisation experiments, results were analysed for normality using the Shapiro-Francia test, after which hypothesis testing was applied in the form of Mann-Whitney tests to assess differences between means of the distributions. For CYP1A1 induction by the compounds in question, outliers were detected using Grubb's test. Following removal of the relevant outliers, normality of the data distributions were evaluated with the Shapiro-Francia test. Hypothesis testing was then performed utilising both Mann-Whitney and ***t***-tests, where applicable, to determine whether any observable differences between means were statistically significant.

Three additional experiments were carried out in duplicate to assess the possible effects that NAC may have on pesticide-induced changes in CYP1A1 activity. These results were also standardised to percentage of control but no preliminary tests (Grubb's and Shapiro-Francia) were performed due to the small sample size (***n*** **=** 6). Normality of distribution of the NAC pre-treatment data could not be established, therefore, Mann-Whitney tests were performed.

4.3. Results

4.3.1. Optimisation

Step 1 of optimisation showed that as the amount of 7-ER, the CYP1A1 substrate, increased from 0.5 - 1000 nM, the amount of resorufin cleaved by the cells also increased in a concentration-dependent manner. From approximately 4 nM up to 1000 nM the fluorescence appeared to increase in a linear fashion as depicted in Figure 4.1. From this figure it is apparent that substrate concentrations of 1 nM and lower were insensitive to changes in enzyme concentration and would yield similar results, which indicated that 7-ER concentrations of ≤ 1 nM are below the limit of quantitation for CYP1A1 activity. The relative fluorescence intensity (mean ± SEM values) of the different concentrations of 7-ER are presented in Table 4.1.

As illustrated in Figure 4.1, background fluorescence increased at 7-ER concentrations of greater than 250 nM. For this reason, further optimisation of the assay was performed at a substrate concentration of 150 nM 7-ER as this would most likely provide the greatest sensitivity and assay range while avoiding increased background fluorescence due to high substrate concentrations.

Table 4.1. Relative fluorescence intensity of resorufin as a result of 7-ER cleavage by untreated HepG2 cells. [7-ER] = concentration (nM) of substrate employed. Compared to 1 nM, all higher concentrations yielded significantly different results with $p < 0.05$.

[7-ER]	0.5	1.0	2.0	3.9	7.8	15.6	31.3	62.5	125.0	250.0	500.0	1000.0
Mean	766	764	829	905	1078	1260	1471	1613	1824	1831	2023	2251
SEM	12	6	8	9	21	43	48	69	62	89	44	63

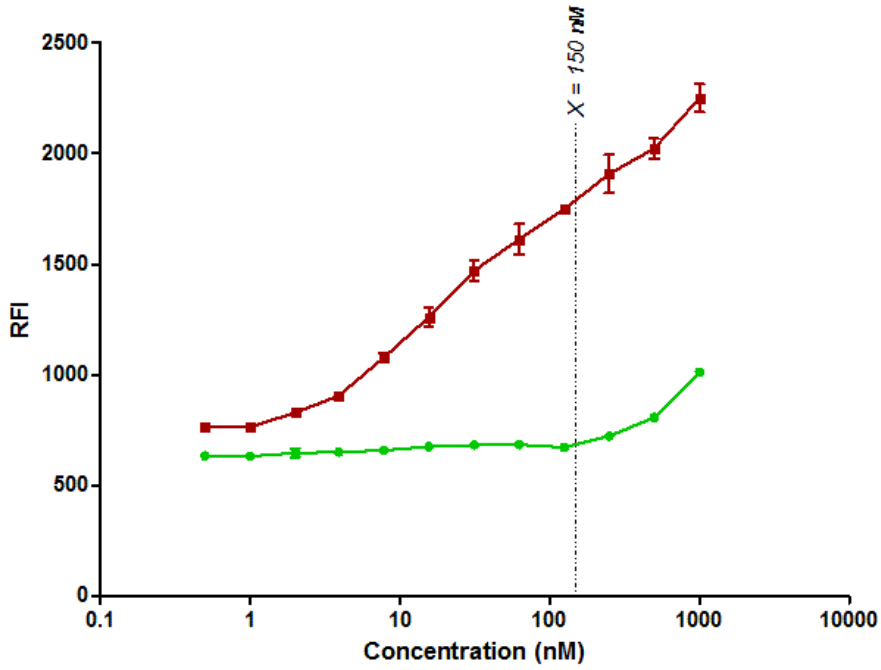

Figure 4.1. **Semi-logarithmic scatterplot of EROD assay optimisation for fluorescence substrate concentration. Following 24 h incubation, after seeding, wells were exposed to different concentrations of 7-ER, ranging from 0.5 - 1000 nM, for 1 h and monitored fluorometrically at λ_{ex} = 520 nm, λ_{em} = 595nm. The green line represents blank wells and the red line wells with untreated cells. The dashed vertical line indicates X = 150 nM and the Y-axis represents relative fluorescence intensity (RFI). Values differed significantly from blanks at all points along the graph with $p < 0.01$.**

Step 2 of the optimisation focused on the use of the cofactor NADPH. Results revealed that the presence of NADPH significantly ($p < 0.001$) decreased fluorescence (Figure 4.2). NADPH also significantly ($p < 0.01$) decreased the relative fluorescence intensity in assay blanks, although the difference was not as large as in the case of treated cells (Table 4.2). The difference in fluorescence intensities (y-axes) between Figures 4.1 and 4.2 are due to treatment with 150 µM omeprazole, which was only applicable in Step 2 of the optimisation.

Table 4.2. Effect of the cofactor NADPH on EROD assay results. In Step 2 of optimisation substrate (150 nM), with or without the addition of 100 nM NADPH was utilised to perform an EROD assay. Results are presented as mean ± SEM of the relative fluorescence intensity. Statistical analyses of these results are shown in Figure 4.2.

	Blank +NADPH	Blank	Cells + NADPH	Cells
Mean	824	863	4469	7631
SEM	± 9	± 8	± 286	± 374

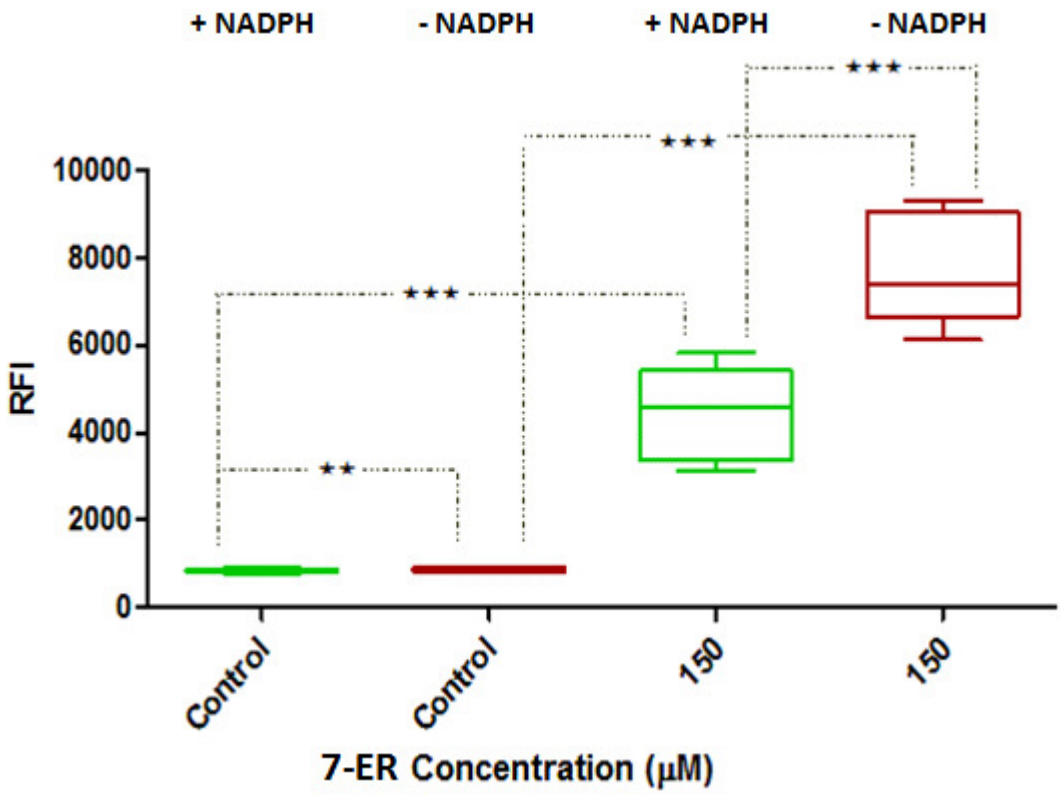

Figure 4.2. Boxplot of EROD assay with the addition or absence of the cofactor NADPH. To induced CYP1A1 activity, cells were exposed to 100 µM omeprazole (except controls) for 24 h before performing the EROD assay. Groups represented on the X-axis were exposed to 0 and 150 nM 7-ER for 1 h and fluorometrically monitored at λ_{ex} = 520 nm, λ_{em} = 595 nm. Red boxplots represent wells that received 7-ER only, while green boxplots represent those that received 7-ER and 100 nM NADPH. Y-axis represents relative fluorescence intensity (RFI), ** indicates $p < 0.01$, *** indicates $p < 0.001$.

4.3.2. CYP1A1 induction

The frequency distributions of data obtained for the CYP1A1 activity in DDT exposed cells shows that the 5 µM data set provides evidence for the presence of outliers, seen as a small peak to the far right of the bulk of the data (Figure 4.3B). Data was subjected to Grubb's test in order to detect outliers (Table 4.3). Testing revealed that three data sets in the DDT group (5, 10 and 100 µM), one in the DDE group (5 µM) and one in the DDD group (Control) contained outliers.

Table 4.3. Grubb's test results for detecting outliers in the observed CYP1A1 data. Values given in the Table are *p*-values. Instances where $p < 0.05$ (*) indicates the presence of outliers.

Concentration	DDT	DDE	DDD
Control	0.53	0.62	0.04*
5 µM	0.00*	0.01*	0.14
10 µM	0.01*	0.31	0.34
50 µM	0.43	0.26	0.43
100 µM	0.02*	0.88	0.35
150 µM	0.06	0.64	0.40

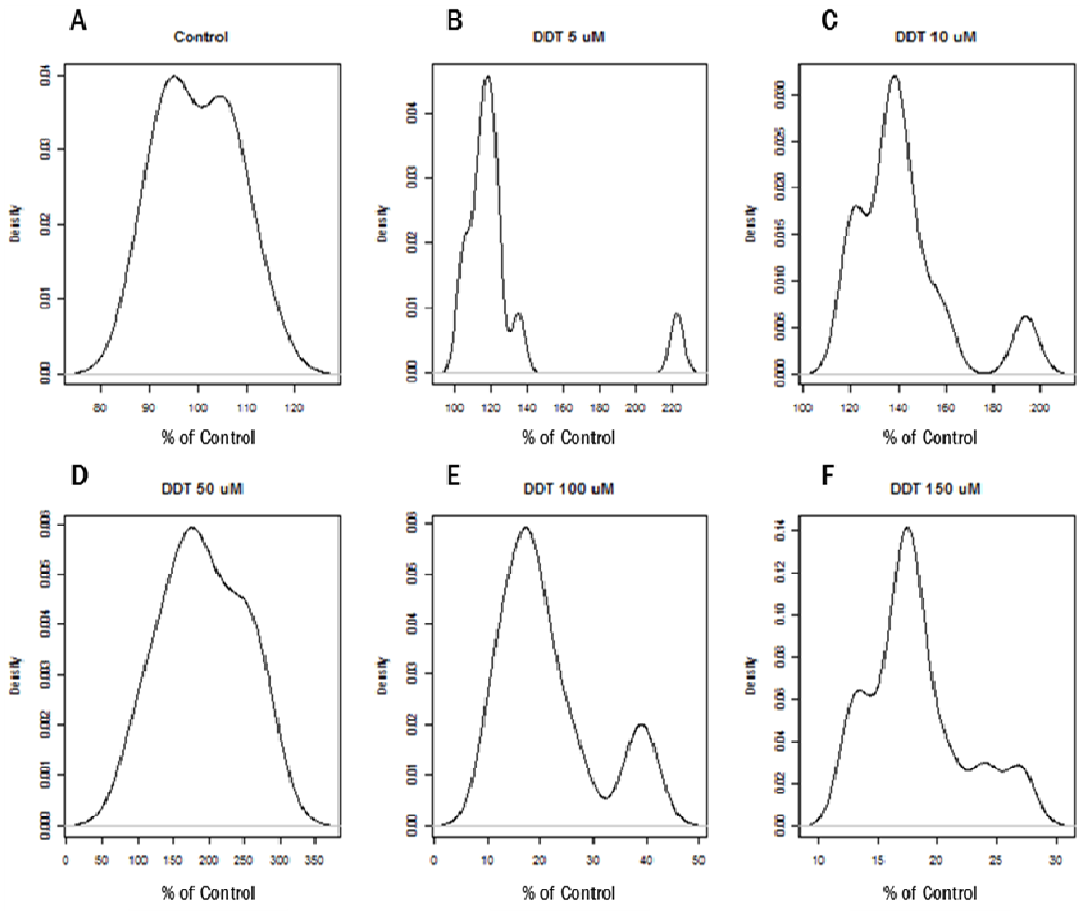

Figure 4.3. Histogram density plots of the observed CYP1A1 data of HepG2 cells exposed to DDT demonstrating the distributions of the collected data, prior to the removal of outliers detected by Grubb's test. The X-axis represents observed values and the Y-axis the count or density.

Relevant outliers were removed and the distributions re-plotted (Figure 4.4). When comparing the distributions in Figures 4.3 and 4.4, a marked difference can be observed as distributions in Figure 4.4 more closely resemble a typical normal distribution. However, visual assessment of graphs alone is not sufficient to claim normality and the data was subjected to a second evaluation of normality, the Shapiro-Francia test. Results from this test are presented in Table 4.4. Normality testing revealed that, after removal of outliers, the DDT group of data sets demonstrated normal distributions, whereas some of the data sets in the DDE (100 µM) and DDD (50 µM) groups still manifested as significantly non-normal. As a result, these two groups of data were tested using non-parametric Mann-Whitney tests, while the DDT group could be tested using unpaired, two-tailed ***t***-tests.

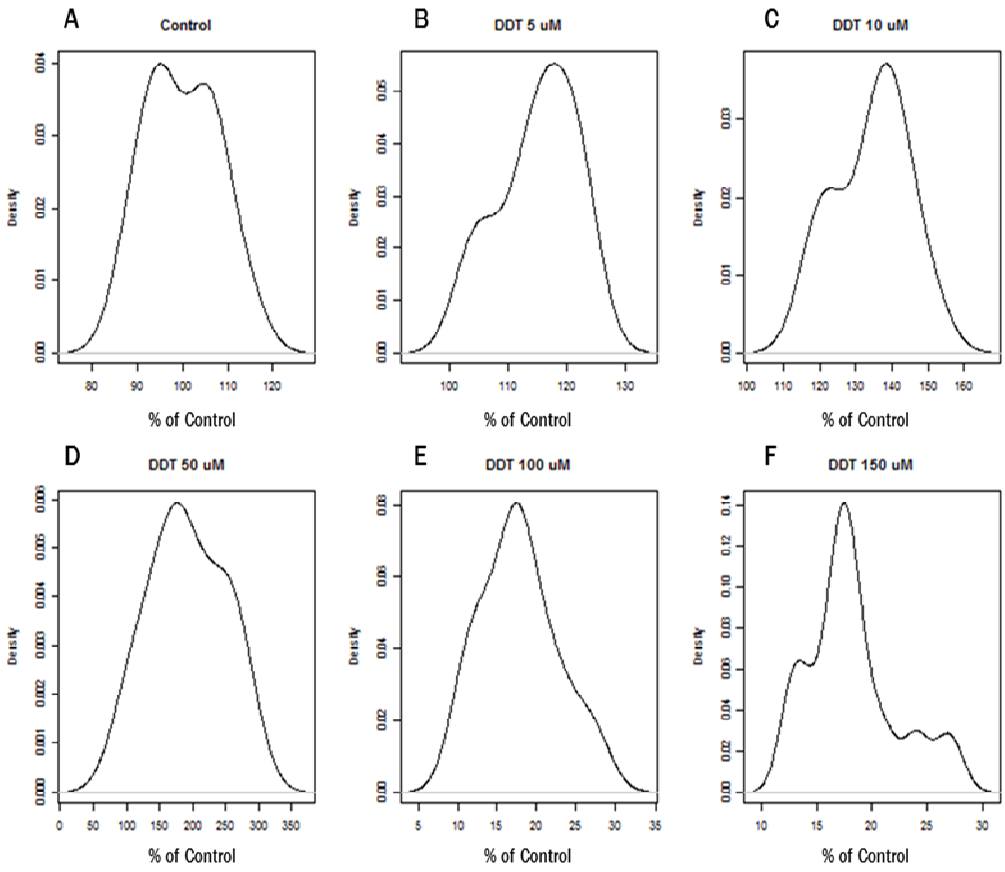

Figure 4.4. Histogram density plots of the observed CYP1A1 data of HepG2 cells exposed to DDT after the removal of outliers detected by Grubb's test. The X-axis represents observed values and the Y-axis the count or density.

Table 4.4. Shapiro-Francia test results for normality of the observed CYP1A1 data after removal of outliers as detected using Grubb's test. Values given in the Table are p-values. Instances where $p < 0.05$ (*) are significantly non-normal.

Concentration	DDT	DDE	DDD
Control	0.67	0.85	0.20
5 µM	0.50	0.75	0.68
10 µM	0.58	0.76	0.70
50 µM	0.87	0.33	0.03*
100 µM	0.80	0.01*	0.76
150 µM	0.25	0.05*	0.72

Relative percentage changes in CYP1A1 activity, as determined using the optimised EROD assay conditions is presented in Table 4.5 and Figure 4.5. Omeprazole, a well-known up-regulator of CYP1A1 activity (Ma and Lu, 2007; Linden *et al.*, 2010; Fujii-Kuriyama *et al.*, 2010), produced a seven-fold increase ($p < 0.001$) in CYP1A1 activity compared to untreated controls. All of the tested compounds significantly ($p < 0.001$) induced CYP1A1 activity up to concentrations of 10 µM. A 50 µM concentration of DDT and DDE caused a further increase in activity ($p < 0.001$), whereas 50 µM DDD did not produce any significant shift in the mean CYP1A1 activity when compared to controls. The highest CYP1A1 activity, other than that from the positive control, was induced by 50 µM DDE (258 ± 21%), representing a two-and-a-half fold increase. High concentrations (100 and 150 µM) of all three test compounds significantly ($p < 0.001$) inhibited CYP1A1 activity.

Table 4.5. Percentage CYP1A1 induction in HepG2 cells following 24 h exposure to DDT, DDE, DDD and the established CYP1A1 inducer, omeprazole (mean ± SEM). * indicates $p < 0.001$ as determined by Mann-Whitney tests.**

Concentration	DDT	DDE	DDD	Omeprazole (150 µM)
Control	100 ± 2	100 ± 1	100 ± 2	
5 µM	126 ± 9***	140 ± 7***	127 ± 5***	
10 µM	141 ± 6***	174 ± 12***	151 ± 6***	692 ± 78***
50 µM	192 ± 16***	258 ± 21***	92 ± 20	
100 µM	21 ± 3***	15 ± 3***	7 ± 1***	
150 µM	18 ± 1***	11 ± 2***	7 ± 1***	

NAC pre-treatment significantly decreased changes in CYP1A1 activity at concentrations of 50 µM for all three test compounds ($p < 0.01$) (Table 4.6 and Figure 4.6). At higher concentrations of the test compounds (100 - 150 µM), NAC pre-treatment showed no significant changes on test compound-induced CYP1A1 activity. In summary, CYP1A1 activity was raised at lower concentrations (5 - 10 µM) of DDT and DDD, however only being

significant in the 5 µM DDT group ($p < 0.05$). Conversely, CYP1A1 activity was decreased at similar concentrations when treated with DDE.

Table 4.6. **Percentage CYP1A1 induction in HepG2 cells by DDT, DDE, DDD relative to untreated controls, with or without 1 h pre-treatment with NAC (mean ± SEM). *, ** and *** represents $p < 0.05$, < 0.01 and < 0.001, respectively (Mann-Whitney tests).**

	DDT		DDE		DDD	
	--	NAC	--	NAC	--	NAC
5 µM	126 ± 9	137 ± 6*	140 ± 7	133 ± 2	127 ± 5	156 ± 14
10 µM	141 ± 6	169 ± 12	174 ± 12	155 ± 7	151 ± 6	201 ± 24
50 µM	192 ± 16	30 ± 9***	258 ± 21	95 ± 12***	92 ± 20	9 ± 3**
100 µM	21 ± 3	22 ± 9	15 ± 23	29 ± 5	7 ± 1	10 ± 3
150 µM	18 ± 1	15 ± 5	11 ± 2	18 ± 4	7 ± 1	10 ± 3

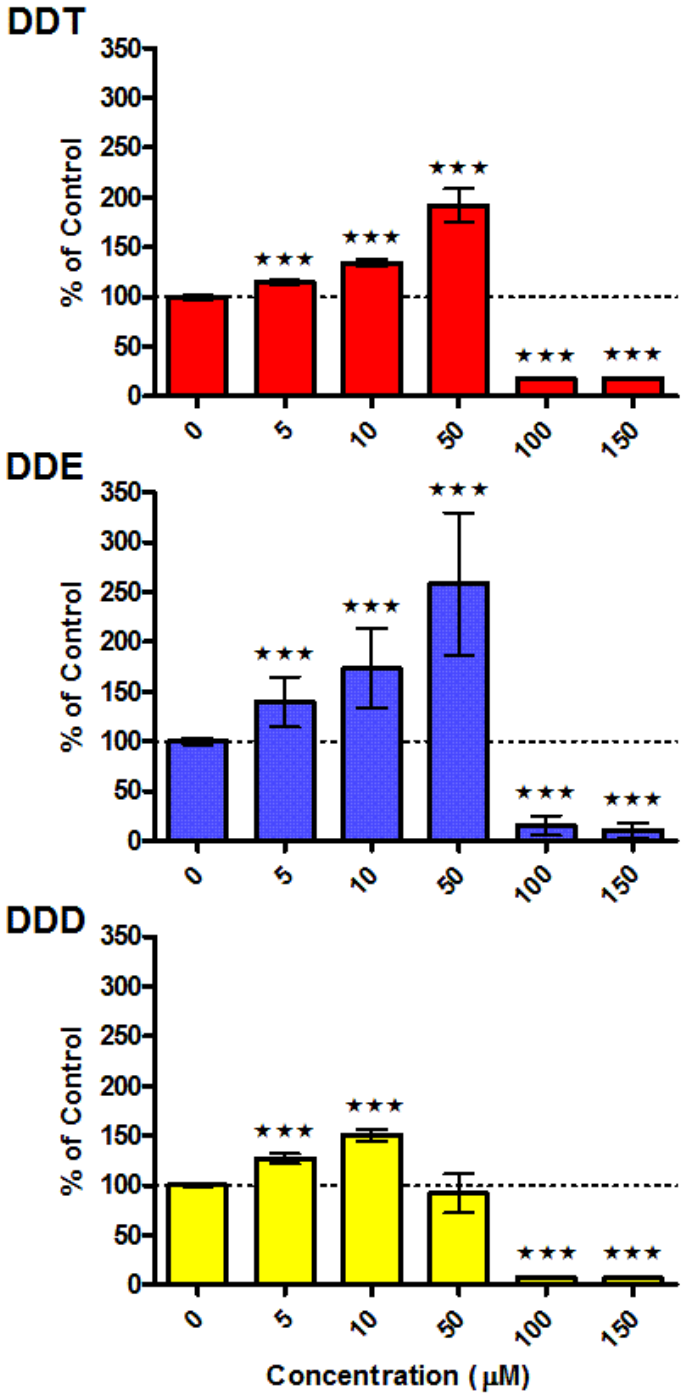

Figure 4.5. Graphical representation of CYP1A1 induction in HepG2 cells exposed to DDT, DDE and DDD mean ± SEM. *** indicates $p < 0.001$ and the dashed horizontal line Y = 100%.

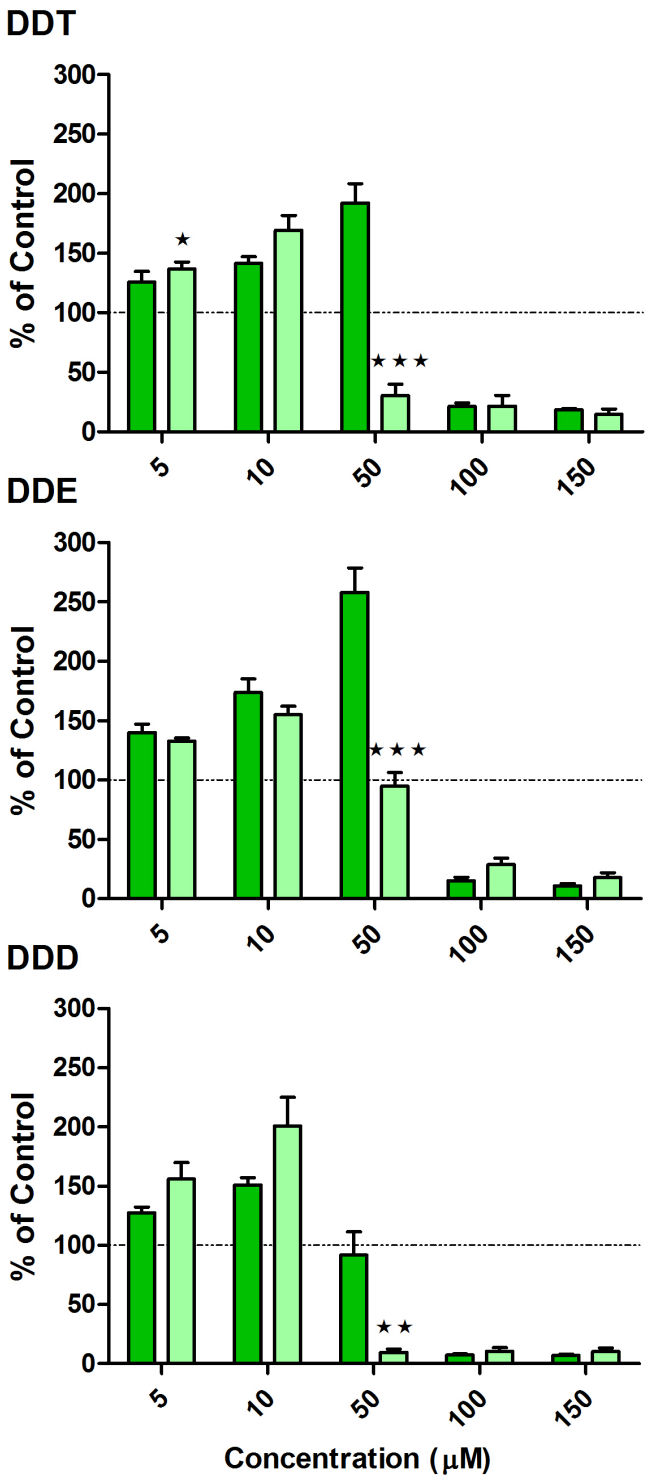

Figure 4.6. CYP1A1 induction in HepG2 cells exposed to DDT, DDE and DDD after 1 h pre-treatment with NAC. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, compared to corresponding dose with no pre-treatment. Dashed horizontal lines show Y = 100%.

4.4. Discussion

4.4.1. Optimisation

Although CYP mRNA levels are much lower in HepG2 cells when compared to primary human hepatocytes, this cell line has been shown to still retain enough CYP function for use in CYP induction studies, specifically CYP1A1, 1A2, 2B6, 2D6, 2E1 and 3A4 (Westerwink and Schoonen, 2007). Initially, tests in the present study for determination of CYP1A1 activity used substrate concentrations of 1 - 10 µM (Alexandre *et al.*, 1999; Dubois *et al.*, 2006; Jonsson *et al.*, 2006), which was not able to detect omeprazole-induced elevations in CYP1A1 activity. The reason for this was excessive CYP1A1 substrate concentrations, which produced considerable background fluorescence, thus limiting the sensitivity of detection of cleaved resorufin and the ability to detect elevated CYP1A1 activity. Optimisation showed that 7-ER concentrations of 2 - 250 nM yielded a steady rise in fluorescence produced by the cells, while the background fluorescence remained essentially constant (Table 4.1 and Figure 4.1). Whether this rise in background fluorescence from 250 - 1000 nM of 7-ER is the result of non-specific release of resorufin or due to direct fluorescence of the substrate itself is not clear and is beyond the scope of this study. Subsequent experiments in the present study have therefore utilised a substrate concentration of 150 nM as this maximised the linear sensitivity and range of the assay while still excluding excessive background fluorescence.

Step 2 of optimisation focused on the use of NADPH as a co-factor in the EROD assay. Results confirmed that performing the EROD assay on whole cell cultures does not require NADPH as a co-factor. The presence of NADPH decreased the sensitivity of the assay by almost 50%. A possible explanation for this may be that the presence of NADPH in the solution quenches the fluorescence. The decrease seen in untreated cells may also be due to quenching but it cannot be excluded that some other biochemical change(s) elicited by excessive NADPH may have occurred. Consequently, NADPH was excluded from the EROD assays in this study when evaluating CYP1A1 induction in intact HepG2 cells by the test compounds.

4.4.2. CYP1A1 activity

Figures 4.3 and 4.4 present the density plots of the observed CYP activity data for DDT. These graphs are shown to demonstrate the presence and effect that outliers may have on a particular data set. Some of the data sets presented in Figure 4.3 clearly show more than one population, especially Figure 4.3B In this graph the majority of observations tend to be around 120% of the control with a second small population that presents with 220% as the mean. After removal of the relevant outliers using Grubb's test, this density plot changes to resemble a more bell-shaped curve, consisting of only one population as seen in Figure 4.4B, illustrating the importance of assessing the data prior to statistical manipulation in order to remove any bias that may be present and in this way decrease the probability of making type I or II errors (false positives and negatives, respectively). The density plots presented in Chapters 3 and 4 were added to demonstrate the importance of assessing data quality. Throughout the rest of the manuscript only the Grubb's and Shapiro-Francia test results will be presented as a measure of data quality.

The three test compounds appeared to induce CYP1A1 activity in HepG2 cells as all three test compounds were able to significantly increase CYP1A1 activity at the lower concentrations assayed (5 - 10 µM). DDT and DDE induced even greater activity at concentrations of 50 µM, yielding approximately 200% and 250% of the activity of the untreated cells, respectively. DDD, on the other hand, did not produce a significant effect at this concentration. At the higher concentrations assayed, a large and statistically significant decrease in CYP1A1 activity was seen for all three test compounds which could be ascribed to cell death due to the toxic effects before CYP 1A1 could be induced. Despite the fact that at test compound concentrations of 50 µM, approximately half of the cells were no longer viable, a significant increase in CYP1A1 activity was still observed.

The up-regulation of CYP1A1 due to DDT exposure has been described *in vitro* (Dehn *et al.*, 2005), *in vivo* (Nims *et al.*, 1998; Sierra-Santoyo *et al.*, 2000) and even in plants when using cultured cells of the common onion, *Allium cepa* (Riffat and Masood, 2006). In contrast to

findings from this study, Dehn *et al.* (2005), who also performed EROD assays on HepG2 cells following DDT exposure, detected no CYP1A1 activity in cells exposed to 10 and 50 µM of DDT, but found a significant increase of CYP1A1 activity from 100 µM up to 1 mM. This may once again be attributed to the considerable difference in cell density used in the two studies, 2×10^5 cells/well (Dehn *et al.*, 2005) *vs.* 2×10^4 cells/well. Additional sources of discrepancy could have resulted from NADPH presence and/or substrate concentrations for 7-ER. However, the authors did not elaborate on their methods, therefore the substrate concentration they used and whether or not they added NADPH as a cofactor, both of which have already been shown to significantly affect EROD assay results, could not be established. Observations from the present study showed that exposure to 100 µM of DDT resulted in a significant decrease in CYP1A1-associated EROD activity, most probably due to cell death. On the other hand, Dehn *et al.* (2005) reported significant increases in EROD activity from concentrations of 100 - 1000 µM of DDT. These findings could implicate that, in the present study concentrations of 100 and 150 µM of DDT may also have induced further CYP1A1 activity, had cell death due to toxicity not been observed. The findings of Dehn *et al*. (2005) that 1 mM of DDT increases CYP1A1 activity is disputable as DDT has a solubility limit of approximately 200 µM in aqueous media at a vehicle solvent concentration of 0.5% (v/v).

Delescluse *et al.* (1998) reported findings similar to the present study in that DDT at 10 µM could induce CYP1A1 activity in HepG2 cells but this increase was found to be statistically insignificant. Following three days incubation with 10 µM of DDT, treated cell cultures were lysed using a freeze-thaw cycle and EROD activity measured in a similar manner to that performed on microsomal fractions with a concentration of 2 µM 7-ER and the addition of 500 µM NADPH. The authors, however, did not report the seeding cell density. The prolonged exposure to DDT, difference in approach to the EROD assay and high substrate concentration may explain why a significant increase was not detected by Delescluse *et al.* (1998), as was the case in the current work. Despite some discrepancies, results from this study and those in literature suggest that DDT does induce CYP1A1 activity *in vitro*, but the concentration at which this occurs seems to be dependent on various experimental conditions when performing the EROD assay.

With regards to the *in vitro* effects of DDE and DDD on CYP1A1, no literature was found in order to compare with the findings from the present *in vitro* study. However, effects on CYP1A1 induced by these two metabolites, as well as DDT, have been described *in vivo*. Nims *et al.* (1998) conducted a study using male F344 rats exposed to dietary DDT, DDE and DDD. Although this was an *in vivo* study, there were some similarities to the observations made in the present study in that these authors also observed dose-dependent increases in hepatic CYP1A1 activity induced by all three test compounds. In addition to this, DDT and DDE were almost equipotent inducers with CYP1A1 activities reaching 3.45- and 3.55-fold increases compared to untreated control animals, whereas DDD was the least potent reaching a maximum CYP1A1 activity of 2.18 times that of untreated control animals. Despite the fact that these experiments were conducted *in vivo*, the potency of the three compounds correlated with what was observed in the present study where (in terms of potency of inducing CYP1A1 activity) the compounds were ranked DDE > DDT > DDD. Dickerson *et al.* (1999) also reported potent, dose-dependent induction of CYP1A1 by DDE *in vivo* while Sierra-Santoyo *et al.* (2000) reported findings that suggest that the CYP1A1 induction by DDT, DDE and DDD may also be gender-dependent.

NAC pre-treatment increased CYP1A1 activity at low concentrations (5 - 10 µM) for both DDT (significant at 5 µM, $\boldsymbol{p} < 0.05$) and DDD but had the opposite effect when cells were exposed to DDE (Table 4.6 and Figure 4.6). However, these observations were not significant. NAC pre-treatment caused significant reductions in CYP1A1 activity at 50 µM for all three test compounds. Previous reports have shown that NAC is able to inhibit heavy metal-mediated CYP1A1 induction (Elbekai and El-Kadi, 2005; Wu *et al.*, 2009). However, results from the present study do not support the fact that NAC inhibited CYP1A1 induction by the test compounds because if this was the case, NAC would have inhibited CYP1A1 induction across the entire test concentration range of the test compounds. Contrarily, the decrease in CYP1A1 activity was only statistically significant at one particular concentration for all three test compounds (50 µM). This observation is more likely to be due to decreases in cell viability as NAC pre-treatment exacerbated the toxic effects of all three test compounds, as was demonstrated in the cytotoxicity assays.

In summary, results from the present study indicate that DDT is capable of up-regulating CYP1A1 activity. Like DDT, its two metabolites also induced CYP1A1 activity, which is expected as all three compounds are similar in structure. In terms of potency of inducing CYP1A1 activity the three test compounds can be ranked as follows: DDE > DDT > DDD. Pre-treating cells with NAC induced CYP1A1 activity at low concentrations of DDT and DDD but not DDE, suggesting that there may be different mechanisms of activation involved. NAC did cause hepatoprotective, significant decreases in CYP1A1 activity at concentrations of 50 µM of all three test compounds, which corresponds with the decreases in cell viability observed earlier.