Chapter 3: *In Vitro* Cytotoxicity

3.1. Background

Cytotoxicity testing is one of the major assays applied during *in vitro* toxin assessment, which focuses mainly on cell death or some measure of growth impairment. This type of testing is designed to evaluate the intrinsic ability of a compound to kill cells (Ferro & Doyle, 2001). Apart from dosage, two other factors play a major role in the toxicology of an entity: the duration of exposure to a compound and the compound's mechanism of toxicity (Riss & Moravec, 2004). At a cellular level, *in vitro* toxicity can manifest in a number of ways including (Horvath, 1980):

1. diminished cellular adhesion
2. dramatic morphological changes
3. a decrease in replication rate or
4. a reduction in overall viability

Many different assays have been developed to determine *in vitro* toxicity such as quantifying cell death/survival by assessing plasma membrane integrity, cell enumeration by total protein content and enumerating viable cells through assessing certain vital functions. Popular assays that are widely used are the total cellular protein assay (sulforhodamine B), the neutral-red uptake assay, the LDH leakage assay, and the tetrazolium dye assays (Murakami, 2000).

The best known of the tetrazolium assays is probably the MTT assay for mammalian cells in which 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazolium salt is reduced by the mitochondria of viable cells to insoluble, purple formazan crystals. Although this is one of the most prevalent viability assays, its weakness lies in yielding false positives in the presence of a number of compounds including albumin and antioxidants such as ascorbic acid, cysteine and glutathione (Funk *et al.*, 2007).

The neutral red uptake (NRU) assay is also widely used to determine cell viability. Neutral red, a supravital (non-toxic) dye, relies on the principle of dye accumulation in the lysosomes and Golgi apparatus of viable, uninjured cells and has the following advantages:

1. it does not rely on a reduction reaction to determine viability, excluding susceptibility to making type I errors (false positives) in the presence of antioxidants or other reductive agents
2. it is cost-effective
3. it is quick
4. is reported to be more sensitive to changes in cell viability of hepatocyte cultures as compared to total protein content determination and the LDH leakage assay (Fotakis and Timbrell, 2006)

In this chapter the effects of DDT, DDE and DDD on hepatocyte viability are presented. Initial cytotoxicity testing was performed in order to establish a concentration range that would be used throughout the study. The concentration range that was selected covered a wide toxicity range, from merely affecting cell function to complete loss of cellular function and viability. This was done in order to assess the incremental effects of the test compounds on different parameters at different levels of toxicity, which may shed light on the optimal concentration range that can be utilised with the procedure developed in the present study.

3.2. Methods

3.2.1. Experimental design

After a 48 h incubation period (to allow cells to adhere to the wells), cells were exposed to DDT, DDE and DDD at concentrations of 5, 10, 50, 100 and 150 µM. Tamoxifen (150µM), which is known to induce cell death through apoptosis in HepG2 cells (Guo *et al.*, 2010), was used as positive control. DMSO (0.5%) was included as vehicle control. After exposure, cells were incubated for 24 h at 37°C until performing the viability assay. To assess the possible

hepatoprotective effects of NAC, three additional experiments were conducted, which included an additional 1 h pre-treatment with 620 nM NAC, prior to test compound exposure.

3.2.2. Viability assay

Exposure medium was aspirated and replaced with 100 µl EMEM containing 100 µg/ml Neutral red dye. Cells were incubated with the dye for 2 h at 37°C, after which medium was discarded and cells were washed with PBS (200 µl). Plates were dried overnight at 40°C and the accumulated dye dissolved by adding 100 µl of Neutral red elution buffer to each well and incubated at room temperature on an orbital shaker for 40 min (Fotakis and Timbrell, 2006). The amount of dye accumulated by the cells in each well was quantified by measuring the absorbance at 540 nm with a reference wavelength of 630 nm (Biotek XL plate reader).

3.2.3. Statistical analyses

Six experiments were carried out in duplicate (***n*** **=** 12) to assess viability response. All observed results were standardised to percentage of control values. Quality of the collected data was assessed by plotting the distributions for the tested concentration of each tested compound. Grubb's test was performed to remove outliers, after which a Shapiro-Francia test was used to assess normality based on a discrete number. Depending on the normality of the data, student's ***t***-tests (parametric) or Mann-Whitney *U*-tests (non-parametric) were performed across the respective concentration ranges to determine whether the different concentrations had any statistically significant influence on HepG2 viability. Three additional experiments were carried out in duplicate to assess the possible effects that NAC may have on the initially observed toxicity. 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), no outliers were removed and normality of the data could not be established. Therefore, all NAC results were analysed using Mann-Whitney tests. The 50% Inhibitory concentrations (IC_{50}) were determined by fitting a Hill equation with variable slope to the observed data. Analyses were performed using GraphPad Prism 5.0 (www.graphpad.com) and the freeware package, R 2.12.1 (www.r-project.org). All reported results are given as mean ± standard error of the mean (SEM), unless stated otherwise.

3.3. Results

Normality testing revealed non-normal distributions, especially for the higher concentrations of the tested compounds. The different distributions for DDT, DDE and DDD are illustrated in Figures 3.1, 3.2 and 3.3, respectively. Non-normality of some distributions was confirmed with the Shapiro-Francia test (Table 3.1). The Shapiro-Francia test is based on hypothesis testing: the null hypothesis states that the collected data originates from a normal distribution, if the ***p***-value drops below the chosen alpha level, the null hypothesis is rejected and the data assumed to originate from a non-normal distribution. Concentrations between 50 µM and 150 µM for DDT and DDD and 100 µM for DDE were found to be significantly non-normal. The Control group in the DDT data set is an example of a perfectly normally distributed data set with ***p*** = 1.00 (Figure 3.1A).

DDT did not have any significant influence on viability up to concentrations of 10 µM but did produce slight cellular proliferation with enumeration results being 105.4 ± 3.5% and 103.4 ± 3.8% for concentrations of 5 µM and 10 µM, respectively (Figure 3.4). Higher concentrations resulted in significant (***p*** < 0.001) decreases in viability. Concentrations of 50, 100 and 150 µM DDT induced 54.3 ± 3.7%, 74.5 ± 4.8% and 74.4 ± 3.8% losses in viability, respectively (Figure 3.4). The Hill equation fitted the observed DDT results well with a coefficient of determination (r^2) of 0.86, meaning that 86% of the variance observed in the results can be accounted for by the fitted model (Figure 3.4). From the fitted equation it was deduced that DDT has an IC_{50} value of 54 ± 1 µM after 24 h exposure, if a cell density of 2×10^4 cells/well is used (Table 3.2).

NAC pre-treatment did not alleviate but rather aggravated DDT toxicity, decreasing the IC_{50} to 40 ± 1 µM, after 24 h exposure (Table 3.2 and Figure 3.4). Compared to DDT exposure alone, NAC pre-treatment significantly decreased the viability at low concentrations of 5 µM ($p < 0.05$) and 10 µM ($p < 0.01$) (Figure 3.4).

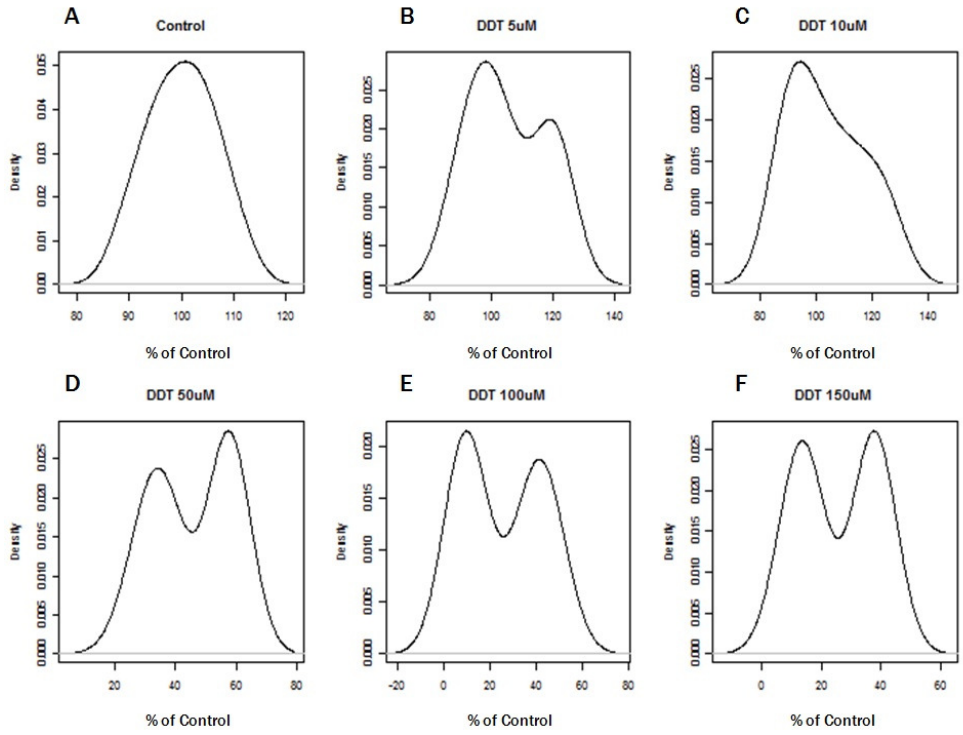

Figure 3.1. Histogram density plots of the observed viability data of HepG2 cells exposed to DDT demonstrating the distributions of the collected data. The control group is a good example of a normal distribution. The observations did not always follow a normal distribution, especially in the higher ranges of viability. X-axis represents observed values and Y-axis, the count.

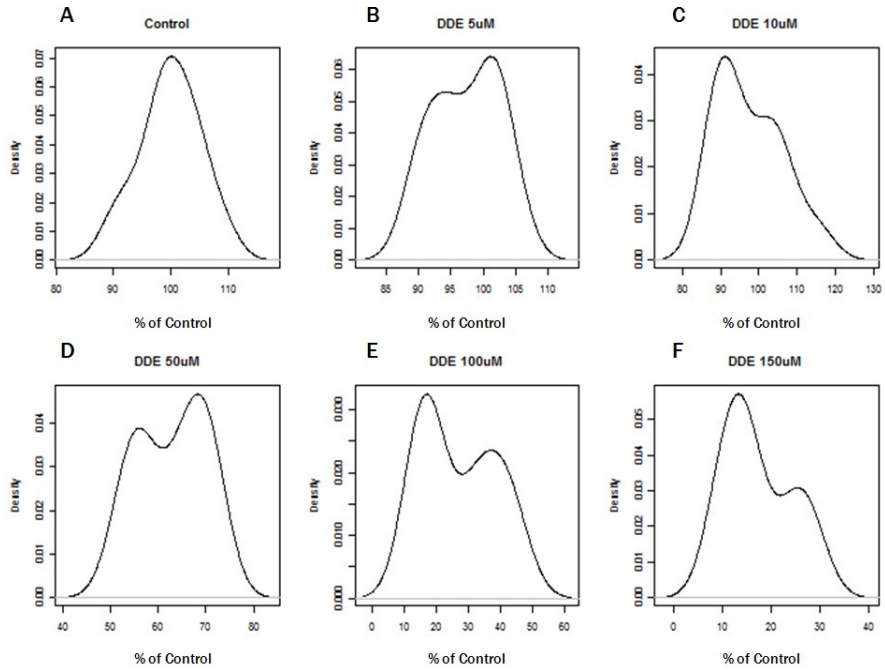

Figure 3.2. Histogram density plots of the observed viability data of HepG2 cells exposed to DDE demonstrating the distributions of the collected data. The results do not follow a normal distribution. X-axis represents observed values and Y-axis, the count.

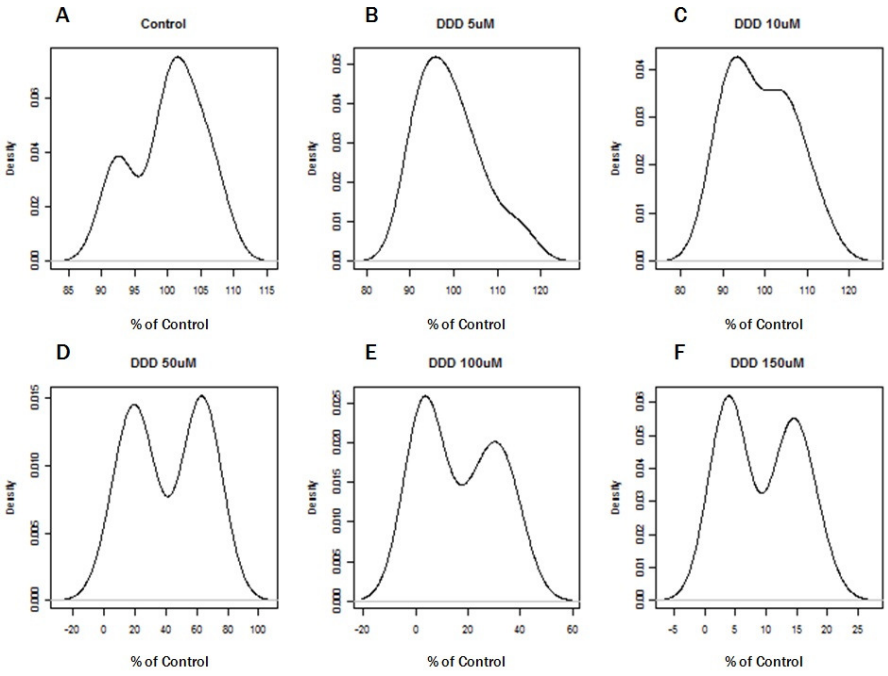

Figure 3.3. Histogram density plots of the observed viability data of HepG2 cells exposed to DDD demonstrating the distributions of the collected data. Data is non-normal as indicated with distinct multiple peaks instead of a single peak. X-axis represents observed values and Y-axis, the count.

Similar to the parent molecule, DDE produced a dose-dependent decrease in viability. Concentrations up to 10 µM did not produce any significant fluctuations in viability. However, unlike DDT, it caused a slight decrease in viability at the 5 µM and 10 µM concentrations, yielding 97.4 ± 1.5% and 97.5 ± 2.5% viability, respectively (Figure 3.4). Concentrations between 50 µM and 150µM produced significant deviation from the control mean ($p < 0.001$). A 50 µM concentration of DDE caused a 37.1 ± 2.0% decrease in viability, while 100 and 150 µM lowered cell viability by 73.1 ± 3.3% and 82.6 ± 2.0% respectively (Figure 3.4). At a cell density of 2×10^4 cells/well, DDE demonstrated an IC_{50} of 64 ± 1 µM ($r^2 = 0.95$) after 24 h exposure (Table 3.2 and Figure 3.4).

Again, NAC pre-treatment did not alleviate the toxicity of the test compound, decreasing the IC_{50} to 58 ± 1 µM, after 24 h exposure (Table 3.2 and Figure 3.4). When compared to DDE exposure alone, NAC pre-treatment significantly ($p < 0.05$) decreased the viability at concentrations of 10 µM and 50 µM (Figure 3.4).

Table 3.1. Shapiro-Francia test results for normality of the observed data. Values given in the table are p-values and instances where p < 0.05 are not normally distributed (*).

Concentration	DDT	DDE	DDD
Control	1.00	0.91	0.54
5µM	0.22	0.39	0.23
10µM	0.33	0.12	0.64
50µM	0.02*	0.33	0.02*
100µM	0.02*	0.03*	0.01*
150µM	0.02*	0.24	0.01*

Table 3.2. **IC_{50} values (±SEM) of cells with/without NAC pre-treatment prior to test compound exposure.**

Test compound	No pre-treatment (µM)	NAC pre-treatment (µM)
DDT	54 ± 1	40 ± 1
DDE	64 ± 1	58 ± 1
DDD	44 ± 1	33 ± 1

Similar to DDT and DDE, DDD also produced a dose-dependent decrease in HepG2 viability. DDD did not significantly affect HepG2 viability up to concentrations of 10 µM, with 5 µM and 10 µM concentrations yielding viabilities of 99.1 ± 2.1% and 99.0 ± 2.3%, respectively. However, concentrations of ≥ 50 µM induced significant ($p < 0.001$) decreases in viability (Figure 3.4). The Hill equation fitted the observed data well ($r^2 = 0.91$). From this analysis, DDD showed an IC_{50} of 44 ± 1 µM after 24 h exposure (Figure 3.4).

NAC pre-treatment produced results similar to those observed with DDT and DDE by not alleviating the toxicity of the test compound at low test compound concentrations. The IC_{50} was decreased to 33 ± 1 µM, after 24 h exposure (Table 3.2 and Figure 3.4). Compared to DDD exposure alone, NAC pre-treatment significantly ($p < 0.001$) decreased the viability at a concentration of 50 µM (Figure 3.4).

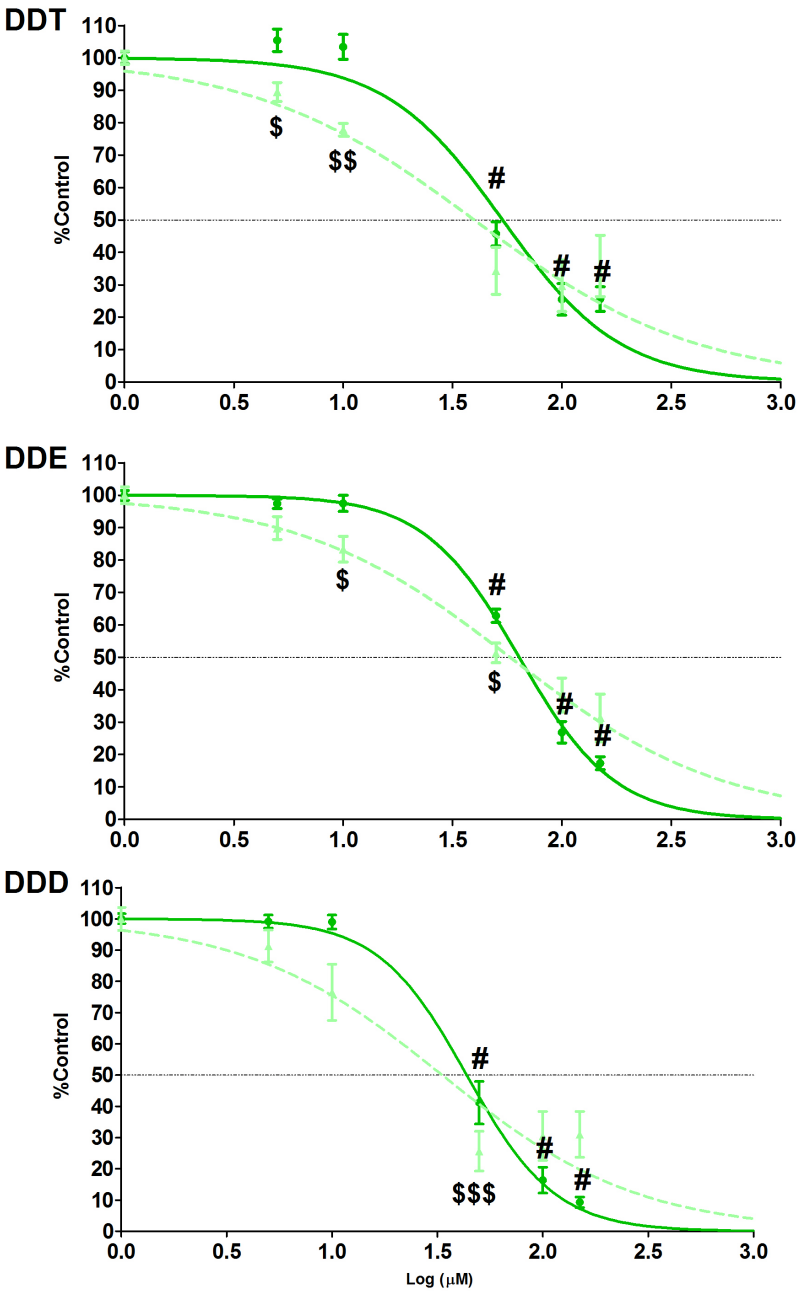

Figure 3.4. Fitted dose-response curves of viability of HepG2 cells after DDT, DDE and DDD treatment (mean ± SEM). Dark green curves represent the test compounds alone and light green curves, cells pre-treated with NAC. Curves were obtained by fitting viability results to a four-parameter Hill equation with variable slope and the following constraints: top = 100 and bottom = 0. Graphs are plotted on semi-logarithmic axes. Dashed horizontal lines represents Y = 50%. **#** = $p < 0.001$, treatment with test compound alone compared to controls. **$** = $p < 0.05$, **$$** = $p < 0.01$, **$$$** = $p < 0.001$, pre-treatment with NAC compared to treatment with test compound alone.

3.4. Discussion

Figures 3.1 - 3.3 present the data distribution plots of the observed data for the different concentrations of DDT, DDE and DDD, respectively. In an ideal situation these curves would resemble a bell-shaped curve. Rather than describing the endpoint, these curves are good for describing the observed data itself and provide guidance for subsequent statistical manipulation. Noticeable from the graphs of some of the data are more than one peak. This is indicative of more than one population being present in a particular set of data. For example, these curves are very closely related to histograms obtained from flow cytometry. If the X-axis of a histogram represents size and the Y-axis count or density, a mixture of oocytes and spermatozoa will present as two distinct populations in terms of size. In the same way, the curves presented here show the number of observations made at a particular value, which in this case would be a % of control value. However, unlike flow cytometry, increasing the number of repetitions would eventually produce a perfect bell-shaped curve.

More than one population indicates variability between experiments. In this way these curves can be interpreted as a measure of the robustness of an assay. However, care should be taken when interpreting these curves as multiple populations may also be the result of outliers. For this reason, outliers identified by the Grubb's test were removed prior to testing for normality (Shapiro-Francia test). Data collected for the three test compounds indicates a trend in that higher concentrations (50 - 150 µM) of all three test compounds demonstrated multiple peaks. This was confirmed with the Shapiro-Francia test (Table 3.1), where most of the 50 - 150 µM data sets proved to be significantly non-normal. The multiple populations that are observed here may have been the result of differences between the preparation of batches of NR dye, buffers or test compounds or differences between cell passages, incubation times, instrument conditions etc. These results should not be considered definitive as the SEMs are also indicative of reproducibility. Also, mean values obtained from the pooled individual assay results yielded good dose-response curves with r^2 values ≥ 0.86, which is acceptable considering it is a non-linear fit. This means that the maximum amount of variability that could not be attributed for was 14% in the case of

DDT, whereas it was 5% and 9% for DDE and DDD, respectively. Taken collectively, the above-mentioned indicates that the NRU assay yielded reproducible results but has its limitations.

With regards to the effects of the test compounds on cell viability, high concentrations of the test compounds were necessary to significantly decrease cell viability after 24 h of exposure. Significant cytotoxicity was only evident at concentrations above 10 µM with IC_{50} values of 40 - 60 µM. Medina-Diaz & Elizondo (2005), who studied the effects of DDT on HepG2 viability, also reported the onset of toxicity at concentrations higher than 10 µM. Results suggest that DDT (but not DDE or DDD) may induce cellular proliferation at sub-toxic concentrations. Although not significant, exposure to 5 µM DDT resulted in a 5% increase in cell number when compared to the control. A possible explanation for this can be found in the work of Kiyosawa *et al.* (2008), who demonstrated the up-regulation of the cell proliferation–related genes *Ccnb1*, *Ccnb2*, *Ccnd1*, *Stmn1*, and *Mdm2* in the livers of rats exposed to DDT. These authors concluded that up-regulation of these genes were facilitated by activation of the constitutive androgen receptor by DDT, which also resulted in the up-regulation of CYP3A4. As the exposure period was only 24 h, it is possible that a longer incubation time could increase proliferation significantly. Cellular proliferation induced by DDT has also been reported in MCF-7 cells (breast adenocarcinoma) (Diel *et al.* 2002). Surprisingly, this proliferative effect decreased at a concentration of 10 µM DDT, which, in the present study, was found to be the concentration that did not adversely affect cell viability. Diel *et al.* (2002) suggested that the decrease in proliferation might be due to the onset of toxicity at these concentrations. Rat mammary gland proliferation induced by DDT has also been reported *in vivo* (Upalla *et al.*, 2005).

In contradiction to the results from the present study, Delescluse *et al.* (1998) observed no toxicity in HepG2 cells exposed to DDT at concentrations as high as 100 µM of DDT. Dehn *et al.* (2005) reported an IC_{50} of 1 mM for DDT in HepG2 cells, which is approximately 20 times greater than the value determined in the present study (54 µM). This difference could possibly be attributed to the ten-fold difference in cell density used between the two

studies: 2×10^5 cells/well (Dehn *et al.*, 2005) *vs.* 2×10^4 cells/well, as well as the vehicle solvent concentration. In the present study the vehicle solvent, DMSO, did not exceed a final concentration of 0.5% (v/v) compared to a 2.5% (v/v) concentration in the study by Dehn *et al*. (2005). It has been reported that the addition of DMSO to culture medium improves the cell viability of isolated primary hepatocytes (Banic *et al.*, 2011), which may also contribute to the difference in IC_{50} values between the two studies.

The IC_{50} values reported for DDT in other hepatocyte cell cultures such as primary hepatocytes and HaCaT cells (rat liver) were 250 µM and 70 µM, respectively (Delescluse *et al.* 1998). These IC_{50} values and that reported in the present study suggest that different hepatocyte cultures may not necessarily respond in the same way to DDT (and other test compounds for that matter). Primary cell cultures are considered the 'gold standard' but as already mentioned, there are many drawbacks in terms of feasibility and predictability when utilising these hepatocytes.

At the highest concentration tested (150 µM) of all three test compounds some cells remained viable. This could demonstrate the resilience of hepatocytes but may also be a drawback of the assay that was employed; in that complete cell death was not achieved. Some of the NR dye did bind to the well surface, mimicking cell numbers. However, because the relevant blanks were included in the assay setup, this should have compensated for any non-specific staining. Nonetheless, the assay yielded good dose-response curves (Figure 3.4) and the results were reproducible (average coefficient of variation < 10), demonstrating the reliability of the NRU assay.

Noticeable from Figure 3.4 is the difference in slope of the dose-response curves of the test compounds alone compared to those of the cells that were pre-treated with NAC. When considering the IC_{50} values, NAC pre-treatment did not protect against toxicity induced by any of the three test compounds. Rather, it seemed to exacerbate it. On examination of the dose-response curves this exacerbating effect can be seen at concentrations of 5 - 50 µM of

all three test compounds. However, at higher concentrations (100 -150 µM), NAC pre-treatment appeared to have alleviated some of the toxicity induced by the three test compounds. Exactly how this may have happened is not obvious but these results suggest that different aspects of cellular physiology are involved. On the one hand, lower concentrations (5 - 10 µM) of the test compounds may appear non-toxic due to some cellular response that allows the cells to cope with the xenobiotics. This crucial response may be counteracted by NAC, which will then decrease the resistance of the cells to the exogenous stressor, causing the loss of cell viability observed at these concentrations.

On the other hand, above some threshold concentration the cells may not be able to counteract the effect of the test compounds, resulting in cell death. NAC may be able to counteract this mechanism of toxicity, which would alleviate some of the toxicity seen above this threshold concentration, as observed for concentrations of 100 - 150 µM of all three test compounds. No literature was found to compare/support any of the NAC pre-treatment results observed in the present study.