In vitro effect of N-acetylcysteine on hepatocyte injury caused by dichlorodiphenyltrichloroethane and its metabolites

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Abstract

The organochlorine pesticide, dichlorodiphenyltrichloroethane (DDT), is still used to combat the spread of malaria in several developing countries despite its accumulation and known hepatotoxic effects that have been demonstrated both *in vitro* and *in vivo*. *N*-Acetylcysteine (NAC) is a recognized hepatoprotective agent that has been reported to reduce hepatotoxicity initiated by many different compounds. The aim of this study was to determine whether NAC could counter *in vitro* hepatocyte injury induced by DDT or its two major metabolites, dichlorodiphenyldichloroethylene and dichlorodiphenyldichloroethane. HepG2 cell cultures were used to assess the following parameters of toxicity: cellular viability, intracellular levels of reactive oxygen species (ROS), mitochondrial membrane potential and initiation of apoptosis. None of the three test compounds induced ROS generation, yet exposure to any of the three compounds produced mitochondrial hyperpolarization, which was countered by NAC pretreatment. All three test compounds also induced apoptotic cell death, which was inhibited by NAC. Despite NAC counteracting some adverse intracellular changes due to organochlorine exposure, it appeared to aggravate the cytotoxic effects of the organochlorine compounds at low test concentrations. As the same outcome may also occur *in vivo*, results from the present study raise concern about the use of NAC as treatment for DDT-induced hepatotoxicity.

Keywords

DDT, hepatotoxicity, mitochondria, *N*-acetylcysteine, ROS, apoptosis

Introduction

The organochlorine pesticide, dichlorodiphenyltrichloroethane (DDT), is still used in many developing countries to combat the spread of malaria. It is primarily applied as an indoor residual spray, leaving behind a residue on the indoor surfaces of homes that will deter/kill disease-carrying mosquitoes.[1–3] After uptake, DDT has a very slow elimination rate by higher organisms due to its accumulation in fatty tissues and is not easily eliminated from the body as it does not readily re-enter the systemic circulation, resulting in high tissue concentrations that may affect higher organisms.[4] *In vivo*, DDT accumulation has been reported to occur from dietary concentrations as low as 1 ppm (1 μg/ml or 2.8 μM).[5] Bioconcentration of DDT through the food chain confirms the oral route of exposure. This implicates the liver as one of the principal targets of toxicity because of first-pass metabolism. Furthermore, this also implies that the liver is the primary target for the major metabolites

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of DDT, dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD). The key function of the liver in the detoxification of xenobiotics partly explains its unique susceptibility to injury from such compounds.[6] Hepatocyte alterations due to DDT have been found to appear after dietary exposure levels as low as 5 ppm (5 μg/ml or 14 μM).[5] Both *in vitro* and *in vivo* studies have demonstrated that DDT is able to induce hepatocyte/liver injury,[7–9] which occurs mainly in the form of cytoplasmic vacuolization and inflammatory cell infiltration, resulting in centrolobullar zonal necrosis and increased relative liver weight due to tissue regeneration. Numerous *in vivo* studies have implicated oxidative stress as a key constituent in organochlorine-induced hepatotoxicity. Organochlorines such as lindane,[10] dieldrin[11] and endosulfan[12] have been shown to produce oxidative hepatotoxicity through lipid peroxidation, DNA adduct formation and depletion of the innate antioxidant defence mechanisms.

N-Acetylcysteine (NAC) first found its use in the clinical setting during the 1960s as a mucolytic agent but is also recognized as the mainstay antidote for acetaminophen/paracetamol poisoning.[13–15] NAC, a thiol, is the acetylated derivative of the amino acid L-cysteine, a precursor of endogenous reduced glutathione. Its mechanism of action in treating acetaminophen overdose is thought to be the replenishment of depleted intracellular glutathione stores or to act as an alternative thiol substrate. In addition to this major role, NAC is also able to reduce disulphide bonds, complex metal contaminants, such as methylmercury in blood and scavenge hydroxyl and peroxyl free radicals.[16–18] NAC is a recognized hepatoprotective agent and has been reported to alleviate hepatotoxicity initiated by various groups of compounds including (1) anaesthetics and other halogenated hydrocarbons like halothane,[19] chloroform[20] and carbon tetrachloride,[21] (2) industrial waste products such as *N*-nitrosodimethylamine,[15] (3) natural products like pyrrolizidine alkaloids[22] and *Amanita* mushrooms,[21] (4) antituberculosis drugs including isoniazid, rifampicin and pyrazinamide[23] and (5) pesticides like paraquat.[21]

As NAC has been proven to protect against hepatotoxicity induced by a variety of compounds, and since exposure to organochlorines continues, the present study is aimed at assessing the efficacy of NAC in preventing *in vitro* hepatocyte injury initiated by exposure to DDT or either of its two major metabolites, DDE or DDD. Different cell-based assays were applied to examine parameters that included cytotoxicity, mitochondrial effects, intracellular reactive oxygen species (ROS) and apoptosis. The HepG2 cell line was used in this study because it is a well-established hepatocyte model that has been used previously to investigate the effects of various chemical entities on the parameters in questions including cytotoxicity,[24] mitochondrial homeostasis,[25,26] oxidative stress[26] and apoptotic cell death via caspase-3 (Cas-3) activation.[27]

Materials and methods

Reagents

Dimethylsulfoxide (DMSO), NAC, neutral red dye, 2′,7′-dichlorodihydrofluoresceindiacetate (DCFH-DA), 2,2′-azo-bis(2-methylpropionamidine) dihydrochloride (AAPH), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1), tamoxifen, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride, β-mercaptoethanol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *N*-acetyl-Asp-Glu-Val-Asp-7-amino -4-methoxycoumarin (Ac-DEVD-AMC), staurosporine and Eagle's minimum essential medium (EMEM) were all purchased from Sigma-Aldrich (St Louis, USA). Foetal calf serum (FCS) was obtained from Highveld Biological (Johannesburg, South Africa). Ethanol and acetic acid was purchased from Illovo Chemicals (Durban, Republic of South Africa) and Merck Chemicals (Darmstadt, Germany), respectively. *p′p′*-DDT, *p′p′*- DDE and *p′p′*-DDD were purchased from Supelco Analytical (Bellefonte, Pennsylvania, USA). Sterile white 96-well plates, suitable for fluorescence, were purchased from Nunc (Roskilde, Denmark).

Cell culture and seeding

HepG2 cells (ATCC HB-8065) were propagated in a carbon dioxide (CO_2) tissue culture incubator (Binder C150, Binder, USA) in EMEM containing non-essential amino acids and fortified with 10% FCS. Cells were grown until 80% confluent before being harvested using trypsin-EDTA and split (1:5) with fresh media. For experimental purposes, cells were harvested and 100 μl were seeded into white 96-well microplates at a density of 2×10^4 cells/well. Cells were then incubated for 48 h under propagation conditions to allow for attachment and acclimatization.

Preparation of test compounds

Stock solutions of all the test compounds were prepared in DMSO at concentrations of 30 mM. Stock solutions of the positive controls, tamoxifen and AAPH, were also made up to 30 mM using DMSO. Staurosporine was made up to 2.14 mM (1 mg/ml) and NAC to 123 mM (20 mg/ml), also in DMSO. Aliquots of all stock solutions were stored at −70°C until use. For cell viability determination, the test compounds were diluted with culture medium to obtain working solutions with concentrations of 10, 20, 100, 200 and 300 μM. For the mitochondrial membrane potential and ROS determination assays, the compounds were diluted with phosphate buffered saline (PBS) to obtain working solutions with concentrations of 10, 20, 100, 200 and 300 μM. In keeping with the test compounds, positive controls were diluted to 300 μM working solutions with either culture medium or PBS. Staurosporine was diluted to a 22 μM working solution in culture medium.

Cell viability assay

After seeding, 100 μl of the working solutions of DDT, DDE or DDD were added to separate wells on a seeded microtiter plate to obtain final exposure concentrations of 5, 10, 50, 100 and 150 μM of the test compounds. The selected concentration range was based on unpublished pilot cell viability experiments. As different cellular events require different timescales to be observed, exposure times to toxins for different endpoint assays ranged from 1 h to 24 h, depending on the parameter being investigated. To assess the effects on cell viability, cells were exposed to the test compounds for 24 h under propagation conditions. Vehicle-treated controls (0.5% DMSO) and relevant blanks were included. Cell viability was determined using the neutral red uptake assay as described by Fotakis and Timbrell.[28]

Reactive oxygen species

Intracellular levels of ROS were measured according to the endpoint method of Zhang et al.[29] with minor modifications. In brief, following acclimatization, cells were loaded with medium containing 6 μM DCFH-DA in culture medium for 1 h at 37°C and 5% CO_2. Thereafter, medium was aspirated and cells were washed with PBS. A volume of 50 μl PBS was immediately added to each well to keep the cells hydrated. This was followed by a further 50 μl of working solutions of the test compounds, diluted in PBS, to yield final exposure concentrations of 5, 10, 50, 100 and 150 μM of DDT, DDE or DDD. Concentrations were selected to coincide with cell viability experiments described above. Cells were then incubated with the test compounds for 3 h to allow any ROS generation to develop, whilst limiting potential metabolism of the parent compound. Following the 3 h exposure, fluorescence intensities were measured with a FluoStar Optima fluorescent plate reader using $\lambda_{ex} = 492$ nm and $\lambda_{em} = 525$ nm at a gain setting of 750. Blanks and vehicle-treated controls (0.5% DMSO) were included in all experiments. AAPH (150 μM) was used as a positive control as it was previously reported to induce ROS generation.[30]

A further set of kinetic experiments was carried out to confirm results initially observed using the endpoint experiments. The same experimental setup was followed as before but with two exceptions: (1) the final exposure concentration range was broadened to include 0.01, 0.1, 1, 10 and 100 μM concentrations of DDT, DDE or DDD and (2) fluorescence intensities were measured over a period of 14 h at 30 min intervals.

Mitochondrial membrane potential

The effects of the test compounds on mitochondria were assessed by measuring the mitochondrial membrane potential ($\Delta\psi_m$) using the ratiometric fluorescent dye, JC-1, according to the method of Nuydens et al.[31] with slight modifications. Briefly, after seeding, cells were loaded with medium containing 10 μM of JC-1 in culture medium for 30 min at 37°C and 5% CO_2. After loading, cells were washed with PBS and 50 μl of PBS was immediately added to each well to keep the cells hydrated. This was followed by addition of 50 μl of the working solutions of DDT, DDE or DDD, diluted in PBS, to yield final exposure concentrations of 5, 10, 50, 100 and 150 μM of the respective test compounds. Concentrations were selected to coincide with cell viability experiments described above. Cells were then exposed to the test compounds for 1 h under propagation conditions. Thereafter, fluorescence intensities were measured with a FluoStar Optima fluorescent plate reader using $\lambda_{ex} = 492$ nm and $\lambda_{em} = 525$ nm to detect the monomeric form of JC-1 and $\lambda_{ex} = 545$ nm and $\lambda_{em} = 595$ nm to detect the aggregate form of JC-1. A gain setting of 1000 was used. The ratio of red/green fluorescence was used as an indication of $\Delta\psi_m$. Blanks

and vehicle-treated controls (0.5% DMSO) were included in all experiments. Tamoxifen (150 μM) was used as a positive control as it was previously reported to induce hyperpolarization of the $\Delta\psi_m$.[32]

Apoptotic cell death

As a measure of apoptotic cell death, Cas-3 activity was fluorometrically determined using the protocol of the kit manufacturer (CAS3F kit, Sigma-Aldrich, St Louis, USA), with modifications. After seeding, 100 μl of the relevant working solutions were added to different wells on a microtiter plate to obtain final exposure concentrations of 5, 10, 50, 100 and 150 μM of DDT, DDE or DDD. Cells were then treated under propagation conditions for 6 h. Thereafter, plates were placed on ice for 20–30 min to cool. As soon as the plates were cold to the touch, medium was aspirated and replaced with 25 μl of ice-cold lysis buffer (10 mM HEPES, 2 mM CHAPS, 5 mM EDTA, 5 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol) and incubated on ice for a further 20 min. Phenylmethylsulfonyl fluoride and β-mercaptoethanol were added into the lysis buffer 30 min prior to using the lysis buffer on the cells. After lysis, 100 μl of assay buffer (20 mM HEPES, 2 mM EDTA, 5 μM Ac-DEVD-AMC, 5 mM β-mercaptoethanol) was added and the plates were incubated overnight at 37°C. Ac-DEVD-AMC and β-mercaptoethanol were added to the assay buffer 30 min prior to initiating the assay. At the end of an approximately 18 h incubation period, the released 7-amino-4-methylcoumarin was measured using a FluoStar Optima fluorescent plate reader with $\lambda_{ex} = 360$ nm and $\lambda_{em} = 460$ nm with a gain setting of 750. Blanks and vehicle-treated controls (0.5% DMSO) were included in all experiments. Staurosporine (11 μM) was used as a positive control as it is known to induce Cas-3 activity.[33]

NAC pretreatment

To investigate the effects that NAC exerts on organochlorine-induced hepatocyte injury, all endpoint experiments were repeated but cells were pretreated for 1 h with 100 μg/ml of NAC in culture medium prior to initiating exposure to the organochlorine test compounds. Pretreating HepG2 cells with 100 μg/ml of NAC has been shown to reverse cytotoxicity induced by cadmium exposure.[34]

Statistical analyses

For initial experiments in which cells were exposed to the individual organochlorine compounds alone, six independent experiments were carried out in duplicate ($n = 12$). Relevant blank values were included in all experiments. For each assay, background noise was deducted from all experimental values before observed values were standardized to the percentage of vehicle-treated controls. Outliers were detected using Grubbs' test and removed (never more than 2, therefore always $n \geq 10$). Normality of the data distributions was evaluated with the Shapiro–Francia test. For experiments in which the cells were pretreated with NAC, three independent experiments were carried out in duplicate ($n = 6$) and no outliers were removed. To determine whether any observable differences between the treatment group means were statistically significant, either Student's *t*-tests (for normal distributed data) or Mann–Whitney tests (for non-normal distributed data) were applied. Results were normalized against the relevant vehicle-treated controls and are presented as mean ± SEM. Dose-dependent inhibitory effects were fitted on semi-logarithmic axes using a four-parameter Hill's equation with no constraints and are described as curve maximum plateau (% of vehicle-treated control), curve minimum plateau (% of vehicle-treated control) and the extrapolated concentration that is estimated to produce a 50% decrease in cell viability relative to vehicle-treated controls (LC_{50}; μM). Results from the kinetic ROS experiments were analysed by two-way analysis of variance. Possible correlations between data sets were evaluated using Pearson's correlation test (only if data sets were shown to have normal distributions). GraphPad Prism v5.0 and the freeware package R v2.13.1 were used for all statistical manipulations and analyses.

Results

Cytotoxicity

Compared to the vehicle-treated controls, DDT did not have any significant influence on cell viability below concentrations of 10 μM. Exposure to higher concentrations resulted in significant ($p < 0.001$) decreases in cell viability. The DDT dose–response curve was calculated to have a maximum relative viability of 104%, a minimum relative viability of 26% and a relative LC_{50} value of 45 μM. NAC pretreatment, decreased the DDT survival curve maximum

relative viability to 91% but increased the minimum relative viability to 32%. The relative LC_{50} showed a substantial change to only 15 μM following NAC pretreatment (Figure 1).

Concentrations up to 10 μM of the metabolite DDE did not produce any significant fluctuations in cell viability, compared to the vehicle-treated controls. However, in a similar response to DDT, exposure to concentrations >10 μM produced significant cytotoxicity ($p < 0.001$). The fitted DDE dose–response curve was calculated to have a maximum relative viability of 99%, a minimum relative viability of 12% and a relative LC_{50} value of 57 μM. NAC pretreatment slightly decreased the DDE curve maximum relative viability to 97% and increased the minimum relative viability to 18%. The relative LC_{50} shifted to 39 μM after NAC pretreatment (Figure 1).

Similar to the other organochlorine test compounds, DDD did not significantly affect HepG2 viability up to concentrations of 10 μM, but exposure to higher concentrations induced significant ($p < 0.001$) decreases in cell viability. The fitted DDD dose–response curve was calculated to have a maximum relative viability of 101%, a minimum relative viability of 7% and a relative LC_{50} value of 41 μM. NAC pretreatment slightly decreased the DDD maximum relative viability to 91% but increased the minimum relative viability to 29%. Again, a noteworthy decrease in relative LC_{50} to 12 μM was observed following NAC pretreatment (Figure 1).

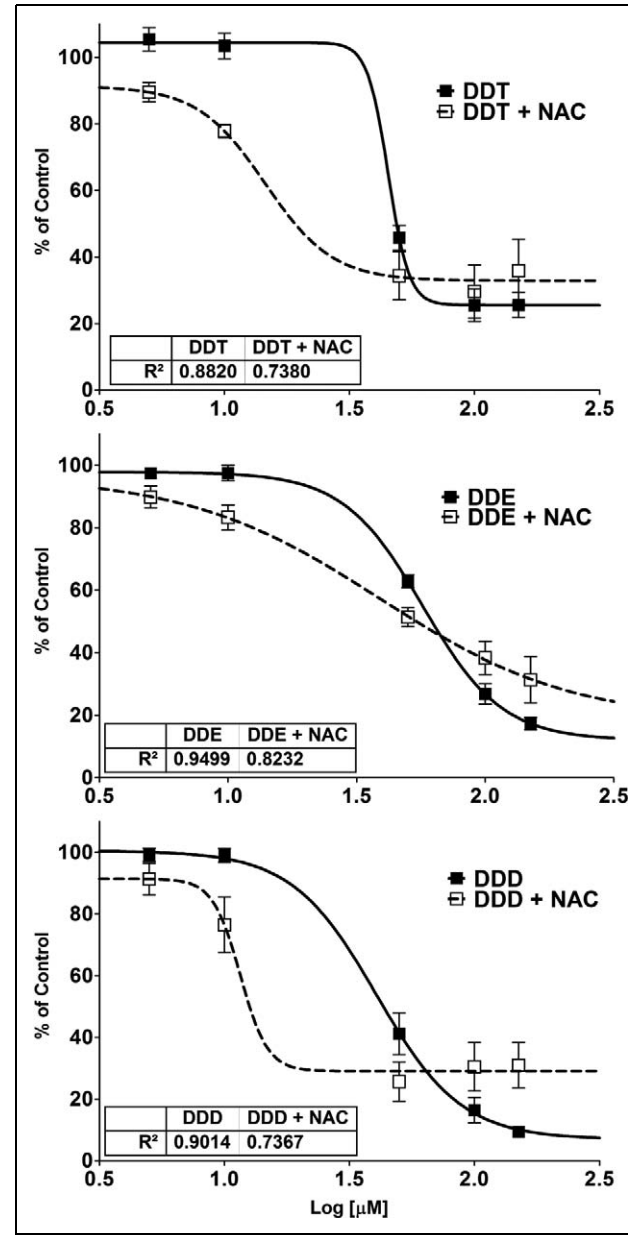

Figure 1. Non-linear regression four-parameter dose–response curves of the cell viability of HepG2 cells following 24 h exposure to 5, 10, 50, 100 and 150 μM concentrations of DDT, DDE or DDD (solid lines). Dashed lines represent fitted dose–response curves of viability of cells pretreated with NAC (100 μg/ml) prior to exposure to the organochlorine test compounds. R^2 = coefficient of determination. *Y*-Axis = percentage viable cells relative to vehicle-treated controls. *X*-Axis = $\log_{10}$ test compound concentration, log-scale axis. DDT: dichlorodiphenyltrichloroethane; DDE: dichlorodiphenyldichloroethylene; DDD: dichlorodiphenyldichloroethane; NAC: *N*-acetylcysteine.

Intracellular ROS

The positive control, AAPH, produced a 4-fold increase in intracellular ROS after 3 h of exposure, compared to vehicle-treated controls. None of the organochlorine test compounds induced any ROS generation in HepG2 cells after 3 h of exposure. However, there was a general trend of decreased intracellular levels of ROS after exposure to the test compounds. DDE had the greatest influence on the degree of intracellular ROS with all but one test concentration (50 μM) significantly ($p < 0.05$) decreasing the intracellular levels of ROS (Figure 2). NAC pretreatment did not produce any significant changes compared to exposure to DDT/DDE/DDD alone (Figure 2).

Kinetic evaluation of intracellular ROS generation induced by the positive control, AAPH, showed a sharp increase in ROS up to 6 h exposure, after which the rate of ROS generation slowly declined to reach a plateau. The maximum fluorescence intensity was approximately 5-fold that of vehicle-treated controls. Compared to the vehicle-treated controls, AAPH

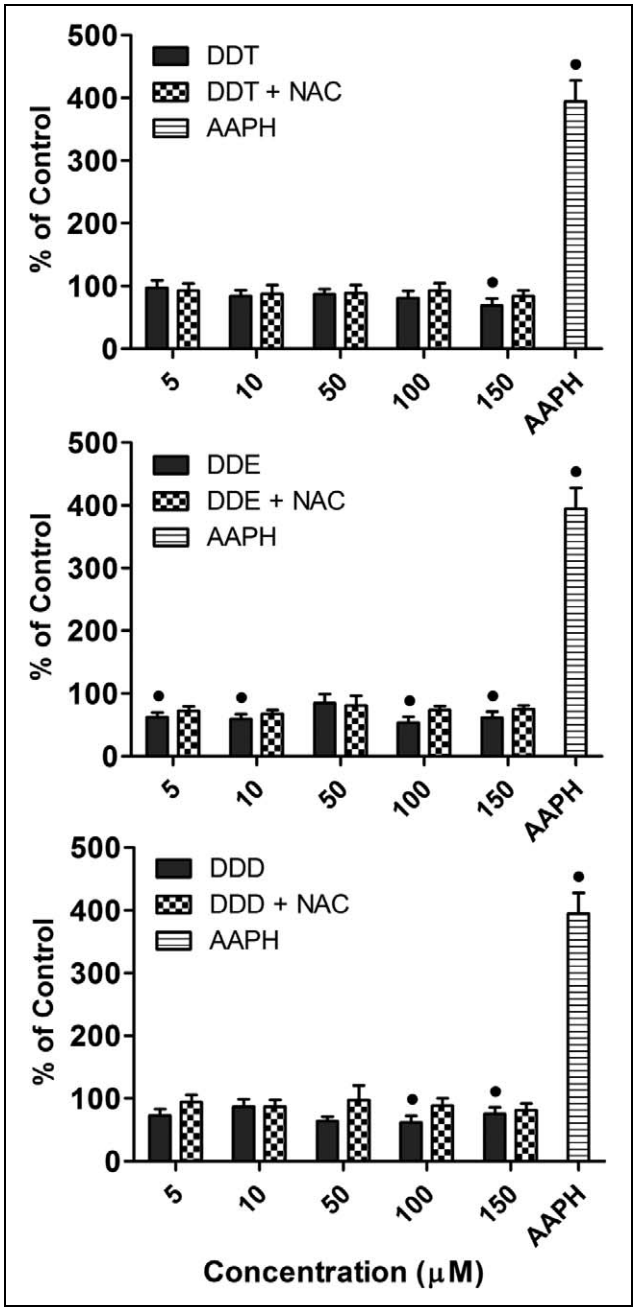

Figure 2. ROS generation in HepG2 cells following 3 h of exposure to DDT, DDE or DDD (mean ± SEM). Solid bars represent cells exposed to the organochlorine test compounds alone. Checkered bars represent cells pretreated with NAC (100 μg/ml) prior to exposure to the organochlorine test compounds. ● = significant deviation from the mean intracellular ROS levels compared to vehicle-treated controls ($p < 0.05$). AAPH (150 μM) was used as positive control to induce ROS generation. *Y*-Axis = intracellular ROS expressed as percentage of vehicle-treated controls. DDT: dichlorodiphenyltrichloroethane; DDE: dichlorodiphenyldichloroethylene; DDD: dichlorodiphenyldichloroethane; NAC: *N*-acetylcysteine; AAPH: 2,2′-azo-bis(2-methylpropionamidine) dihydrochloride; ROS: reactive oxygen species.

significantly ($p < 0.01$) elevated intracellular ROS from 2 h of exposure and longer. No ROS generation was observed when cells were exposed to DDT, DDE or DDD for a period of up to 14 h, but all tested concentrations of DDT, DDE and DDD decreased the intracellular levels of ROS, relative to vehicle-treated controls, from approximately 2 h of exposure onwards. These observations of decreased intracellular levels of ROS were not statistically significant. Results from kinetics experiment are not graphically presented but were used to confirm initial results from endpoint experiments.

Effects on $\Delta\psi_m$

The positive control, tamoxifen, caused significant ($p < 0.001$) hyperpolarization of the mitochondrial membrane, with an almost 7-fold increase in $\Delta\psi_m$, compared to the vehicle-treated controls. All three of the organochlorine test compounds caused slight, non-significant depolarization of $\Delta\psi_m$ at the lower concentrations of 5 and 10 μM. However, DDT exposure resulted in a significant ($p < 0.001$) increase in $\Delta\psi_m$ at concentrations >50 μM (Figure 3). A similar trend was seen with DDE, although the increases were significantly greater than those induced by equivalent dosages of DDT ($p < 0.001$) (Figure 3). Of the three organochlorine compounds, DDD induced the most profound effects, with significant ($p < 0.001$) increases in $\Delta\psi_m$ at concentrations ≥50 μM (Figure 3). At 50 μM, DDD caused an increase equivalent to that produced by 100 μM of DDT (46 *vs.* 45%). At 100 and 150 μM concentrations, DDD yielded 201% and 240% increases in $\Delta\psi_m$ compared with the vehicle-treated controls ($p < 0.001$).

Pretreating cells with NAC countered changes in $\Delta\psi_m$ brought about by the organochlorine compounds. At low concentrations (≤10 μM), where the test compounds induced depolarization, NAC pretreatment caused slight hyperpolarization. At higher concentrations (≥50 μM), where the test compounds induced significant hyperpolarization, NAC significantly reduced $\Delta\psi_m$ (Figure 3).

Apoptosis

Staurosporine, a general inducer of apoptosis that was used as positive control in this study, significantly ($p < 0.001$) induced Cas-3 activity at 11 μM with a mean increase of 97% compared with the vehicle-treated controls. All the tested concentrations of DDT (5–150 μM) were found to significantly ($p < 0.001$)

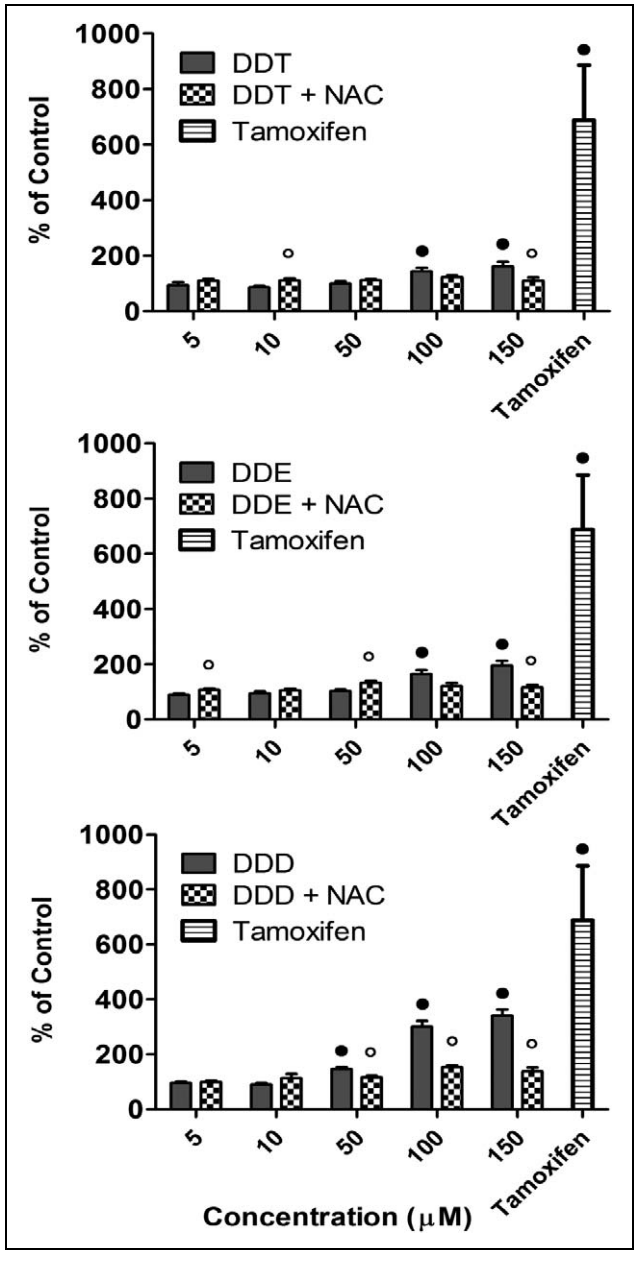

Figure 3. Changes in the $\Delta\psi_m$ of HepG2 cells following 1 h of exposure to DDT, DDE or DDD (mean ± SEM). Solid bars represent cells exposed to the organochlorine test compounds alone. Checkered bars represent cells pretreated with NAC (100 μg/ml) prior to exposure to the organochlorine test compounds. ● = significant increase of $\Delta\psi_m$ induced by organochlorine compounds compared with vehicle-treated controls ($p < 0.05$). **o** = significant effect of NAC pretreatment on change induced by organochlorine test compound ($p < 0.05$). Tamoxifen (150 μM) was used as positive control to induce hyperpolarisation. *Y*-Axis = relative $\Delta\psi_m$ expressed as percentage of vehicle-treated controls. $\Delta\psi_m$: mitochondrial membrane potential; DDT: dichlorodiphenyltrichloroethane; DDE: dichlorodiphenyldichloroethylene; DDD: dichlorodiphenyldichloroethane; NAC: *N*-acetylcysteine.

increase Cas-3 activity in a dose-dependent manner, ranging from a 21% increase (5 μM) to a 63% increase (150 μM) (Figure 3). DDE also significantly ($p < 0.001$) raised Cas-3 activity in a dose-dependent manner at concentrations ≥50 μM. DDD yielded results similar to that of DDT, causing significant ($p < 0.01$) increases in Cas-3 activity at all tested concentrations (5–150 μM) (Figure 4). However, at concentrations of 100 and 150 μM, DDD caused highly significant ($p < 0.001$) elevations in Cas-3 activity compared to the vehicle-treated controls and both of the other two test compounds at the same concentrations.

Pretreating cells with NAC significantly ($p < 0.05$) reduced Cas-3 activity when cells were exposed to concentrations of 50–150 μM of DDT and DDE. Only in cells treated with 150 μM of DDD did NAC pretreatment significantly ($p < 0.001$) reduce Cas-3 activity (Figure 4).

Discussion

High concentrations of the three test compounds were required to significantly decrease cell viability after 24 h of exposure. Significant decreases in hepatocyte viability were only evident at concentrations >10 μM (approximately 3.5 μg/ml or 3.5 ppm) of the test compounds. This observation relates to a previous report where HepG2 cells that were also exposed to DDT for 24 h showed the same toxicity threshold concentration of 10 μM.[9] Interestingly, in rats exposed to DDT, hepatocyte alterations were found to occur from dietary exposure levels of 5 ppm (approximately 14 μM), which closely correlates to the 10 μM *in vitro* toxicity threshold reported here and by Medina-Diaz et al.[9] This same toxicity threshold of 10 μM was also observed in MCF-7 breast adenocarcinoma cells,[35] which suggests some 'basal' mechanism of toxicity involving receptors/pathways conserved across different cell types. In one acute *in vitro* toxicity study using HepG2 cells, approximately 50% cell death was reported at a concentration of 1 mM of DDT,[36] which is a noteworthy difference in toxic response despite using the same cell line. The concentration range used in the present study was selected based on the insolubility of the test compounds at concentrations of 200 μM using 0.5% (volume per volume (v/v)) DMSO in culture medium. Dehn et al.[36] used a 2.5% (v/v) concentration of DMSO, which has been reported to improve the viability of hepatocyte cultures.[37] This could, to some degree, explain the

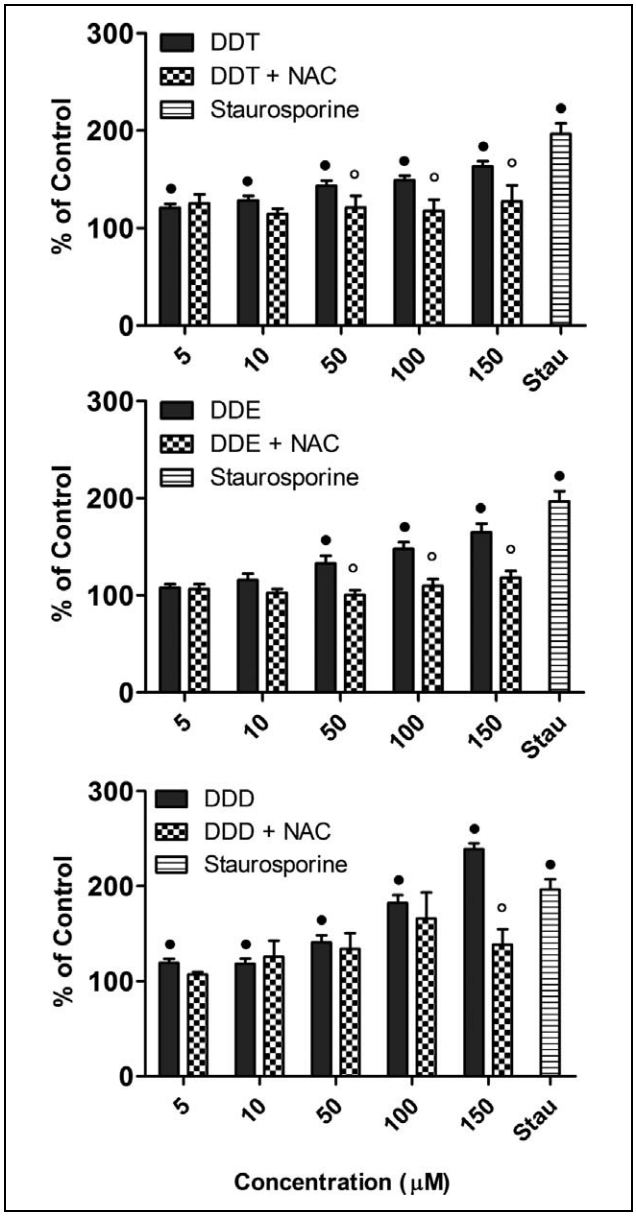

Figure 4. Caspase-3 activity in HepG2 cells following 6 h of exposure to DDT, DDE or DDD (mean ± SEM). Caspase-3 activity was used as a measure of cell death by apoptosis. Solid bars represent cells exposed to the organochlorine test compounds alone. Checkered bars represent cells pretreated with NAC (100 μg/ml) prior to exposure to the organochlorine test compounds. ● = significant increase in caspase-3 activity induced by organochlorine compounds compared with vehicle-treated controls ($p < 0.05$). **o** = significant inhibition of caspase-3 activity induced by organochlorine test compound ($p < 0.05$). Staurosporine (Stau) at a concentration of 11 μM was used as positive control to induce caspase-3 activity. *Y*-Axis = relative caspase-3 activity expressed as percentage of vehicle-treated controls. DDT: dichlorodiphenyltrichloroethane; DDE: dichlorodiphenyldichloroethylene; DDD: dichlorodiphenyldichloroethane; NAC: *N*-acetylcysteine.

differences in response noted between the two studies regarding the effect of DDT on cell viability. Another factor that could contribute to the discrepancy in observations is the 10-fold difference in cell density used between the two studies, 2×10^5 cells/well for Dehn et al.[36] versus 2×10^4 cells/well in the present study. The higher cell density used by Dehn et al.[36] may require a higher concentration of DDT to produce a similar degree of cell death.

The fitted dose–response curve of DDT showed a slight increase in the calculated curve maximum viability (Figure 1), which suggests that DDT may have some proliferative effect at lower test concentrations (<10 μM). This may be due to the upregulation of the cell proliferation–related genes *Ccnb1*, *Ccnb2*, *Ccnd1*, *Stmn1* and *Mdm2*, which have been reported in the livers of rats exposed to DDT.[38] The authors concluded that the upregulation was facilitated by activation of the constitutive androgen receptor by DDT, which also resulted in the upregulation of CYP3A4 enzyme. As the exposure period in the present study was only 24 h, it is possible that a longer incubation time may have resulted in significantly increased proliferation. Cellular proliferation induced by DDT has also been reported in MCF-7 cells.[35] Exposure to DDE and DDD did not exhibit the same proliferative effect as DDT at low test concentrations.

NAC pretreatment reduced the toxicity threshold concentration of all three organochlorine test compounds. This is evident from the fitted curves of DDT and its metabolites, where NAC pretreatment decreased the curve maximum viabilities of the individual test compounds by between 2 and 13% (Figure 1). This effect was more profound with DDT and DDD than with DDE exposure. NAC pretreatment also decreased the relative LC_{50} values of all three organochlorine compounds by between 18 and 30 μM. In stark contradiction to these observations, NAC pretreatment alleviated hepatocytotoxicity brought about by all three organochlorine compounds at the higher concentrations tested (100–150 μM). NAC appeared to increase the minimum relative cell viabilities by between 6 and 22% (Figure 1). Results from the cell viability endpoint assays indicate that, rather than acting as a hepatoprotective agent, NAC used prophylactically may aggravate hepatocyte injury initiated by exposure to DDT or its metabolites, especially at lower concentrations of the test compounds.

These results may seem conflicting to a presumed hepatoprotective effect. However, the mechanism of

hepatoprotection of NAC is typically linked to its ability to counteract oxidative stress.[39] In the present study, no significant ROS generation was observed following exposure to any one of the organochlorine test compounds, which may explain why NAC did not demonstrate any protective effect against lower concentrations of any of the test compounds. In fact, the test compounds significantly decreased intracellular ROS (Figure 2). This finding contradicts reports in the literature that used cultured human[40] and fish primary hepatocytes,[41] where increased ROS generation was reported after DDT exposure. For this reason, a set of kinetics experiments was performed at an extended concentration range of DDT, DDE and DDD, which included concentrations that are reported to induce ROS generation in hepatocytes,[40,41] in order to confirm the results obtained from endpoint experiments. Still, no ROS generation was observed over a period of 14 h of exposure. Rather, all three test compounds decreased the levels of intracellular ROS, although not significantly. The decreases in ROS levels were further confirmed using flow cytometry (results not shown). The decrease in intracellular ROS levels observed after treatment with DDT is in agreement with the findings on fish primary hepatocytes.[42] It should be noted that the study that reported decreased intracellular ROS[42] used hepatocytes from a different fish species than that of the one which showed ROS generation.[41] These conflicting observations by the same research group could be interpreted as a species-specific response to DDT regarding intracellular ROS generation. This may also explain why ROS generation was not seen in HepG2 cells in the present study but was reported in L-02 hepatocytes.[40] Although both these cell lines are of human origin, the former is cancerous (HepG2), while the latter is not (L-02),[43] thereby possessing different genotypic and phenotypic features. Furthermore, it is well established that HepG2 cells do not possess the metabolic capacity of their innate *in vivo* counterparts,[44] which may also explain the results of the present study. ROS generation caused by exposure to polychlorinated biphenyls (PCBs) is related to uncoupling of the cytochrome P450 enzymes as demonstrated by Green et al.[45] in the metabolically inactive V79MZ cell line. The study compared PCB-induced ROS generation in metabolically incompetent cells and cells transfected with human cytochrome P4501B1. From the results, it was evident that metabolically incompetent cells did not yield any ROS generation following 1 h of exposure to three different PCBs, whereas their cytochrome P450-transfected counterparts produced significant ROS generation. For this reason, the lack of metabolism of HepG2 cells may be responsible for not observing any ROS generation in the present study. Interestingly, Green et al.[45] also reported that the degree of ROS generation positively correlated with the degree of chlorination of the test PCBs.

To our knowledge, this is the first report of the effects of DDT, DDE and DDD on the $\Delta\psi_m$ of intact, cultured hepatocytes. Mitochondrial hyperpolarization observed in this study is not likely to be the result of stimulation of the electron transport chain since previous studies have shown that DDT is capable of inhibiting certain complexes of the respiratory chain, including complexes II[46] and III[46,47] of the electron transport chain. Similarly, DDE has been reported to inhibit these respiratory complexes in mitochondria isolated from rat liver.[48] The observed elevations in $\Delta\psi_m$ may have occurred due to decreased hydrogen ion (H^+) re-entry into the mitochondrial cytosol via ATP synthase. Several authors have reported that DDT exerts an inhibitory effect on mitochondrial ATP synthase.[46,47,49–51] A similar ATP synthase inhibitory action has also been described for DDE in liver mitochondria.[48,50] Another reason for mitochondrial hyperpolarization could be the inhibition of the H^+ ion leak, which may cause congestion by preventing H^+ ion from re-entering the mitochondrial cytosol, thereby increasing the electrochemical gradient and subsequently $\Delta\psi_m$. Such an inhibition of in the H^+ leak has been observed in rat liver mitochondria exposed to nanomolar concentrations of DDE.[48] A structure outside the electron transport chain that may also play a role in hyperpolarizing the $\Delta\psi_m$ is the voltage-dependent anion channel (VDAC), which is located on the outer mitochondrial membrane.[52] To date, no evidence exists proving that DDT affects the VDAC in any manner. Although the exact mechanism of induced hyperpolarization is not clear, results from the present study indicate that all three compounds significantly increase the concentration of H^+ ion in the mitochondrial intermembrane space.

NAC pretreatment induced significant increases in $\Delta\psi_m$ at low concentrations (5 and 10 μM) of all three organochlorine compounds. At higher concentrations of 100 and 150 μM of the test compounds, NAC pretreatment had the opposite effect, significantly decreasing organochlorine-induced hyperpolarization (Figure 3). It is possible that NAC exerts a partial agonistic hyperpolarizing effect on $\Delta\psi_m$. At low concentrations of the test compounds, NAC may produce

hyperpolarization that the test compounds cannot counteract. This is supported by previous research where NAC on its own has been reported to hyperpolarize the $\Delta\psi_m$ in A549 lung epithelial cells.[53] These authors further showed that NAC pretreatment was also able to reverse changes in $\Delta\psi_m$ induced by another pesticide, paraquat. In the present study NAC pretreatment alleviated toxin-induced cellular death only at high concentrations of the test compounds (100–150 μM). These are the same concentrations at which NAC inhibited the extensive hyperpolarization of the $\Delta\psi_m$ caused by exposure to any one of DDT, DDE or DDD, which advocates inhibition of mitochondrial toxicity as the mechanism by which NAC counteracts hepatocyte injury caused by exposure to high concentrations of DDT, DDE or DDD.

All three test compounds induced dose-dependent activation of Cas-3, implying an apoptotic mode of cell death. DDD proved to be the most potent inducer of apoptosis, causing a significant increase in Cas-3 activity, which was higher than both DDT and DDE at concentrations of 100 μM (almost equal to +30%) and 150 μM (almost equal to +70%). In terms of potency of inducing apoptosis, the following was observed: DDD > DDT > DDE. These results were found to correlate well with the relative LC_{50} values determined in cell viability experiments, where the same trend was noted. The correlation between Cas-3 activity and cell viability for all three test compounds was found to be significant with $p < 0.05$ and a negative Pearson's correlation coefficient (r) of −0.93, that is, the higher the Cas-3 activity, the less viable the cells are observed. This observation provides further support for an apoptotic mode of cell death. It has been reported that DDT exposure also causes apoptosis in human peripheral blood mononuclear cells,[54] rat thymocytes[55] and murine embryos,[56] which further supports the concept of some 'basal' mechanism of toxicity involving receptors/signalling cascades present in most cell types. DDE has also been reported to cause apoptosis *in vitro* in peripheral blood mononuclear cells[46] and rat Sertoli cells[57] as well as *in vivo* in the testes of DDE-exposed rats.[58] Apoptosis due to DDD exposure has only been noted in peripheral blood mononuclear cells.[54] No literature describing DDE- or DDD-induced apoptosis in cultured hepatocytes could be found. The fact that elevated Cas-3 activity was observed after 6 h of exposure to the test compounds, with no ROS generation during this period, implies that disruption of mitochondrial homeostasis (after only 1 h of exposure) is mainly responsible for the observed apoptotic cell death/hepatocyte injury. This is supported by a significant positive correlation between changes in $\Delta\psi_m$ and Cas-3 activity for each of DDT ($p < 0.05$, $r = 0.90$), DDE ($p < 0.05$, $r = 0.96$) and DDD ($p < 0.05$, $r = 0.96$). No significant correlation was found between intracellular ROS levels and Cas-3 activity, further indicating that disruption of mitochondrial homeostasis is likely to be responsible for the induction of apoptosis.

NAC is believed to be an effective inhibitor of apoptosis initiated by a range of *in vitro* stressors.[59] This is in accord with the results from the present study where pretreating cells with NAC significantly reduced Cas-3 activity at higher concentrations of the organochlorine compounds. This could be the result of NAC counteracting mitochondrial hyperpolarization in the initial stages of exposure, thus inhibiting the subsequent apoptotic cascade. Similarly, NAC has been found to inhibit Cas-3 cleavage following clivorine treatment in L-02 hepatocytes,[22] which was related to the mitochondrial pathway of apoptosis induction. The authors reported that NAC maintained the levels of the anti-apoptotic protein Bcl-xL and inhibited cytochrome C release from mitochondria, which halted the subsequent formation of the apoptosome. It is possible that the same mechanism of inhibition of apoptotic cell death occurred in the present study.

In summary, observations from the present study demonstrate that DDT, DDE and DDD hepatocyte toxicity initiates cell death by apoptosis. This is thought to be the result of disrupted mitochondrial homeostasis, which has been described as an early apoptotic event.[60] Although NAC pretreatment exacerbated hepatocyte injury induced by low concentrations of these organochlorine test compounds, it did inhibit toxicity exerted at higher concentrations of the test compounds. No intracellular ROS generation was observed following exposure to any of the organochlorine compounds, which may explain the lack of hepatoprotection offered by NAC pretreatment. NAC was able to inhibit elevations in Cas-3 activity brought about by DDT, DDE or DDD, which may be attributed to the ability of NAC to counteract test compound-induced mitochondrial hyperpolarization. From the results, it is evident that NAC aggravates DDT-induced hepatocyte injury at low concentrations. This study provides *in vitro* observations that raise concern for the use of NAC as prophylaxis against, or as treatment for DDT-induced hepatotoxicity. *In vivo*

studies are required to confirm these observations in an intact physiological system.

Conflict of Interest

The authors declared no conflict of interest.

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