Low dose induction of micronuclei by lindane

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Environmental contaminants possessing hormonal activity have long been suspected of playing a role in cancer causation. What is unclear is whether such agents elicit their effects through genotoxic and/or epigenetic mechanisms. γ-Hexachlorocyclohexane (γ-HCH, lindane) was tested in the 10^-12-10^-4 M range. Chromosomal damage in MCF-7 breast cells and PC-3 prostate cells was assessed using the cytokinesis block micronucleus assay. Micronuclei (MNi) were scored in 1000 binucleate cells per treatment. Cell viability and cycle kinetics were also assessed, along with immunocytochemical and quantitative gene expression analyses of CDKN1A (P21WAF1/CIP1), BCL-2 and BAX. Following 24 h treatment, lindane (10^-12-10^-10 M) induced increases (up to 5-fold) in MNi in both cell lines. Increases in MNi occurred in the absence of DNA single-strand breaks or cytotoxicity and, compared with lines. Increases in MNi occurred in the absence of DNA single-strand breaks or cytotoxicity and, compared with

Introduction

1,2,3,4,5,6-Hexachlorocyclohexane (HCH) belongs to the organochlorine pesticide family that, whilst banned in many countries such as the UK, are still widely used elsewhere (1,2). HCH consists of eight separable stereoisomers and lindane, the α isomer, possesses the most significant insecticidal activity (2). The other isomers include the α and β forms. Mixtures of HCH isomers have been used extensively since the 1940s to control plasmodial mosquitoes and lindane is still used in the USA as a component of pediculicide shampoos for head lice and scabies (3). β-HCH is the most persistent and, therefore, accumulative isomer, whilst the α and γ isomers are mostly converted to the β isomer in biological systems (4). However, between 1970 and 1996, usage of the α and β isomers has fallen more rapidly than that of lindane itself (2). Whilst lindane poisoning may result in tremors, ataxia, convulsions, stimulated respiration, prostration and, in especially severe cases, degenerative hepatic and renal tubule changes, there has been speculation that such agents may also play a role in the aetiology of cancer (5). The primary route of exposure in the general population is through dietary intake (6), particularly via meat and dairy products (7). Lifetime feeding studies in mice revealed that technical grade HCH and some of its isomers, including lindane, increased the incidence of hepatocellular tumours (8). In such animal models lindane-induced damage may result from the generation of superoxide anion radicals (O^-2) (9,10) and/or DNA single-strand breaks (SSBs) (11) or via epigenetic mechanisms (12). Surprisingly little is known regarding the mutagenic and/or carcinogenic potential of lindane, although it has been shown to induce chromosomal aberrations in human peripheral lymphocytes in vitro (13) and micronucleus (MN) formation in bone marrow in vivo (14).

Incidence rates for cancers of both breast and prostate, which are hormone-responsive tissues, are higher in more developed countries than in less-developed regions (15). Factors that influence hormonal exposures may modulate risk associated with these cancers (16). Lindane interferes with reproductive activity in animals, an effect that may be mediated through a direct inhibition of adrenal and gonadal steroidogenesis (17,18). This chemical also interferes with gap junction intercellular communication (19) and induces cytochrome P450 metabolic enzymes (20), factors that may each play a significant role in tumour-promoting activity (8). As a lipophilic agent, lindane becomes concentrated in the ovary and testis, which could be relevant to the increasing incidence of testicular cancer (21).

Hitherto, hormonal agents have not tested positive in many classical bacterial and mammalian cell gene mutation assays (22). Similarly, lindane has been found to be relatively inactive in such test systems (8,23). Epigenetic mechanisms implicated in cancer causation may include stimulation of cell proliferation, spontaneous induction of replication errors or disruption

Abbreviations: ara-C, cytosine arabinoside; BP, benzo[a]pyrene; BSAT, 0.2% bovine serum albumin in Tris-buffered saline (pH 7.6); CBMN, cytokinesis block micronucleus; CTL, comet tail length; DAB, 3,3'-diaminobenzidine; DMSO, dimethyl sulphoxide; HCH, 1,2,3,4,5,6-hexachlorocyclohexane; HU, hydroxyurea; IMS, industrial methylated spirits; MN, micronucleus; MNi, micronuclei; O^-2, superoxide anion radicals; PBS, phosphate-buffered saline; PhiIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; RT, reverse transcription; SSBs, single-strand breaks; TBS, Tris-buffered saline.

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of spindle formation and subsequent induction of aneuploidy (24,25). However, endocrine disruptors may serve to enhance the sensitivity of target epithelial cell populations to other genotoxins (26). Lindane has been found to induce increases in SSBs in the DNA of treated cells (11,23,27), but the concentrations employed to elicit these effects were high.

Because of the unresolved issues regarding the genotoxicity of lindane, we have investigated the effects of this hormonal compound in the estrogen receptor-positive breast carcinoma MCF-7 cell line and in the androgen-independent prostate carcinoma PC-3 cell line. Levels of genotoxicity were assessed primarily using the cytokinesis block micronucleus (CBMN) assay but also in the alkaline single cell gel electrophoresis (Comet) assay. Cell viability and cell cycle kinetics, along with immunocytochemical and quantitative gene expression analyses of CDKN1A (P21WAF1/CIP1), BCL-2 and BAX, were also assessed. Experiments were carried out at ‘low dose’ and ‘high dose’ concentrations in order to determine whether differential effects could be observed.

Materials and methods

Chemicals

Chemicals were obtained from Sigma Chemical Co. (Poole, UK) unless otherwise stated. Cell culture consumables were obtained from Invitrogen Life Technologies (Paisley, UK) unless otherwise stated. Antibodies were obtained from DakoCytomation (Ely, UK).

Cell culture

The human mammary carcinoma MCF-7 cell line was grown in Dulbecco’s modified essential medium supplemented with 10% heat-inactivated foetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). The human prostate carcinoma PC-3 cell line was grown in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were grown in 5% CO2 in air at 37°C in a humidified atmosphere and disaggregated using a trypsin (0.05%)/EDTA (0.02%) solution, to form single cell suspensions prior to sub-culture or incorporation in experiments. Test agents were added as solutions in dimethyl sulphoxide (DMSO) and DMSO was used as a vehicle control: DMSO concentrations did not exceed 1% (v/v).

The CBMN assay

 Routinely cultured cells were disaggregated and resuspended in complete medium prior to seeding aliquots (3 ml, ~1 × 105 cells) into 30 mm Petri dishes containing 20 mm coverslips (22). After 24 h, attached cells were then treated for a further 24 h, as indicated. Medium was then replaced with fresh medium, without test agent but containing cytochalasin B (2 µg/ml). Micro- nuclei (MNI) in 1000 binucleate cells from a minimum of three experiments were scored as either micronucleated binucleate cells, total numbers of MNi or MNi distributions in binucleate cells.

The alkaline single cell gel electrophoresis (Comet) assay

Alkaline lysis followed by alkaline gel electrophoresis was employed in order to detect DNA SSBs (11,28,29). Cells were incubated at 37°C for 2 h in the presence or absence of a test agent, as indicated, with or without the DNA repair inhibitors hydroxyurea (HU) (1 mM) and cytosine arabinoside (ara-C) (120 µM). Single cell suspensions in low melting point agarose were then evenly applied to microscope slides and allowed to set on a cold surface for 5 min. The slides were subsequently submerged in cold lysis solution (2.3 M NaCl, 100 mM EDTA disodium salt, 10 mM Tris, 1% Triton X-100 and 10% DMSO), protected from light and stored at 4°C for at least 1 h. Then the slides were transferred to a light-tight container and covered in electrophoresis solution (0.3 M NaOH, 1 mM EDTA, freshly prepared, pH > 13) and stored for 40 min to allow DNA unwinding. Finally, slides were transferred to a horizontal electrophoresis tank and covered in fresh electrophoresis solution prior to electrophoresis at 0.5 V/cm and 300 mA for 24 min. After electrophoresis, slides were neutralized (0.5 M Tris, pH 7.5) and stained with ethidium bromide (20 ng/ml) after which comet tail length (CTL) (µm) was visualized by epifluorescence using a Leitz Dialux 20 EB microscope. A total of 100 digitized images/data point, 50 from each of two duplicate slides, was measured in each experiment. Experiments were repeated independently

Table 1. Primers used for quantitative real time RT–PCR analyses

<table>
<thead>
<tr>
<th>Assay</th>
<th>Name</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P21WAF1/CIP1</td>
<td>P21WAF1/CIP1</td>
<td>GAC CAC CAT GAG ACA TTT CCA</td>
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<tr>
<td></td>
<td>P21WAF1/CIP1-F</td>
<td>TTT CAT GGA CCT GAT TAG</td>
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<tr>
<td>BCL-2</td>
<td>BCL-2-F</td>
<td>GGC TGG GAT GTC TTT</td>
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<tr>
<td></td>
<td>BCL-2-R</td>
<td>GCG AAA CTG AGA AGC GTC TTT</td>
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<tr>
<td>BAX</td>
<td>BAX-F</td>
<td>CTT GGC TGT AAG CTA</td>
</tr>
<tr>
<td></td>
<td>BAX-R</td>
<td>GTG CAC AGG GCC TGT AAT</td>
</tr>
<tr>
<td>β-ACTIN</td>
<td>β-ACTIN-F</td>
<td>CTT GGC ACC CAG CAC AAT</td>
</tr>
<tr>
<td></td>
<td>β-ACTIN-R</td>
<td>GCC GAT CCA CAC GGA GTA</td>
</tr>
</tbody>
</table>

F, forward primer; R, reverse primer.
**β-ACTIN** amplification 5 ng cDNA template, made up to a total volume of 25 μl with sterile H2O. Thermal cycling parameters included activation at 95°C (10 min) followed by 40 cycles each of denaturation at 95°C (15 s) and annealing/extension at 60°C (1 min). Each reaction was performed in triplicate and ‘no template’ controls were included in each experiment. Dissociation curves were run to eliminate non-specific amplification, including primer-dimers.

**The cell growth assay**

Following disaggregation, cells were resuspended in complete medium prior to seeding aliquots (5 ml, ~0.5–1 x 10⁵ cells) into 25 cm² flasks. Cells were allowed to attach for 24 h prior to addition of lindane, as indicated. Following a further 24 h treatment, medium was replaced with fresh lindane-free medium. At the time points indicated the cells were disaggregated, resuspended in PBS and applied to a haemocytometer with a coverslip prior to obtaining a cell count.

**Flow cytometry**

Cells were resuspended in aliquots of complete medium (10 ml, ~1 x 10⁶ cells), seeded into 75 cm² flasks and allowed to attach for 24 h prior to treatment, as indicated below. Following disaggregation, cell aliquots were washed twice with PBS prior to fixation with ice-cold ethanol (70%, aqueous) and storage overnight at -20°C. Cell aliquots were again washed twice with PBS prior to incubation with RNase A (10 μg/ml) and propidium iodide (50 μg/ml) for 60 min at 37°C. DNA content of 10 000 events/treatment was analysed using a Becton Dickinson FACSCaliber flow cytometer and the CELLQuest software version provided by the manufacturer. Cell cycle analysis was carried out using ModFitLT for Mac v2.0.

**The clonogenic assay**

Following disaggregation, cells were resuspended in complete medium (1 x 10⁶ cells in a 5 ml aliquot) and seeded into 25 cm² flasks in the presence or absence of lindane for 24 h, as indicated. The medium was then replaced with fresh lindane-free medium. Cells were cultured undisturbed for a further 7 days prior to removal of medium and fixation with 70% ethanol. Colonies were then stained with 5% Giemsa and counted and percentage plating efficiencies calculated.

**Results**

Lindane-induced MN-forming activity in MCF-7 and PC-3 cells is compared with that of BP and PhIP in Figures 1 and 2. In MCF-7 cells, BP treatment at concentrations of 10⁻⁸, 10⁻⁷, 10⁻⁶ and 10⁻⁵ M resulted in levels of 17, 24, 73 and 87 micronucleated binucleate cells/1000, respectively, as compared with a background level of 12 micronucleated binucleate cells.

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**Fig. 1.** Micronucleus-forming activity in MCF-7 cells of BP, lindane and PhIP induced by a 24 h treatment. Cell suspensions (3 ml, ~1 x 10⁴ cells) in 30 mm Petri dishes were prepared as described in Materials and methods. Following treatment, the cells were blocked at cytokinesis by replacement with fresh medium containing 2 μg/ml cytochalasin B and cultured for a further 24 h, prior to fixation and staining with 5% Giemsa. Micronucleus formation was scored in 1000 binucleate cells.
cells/1000 (Figure 1). Treatment with PhIP at concentrations of $5 \times 10^{-11}$, $5 \times 10^{-10}$, $5 \times 10^{-9}$, and $5 \times 10^{-8}$ M resulted in 22, 34, 44, and 93 micronucleated binucleate cells/1000, respectively (Figure 1). However, lindane concentrations of $10^{-12}$, $2 \times 10^{-11}$, $10^{-11}$, and $2 \times 10^{-11}$ M resulted in 32, 47, 57, and 54 micronucleated binucleate cells/1000, respectively (Figure 1). Similar effects were induced in PC-3 cells in which a background level of 56 micronucleated binucleate cells/1000 was recorded (Figure 2). BP treatment at concentrations of $10^{-7}$, $10^{-6}$ or $10^{-5}$ M resulted in levels of 84, 137 and 181 micronucleated binucleate cells/1000, respectively (Figure 2). PhIP treatment at concentrations of $5 \times 10^{-8}$, $5 \times 10^{-7}$ and $5 \times 10^{-6}$ M resulted in 66, 84 and 121 micronucleated binucleate cells/1000, respectively (Figure 2). However, lindane concentrations of $10^{-12}$, $10^{-11}$ and $10^{-10}$ M resulted in 125, 222 and 133 micronucleated binucleate cells/1000, respectively (Figure 2). In both MCF-7 and PC-3 cells the MN-forming activity of lindane was associated with increases in binucleate cells containing multiple MNi (Figures 1 and 2).

Table II shows that lindane caused more MNi in MCF-7 cells than the $\alpha$ and $\beta$ isomers of HCH with concentrations as low as $10^{-12}$ M (1 pM) doubling the levels of MNi in comparison with background levels. The $\alpha$ isomer appeared to be the least MN-forming whilst the $\beta$ isomer induced an intermediate level of MNi. In comparison, a concentration of $10^{-7}$ M BP was required to induce levels of MN formation similar to those caused by $10^{-12}$ M lindane (Table II).

Figure 3 compares the comet-forming activity of the $\alpha$, $\beta$ and $\gamma$ isomers of HCH in MCF-7 cells. Following treatment with lower concentrations of lindane, no comet-forming effects were observed (data not shown). However, $10^{-4}$ M treatment with lindane resulted in significant increases in SSBs in either the absence or presence of HU/ara-C; the $\alpha$ and $\beta$ isomers of HCH were similarly comet-forming. In the absence of DNA repair inhibitors, $\alpha$-HCH induced an increase...
in median CTL to 58.45 µm ($P < 0.0001$) compared with a control median CTL of 14.08 µm; in their presence, an increase ($P < 0.0001$) in median CTL to 68.25 µm was observed as compared with a control median CTL of 17.92 µm. Lindane appeared to be less comet-forming: in the absence or presence of HU and ara-C, resulted in significantly enhanced levels of repair inhibitors. Incorporation of the DNA repair inhibitors, from BP-treated and control MCF-7 cells, respectively. Comet increases ($P < 0.0005$) in the percentage of cells positive for the anti-apoptotic protein Bcl-2 were observed following low dose lindane treatment (Figure 5A). No effect on the percentage of cells positive for the pro-apoptotic protein Bax was observed. However, following $10^{-4}$ M lindane treatment, the percentage of cells positive for Bcl-2 fell to control levels whereas the percentage of cells positive for Bax increased significantly ($P < 0.0001$). In contrast, in the androgen-independent PC-3 cell line no ‘low dose’ lindane effects were observed on the percentage of cells staining positive for Bcl-2. Following $10^{-4}$ M lindane treatment, a significant reduction ($P < 0.05$) in Bcl-2-positive cells was observed. At lindane concentrations of $10^{-11}$ and $10^{-10}$ M significant increases ($P < 0.0005$) in the percentage of Bax-positive PC-3 cells were observed (Figure 5B). Paradoxically, following $10^{-4}$ M lindane treatment, the percentage of cells positive for Bax was reduced to control levels.

In MCF-7 cells, high dose ($10^{-4}$ M) lindane treatment for 24 h resulted in more than a 3-fold increase in quantitative $P21\text{WAF1/CIP1}$ and a 1.6-fold increase in BAX whilst no marked effect on BCL-2 expression was observed (Table III). Low dose ($10^{-11}$ M) lindane treatment did not markedly alter gene expression in MCF-7 cells. Likewise, no marked alterations in quantitative gene expression of $P21\text{WAF1/CIP1}$, BCL-2 and BAX were observed in PC-3 cells, either following low dose or high dose lindane treatment (Table III).

The effects of lindane on the growth kinetics of MCF-7 and PC-3 cells are shown in Figure 6A and B. Following a 24 h treatment, low dose concentrations ($10^{-12}$, $10^{-11}$ and $10^{-10}$ M) resulted in a consistent reduction in the normal growth characteristics of MCF-7 cells; an effect that was still apparent 24 h after lindane had been removed. Whilst these lindane concentrations also reduced PC-3 cell numbers for up to 24 h post-treatment, the effects were not as marked as with MCF-7 cells. After a further 24 h, i.e. 48 h after removal of lindane, both cell lines had attained cell numbers close to control levels, although in all cases lindane treatment resulted in a reduction in cell number at this time point. High dose lindane treatment ($10^{-4}$ M, 100 µM) for 24 h resulted in profound decreases in growth kinetics; for example, 24 h after removal of lindane-containing medium cell numbers were still markedly reduced and even at 48 h they were still only some 50% of control values (Figure 6A and B).

In exponentially growing MCF-7 and PC-3 cells, control percentage cell cycle distributions were 25.85 ± 0.46 and 37.94 ± 0.36 in G$_0$/G$_1$, 57.86 ± 0.31 and 36.87 ± 0.61 in S phase and 25.86 ± 0.46 and 25.20 ± 0.27 in G$_2$/M, respectively (Table IV). β-HCH did not appear to disrupt cell cycle kinetics. Low doses of α-HCH or lindane also did not appear to effect the cell cycle kinetics of either MCF-7 or PC-3 cells (data not shown). However, $10^{-4}$ M α-HCH resulted in increases in percentage of cells in G$_0$/G$_1$ (41.08 ± 0.15 and 50.22 ± 0.61) and decreases in percentage of cells in G$_2$/M (0.27 ± 0.16 and 18.83 ± 0.28) with no changes being observed in the percentage of cells in S phase (58.65 ± 0.19 and 30.95 ± 0.89) in MCF-7 and PC-3 cells, respectively. High dose lindane ($10^{-4}$ M) induced profound increases in percentage of cells in G$_0$/G$_1$ (70.55 ± 0.19 and 82.72 ± 0.23) coupled with corresponding decreases in percentage of cells in S phase (27.29 ± 0.65 and 8.11 ± 0.74) and percentage of cells in G$_2$/M (2.17 ± 0.48 and 9.17 ± 0.57) in both MCF-7 and PC-3 cells, respectively. The induction by lindane of a G$_1$ arrest was found to be reversible; 24 h after removal of $10^{-4}$ M lindane.

Table II. Micronucleus formation in MCF-7 cells

<table>
<thead>
<tr>
<th>Conc. of test agent</th>
<th>Micronuclei Treatment</th>
<th>BP</th>
<th>α-HCH</th>
<th>β-HCH</th>
<th>γ-HCH</th>
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<tbody>
<tr>
<td>Background M</td>
<td>MN</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>TMN</td>
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<tr>
<td>10$^{-12}$ M</td>
<td>MN</td>
<td>-</td>
<td>24</td>
<td>35</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>TMN</td>
<td>-</td>
<td>27</td>
<td>44</td>
<td>64</td>
</tr>
<tr>
<td>10$^{-11}$ M</td>
<td>MN</td>
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<td>37</td>
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<td>47</td>
</tr>
<tr>
<td></td>
<td>TMN</td>
<td>-</td>
<td>46</td>
<td>35</td>
<td>57</td>
</tr>
<tr>
<td>10$^{-8}$ M</td>
<td>MN</td>
<td>24</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>TMN</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10$^{-7}$ M</td>
<td>MN</td>
<td>37</td>
<td>-</td>
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<td>-</td>
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<tr>
<td></td>
<td>TMN</td>
<td>50</td>
<td>-</td>
<td>-</td>
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<tr>
<td>10$^{-6}$ M</td>
<td>MN</td>
<td>74</td>
<td>-</td>
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<td></td>
<td>TMN</td>
<td>143</td>
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<tr>
<td>10$^{-5}$ M</td>
<td>MN</td>
<td>96</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TMN</td>
<td>170</td>
<td>-</td>
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</tbody>
</table>

MCF-7 cells were treated for 24 h in the presence or the absence of the test agents (α, β or γ isomers of HCH or BP), as indicated, prior to cytokinesis block with cytochalasin B (2 µg/ml). After a further 24 h incubation cells were fixed and stained with 5% Giemsa as described in Materials and methods. MN, micronucleated cells/1000 binucleate cells; TMN, total number of micronuclei/1000 binucleate cells; –, not determined.
MCF-7 and PC-3 cells re-attained control cell cycle distributions (data not shown).

Figure 7 shows the effects of treatment with lindane ($10^{-12}$ - $10^{-4}$ M) for 24 h on the plating efficiency of MCF-7 cells. In control flasks, a plating efficiency of 43.7 ± 1.7% was observed. Whilst lower lindane concentrations ($10^{-12}$ and $10^{-11}$ M) did not affect plating efficiencies significantly, reductions ($P < 0.05$) were observed following treatment with $10^{-10}$, $10^{-8}$ and $10^{-6}$ M lindane. At a lindane concentration of $10^{-4}$ M plating efficiency fell to 17.7 ± 0.7% ($P < 0.005$).

Discussion

The role that endocrine disrupters such as the insecticide lindane may play in the aetiology of human cancer remains
at best enigmatic. Certainly, there have been several epidemiological studies that have shown elevated cancer incidence rates amongst exposed groups (31) and, recently, positive associations between lindane exposure and the incidence of prostate cancer (32) and of non-Hodgkin’s lymphoma (33) have been reported. These highly persistent, lipophilic chemicals can accumulate in human fat stores to levels in excess of 1 p.p.m. (1 mg/g) (34) whereas adipose concentrations of fat-soluble carcinogens probably never exceed 1 p.p.b. (1 ng/g) (35,36). However, other studies appear to dissociate organochlorine exposure from increased breast cancer risk (37). We have investigated low and high dose effects of lindane to determine whether environmental concentrations induce genomic alterations in the form of MNi or interfere with cell survival mechanisms.

Previously we demonstrated the genotoxicity, at nanomolar concentrations, of endogenous oestrogens (β-oestradiol, oestrone and oestriol) in both MCF-7 cells and primary breast milk cells using the CBMN and Comet assays (22). Following β-oestradiol treatment, MN-forming effects observed at lower concentrations were not apparent at higher concentrations and it was possible to dissociate these observations from hormone-induced proliferative effects (22). β-Oestradiol is also known to interfere with the control of apoptosis (38). Genotoxic effects in cells that survive and proliferate are potentially of importance for the carcinogenic process.

Fig. 4. Representative photomicrographs of cells visualized in the different protocols employed in this study. (A) Micronucleated binucleate MCF-7 cells resulting from treatment with 10⁻¹¹ M lindane; (B) MCF-7 cells stained for p53 following 24 h treatment with 1.0 μM BP; (C) Nucleus resulting from a 2 h 1.0 μM BP treatment of an MCF-7 cell following electrophoresis in the Comet assay; (D) nucleus from a control MCF-7 cell following electrophoresis in the Comet assay; (E) MCF-7 cells stained for p21⁷⁰⁵⁴ following treatment with 1.0 μM BP; (F) MCF-7 cells stained for Bcl-2 following treatment with 10⁻¹¹ M lindane; (G) MCF-7 cells stained for Bax following treatment with 10⁻⁴ M lindane; (H) PC-3 cells stained for p21⁷⁰⁵⁴ following treatment with 10⁻⁴ M lindane; (I) PC-3 cells stained for Bcl-2 following treatment with 10⁻¹¹ M lindane; (J) PC-3 cells stained for Bax following treatment with 10⁻¹¹ M lindane. For immunohistochemical analyses, cells were treated, as indicated, for 24 h on coverslips, after which they were analysed for protein expression as described in Materials and methods.
The present study shows that, in the $10^{-12}$ to $10^{-10}$ M concentration range, lindane-induced increases in MNi occur in MCF-7 and PC-3 cells; these concentrations are lower than the concentrations of BP and PhIP required to produce the same effect (Figures 1 and 2 and Table II). As previously observed with $\beta$-oestradiol (22), higher lindane concentrations do not appear to be as MN-forming (data not shown). Whilst low dose lindane did not appear to reduce colony-forming ability, as measured by reductions in plating efficiency (Figure 7), decreases in the growth kinetics of both MCF-7 and PC-3 cells were apparent (Figure 6). These growth-inhibiting effects persist for 24 h following the removal of lindane-containing medium, after which cell doubling times increase to more closely match controls. This is in contrast to the significant reductions in colony-forming ability and in cell growth characteristics that follow $10^{-4}$ M lindane treatment (Figures 6 and 7). In line with these observations, $10^{-4}$ M lindane induced a profound $G_1$ arrest in both MCF-7 and PC-3 cells. However, at lower concentrations no effects on cell cycle kinetics were observed (data not shown).

Lindane, at low concentrations, clearly induced MN formation (Figures 1 and 2) whilst being inactive in the Comet assay (data not shown). The most obvious suggestion is that the

![Fig. 5. Immunohistochemical analysis of (A) MCF-7 breast cells or (B) PC-3 prostate cells. Cells were treated, as indicated, for 24 h on coverslips, after which they were analysed for protein expression as described in Materials and methods. The antibodies employed were: p53 mouse monoclonal (DO-7, isotype IgG2b), p21$^{\text{WAF1/CIP1}}$ mouse anti-human monoclonal (SX118, isotype IgG1), Bcl-2 mouse anti-human monoclonal (124, isotype IgG1) and Bax rabbit anti-human polyclonal. The percentages of cells staining positive were determined following five separate counts of 100 cells and are presented as the means ± SD. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$ (treatment versus control) as determined by an unpaired $t$-test with Welch’s correction.

![Fig. 6. Growth curves in the presence of lindane treatment in (A) MCF-7 cells or (B) PC-3 cells. Cells ($0.5$ to $10^5$) were seeded into 75 cm$^2$ flasks and allowed to attach for 24 h. As described in Materials and methods, cells were treated with lindane and incubated for up to 24 h. Following replacement with lindane-free medium, cells were cultured at $37^\circ$C and 5% CO$_2$ in air in a humidified atmosphere for up to 72 h. Cell numbers were estimated using a haemocytometer and the mean value of duplicate counts for each experimental condition was obtained in individual experiments. Each value for each experimental condition then contributed to the mean ± SD of three separate experiments.

Table III. Relative gene expression measured by quantitative real time RT-PCR

<table>
<thead>
<tr>
<th>Lindane conc.</th>
<th>$P21^{\text{WAF1/CIP1}}$</th>
<th>$BCL-2$</th>
<th>$BAX$</th>
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<tbody>
<tr>
<td>MCF-7 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1 (c)</td>
<td>1 (c)</td>
<td>1 (c)</td>
</tr>
<tr>
<td>$10^{-11}$ M</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>$10^{-9}$ M</td>
<td>3.3 ± 2.2</td>
<td>1.4 ± 0.4</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>PC-3 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1 (c)</td>
<td>1 (c)</td>
<td>1 (c)</td>
</tr>
<tr>
<td>$10^{-11}$ M</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.4</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>$10^{-9}$ M</td>
<td>1.5 ± 0.9</td>
<td>1.1 ± 0.6</td>
<td>1.2 ± 0.3</td>
</tr>
</tbody>
</table>

(c), calibrator, which for the purposes of these experiments were untreated controls.

Effect of lindane treatment ($10^{-11}$ or $10^{-9}$ M) following 24 h exposure on gene expression of $P21^{\text{WAF1/CIP1}}$, $BCL-2$ and $BAX$ in MCF-7 cells and PC-3 cells. Following reverse transcription of total RNA, amplification was carried out using primers as described in Materials and methods. Within each experiment reactions were performed in triplicate and ‘no-template’ controls were included. Averaged threshold cycle ($C_T$) values for each reaction were normalised to $\beta$-ACTIN values thus giving $\Delta C_T$ values. Alterations in gene expression were determined by comparing treatment groups with the calibrator, giving $\Delta \Delta C_T$ values. Results are the means ± SD of three separate experiments.
Table IV. Hexachlorocyclohexane (HCH) effects on cell cycle distribution

<table>
<thead>
<tr>
<th>HCH conc.</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCF-7</td>
<td>PC-3</td>
<td>MCF-7</td>
</tr>
<tr>
<td>Background</td>
<td>25.85 ± 0.46</td>
<td>37.94 ± 0.36</td>
<td>57.86 ± 0.31</td>
</tr>
<tr>
<td>α-HCH 1 × 10^{-4} M</td>
<td>41.08 ± 0.15</td>
<td>50.22 ± 0.61</td>
<td>58.65 ± 0.19</td>
</tr>
<tr>
<td>β-HCH 2 × 10^{-4} M</td>
<td>27.67 ± 0.86</td>
<td>42.08 ± 0.27</td>
<td>57.11 ± 1.19</td>
</tr>
<tr>
<td>γ-HCH 1 × 10^{-4} M</td>
<td>70.55 ± 0.19</td>
<td>82.72 ± 0.23</td>
<td>27.29 ± 0.65</td>
</tr>
</tbody>
</table>

Effects of stereoisomers of HCH (α, β and γ isomers) on the cell cycle distribution of MCF-7 and PC-3 cells. Cells were seeded into flasks and incubated at 37°C and 5% CO2 for 24 h prior to treatment, for a further 24 h. DNA content of 10 000 events/treatment was analysed using a Becton Dickinson FACS calibre flow cytometer as described in Materials and methods. Results are the means ± SD of triplicate measurements.

Fig. 7. Effects of lindane on plating efficiency in MCF-7 cells. Cells (1 × 10^4) were seeded in 25 cm² flasks in the presence or absence of lindane and incubated for 24 h, as described in Materials and methods. Following replacement with lindane-free medium, cells were cultured undisturbed at 37°C and 5% CO2 in air in a humidified atmosphere for 7 days. Surviving colonies were fixed and stained, and percentage plating efficiency was calculated from the percentage of colonies counted and the number of cells initially seeded. *P < 0.05, **P < 0.005 (treatment versus control) as determined by an unpaired t-test with Welch’s correction.

Lindane was not found to induce alterations in the levels of cells staining positive for either p53 or p21^{Waf1/Cip1} at concentrations of 10^{-12}, 10^{-11} and 10^{-10} M (Figure 5A). However, these low concentrations induced significant increases in the percentage of MCF-7 cells staining positive for Bcl-2 (69.2 ± 3.3 in 10^{-11} M lindane-treated versus 35.8 ± 4.4 in control MCF-7 cells), giving rise to a Bcl-2:Bax ratio of 3.1 in vehicle control cells and 5.5 in 10^{-11} M lindane-treated MCF-7 cells. Treatment with 10^{-4} M lindane resulted in a percentage of 40.4 ± 5.7 cells stained positive for Bcl-2 and whilst no changes in the percentages of cells stained positive for Bax were observed at lower lindane concentrations, significant increases were observed at the higher concentration (39.4 ± 4.7 in 10^{-4} M lindane-treated cells versus 11.4 ± 4.9 in control MCF-7 cells), resulting in a Bcl-2:Bax ratio of 1.0. Such an effect would be expected to favour the induction of apoptotic mechanisms within cells.

In p53-null PC-3 cells a different profile of effects was observed (Figure 5B). Whilst no changes in the percentage of cells stained positive for p21^{Waf1/Cip1} were observed at lower concentrations, a significant increase was observed at higher concentrations (47.8 ± 6.7 in 10^{-4} M lindane-treated cells versus 12.6 ± 3.0 in control PC-3 cells). However, an elevation in p21^{Waf1/Cip1} expression was observed only in MCF-7 cells (Table III). The failure to observe a similar increase in percentage of MCF-7 cells staining positive for p21^{Waf1/Cip1} (Figure 5A) could be due to increased protease-mediated degradation (40).

Overall, low dose lindane concentrations did not alter the percentage of PC-3 cells stained positive for Bcl-2, following 10^{-4} M treatment a significant reduction was apparent (21.6 ± 6.2 in 10^{-4} M lindane-treated cells versus 46.4 ± 9.1 in control PC-3 cells). In contrast to MCF-7 cells (Figure 5A), in PC-3 cells increases in the percentage of cells stained positive for Bax were observed (88.4 ± 5.0 in 10^{-11} M lindane treated versus 49.2 ± 5.0 in control PC-3 cells) (Figure 5B). In PC-3 cells a Bcl-2:Bax ratio of 0.9 in control cells and 0.6 in 10^{-11} M lindane-treated cells was observed. Such observations would suggest that lindane induces pro-apoptotic mechanisms in PC-3 cells.

The effects produced in cells treated with low doses of lindane in the present study may be important in the context of considering the effects of environmentally relevant concentrations. Mechanisms through which these hormone-like compounds act are currently under investigation.

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References


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