

Enhanced Micronucleus Formation and Modulation of Bcl-2:Bax in MCF-7 Cells after Exposure to Binary Mixtures

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BACKGROUND: Within mixtures, interactions between different xenobiotics may occur to give rise to additive, synergistic, inhibitory and/or stimulatory effects in target cells. The role that xenobiotics individually or in mixtures, and at environmental concentrations, play in the etiology of common human diseases often remains obscure.

METHODS: In the presence or absence of lindane, chromosomal aberrations were detected in MCF-7 cells after 24-hr treatment with benzo[*a*]pyrene (B[*a*]P) or 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) using the cytokinesis-block micronucleus assay. Micronuclei were scored in 1,000 binucleate cells/treatment. We investigated intracellular responses using quantitative gene expression analyses of cyclin-dependent kinase inhibitor 1A [*CDKN1A* (*P21*^{WAF1/CIP1})], B-cell leukemia/lymphoma 2 (*BCL-2*), BCL-2-associated X (*BAX*), and isoforms of cytochrome P450 (CYP), *CYP1A1*, *CYP1A2*, and *CYP1B1*. Immunocytochemical analyses of p53, p21^{Waf1/Cip1}, Bcl-2 and Bax protein expression in MCF-7 cells were also carried out.

RESULTS: After exposure to binary mixtures of B[*a*]P plus lindane or PhIP plus lindane, a 10-fold increase in micronucleus formation resulted; these test agents individually induced 2- to 5-fold increases. Lindane increased the ratio of Bcl-2:Bax, as did 17 β -estradiol (E₂). Although treatment with B[*a*]P alone was found to elevate expression of *P21*^{WAF1/CIP1} and CYP isoenzymes, it reduced the ratio of *BCL-2:BAX* mRNA transcripts. Treatment with a binary mixture of 10⁻⁸ M B[*a*]P plus 10⁻¹² M lindane or 10⁻¹⁰ M E₂ reversed B[*a*]P-induced reductions in the ratio of Bcl-2- to Bax-positive cells. In contrast, treatments with PhIP (known to possess hormonelike properties) plus lindane or E₂ resulted in profound reductions in Bcl-2:Bax ratio.

CONCLUSIONS: Our results suggest that low-dose treatments (i.e., close to environmental levels) may increase DNA damage while influencing survival in exposed cells and that these effects may depend on the endocrine activity of test agents.

KEY WORDS: 17 β -estradiol, BAX, Bcl-2, benzo[*a*]pyrene, binary mixture, clonogenic assay, cytokinesis-block micronucleus assay, lindane, micronucleus, PhIP. *Environ Health Perspect* 115(suppl 1):129–136 (2007). doi:10.1289/ehp.9361 available via <http://dx.doi.org/> [Online 8 June 2007]

Environmental exposures are likely to be a significant factor in the etiology of several diseases including neurodegenerative disease (Kamel and Hoppin 2004), coronary heart disease (Zhang et al. 1998), cancer (Grover and Martin 2002; Ragavan et al. 2006), and diabetes (Cranmer et al. 2000). Epidemiologic studies examining demographic differences in disease incidence and altered risk patterns among migrant populations (Peto 2001) lends weight to the notion that xenobiotics play an important role in disease causation (Safe 2004). Humans are variously and continuously exposed to mixtures of disease-implicated xenobiotics via environment and/or diet (Kalantzi et al. 2004c). Toxicity testing employing high-dose treatments with individual agents may be an inappropriate means of assessing low-dose exposures (Welshons et al. 2003). Different damage-induction mechanisms at low-dose versus high-dose concentrations may occur (Joosten et al. 2004; Kalantzi et al. 2004b). Following exposures to mixtures, additive, synergistic, inhibitory and/or stimulatory interactions between different xenobiotics may occur.

Candidate and ubiquitous xenobiotics include polycyclic aromatic hydrocarbons

(PAHs) [e.g., benzo[*a*]pyrene (B[*a*]P)], heterocyclic aromatic amines [e.g., 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)] and persistent organic pollutants (POPs) [e.g., lindane; γ -hexachlorocyclohexane (γ -HCH)] (Kalantzi et al. 2004b). B[*a*]P is a procarcinogen that requires metabolic activation to 7,8-diol 9,10-oxides (Sims et al., 1974); these ultimate carcinogenic and electrophilic species bind covalently with the N2 position of guanine (Cheng et al. 1989; Rubin 2001). PhIP is a protein-derived mutagen that appears to possess significant estrogenic properties (Felton et al. 2004; Gooderham et al. 2002; Lauber et al. 2004). It is metabolically activated via a two-step mechanism initially involving conversion to *N*-hydroxy-PhIP (Crofts et al. 1998; Zhao et al. 1994) followed by esterification to a metabolite that binds to the C8 position of guanine (Schut and Snyderwine 1999). Lindane is a lipophilic pesticide that moves through environmental compartments (Martin and Semple 2004) and may induce genomic alterations in the form of micronuclei (Kalantzi et al. 2004b).

Biotransformation is often facilitated by oxidative metabolism via the cytochrome

P450 (CYP) mixed-function oxidase system. The CYP multigene family consists of constitutively-expressed, inducible isoenzymes that are found mainly in the liver but also to varying degrees in extrahepatic sites (Ragavan et al. 2004). The proteins of the B-cell leukemia/lymphoma-2 (Bcl-2) family regulate the permeability of the outer mitochondrial membrane (Vaux and Korsmeyer 1999) and apoptosis (Adams and Cory 1998), and also appear to be modulators of oncogenic progression (Theodorakis et al. 2002). Through a modulation of Bcl-2:Bcl-2-associated X (Bax), PhIP appears to delay the involution of the rat mammary gland (Venugopal et al. 1999); dysregulation effects that may not be dissimilar to those induced by 17 β -estradiol (E₂) (Leung and Wang 1999).

In previous studies, we demonstrated the genotoxicity of nanomolar concentrations of endogenous estrogens (E₂, estrone, or estriol) (Yared et al. 2002) and low-dose lindane (Kalantzi et al. 2004b). Subsequently, it was shown that endogenous oestrogens or human milk-fat extracts, known to contain organochlorinated and brominated xenobiotics, markedly enhanced B[*a*]P-induced genotoxicity in clonogenic MCF-7 cells (Davis et al. 2002; Kalantzi et al. 2004a). Such exposures were associated with an elevation in the ratio of the anti-apoptotic Bcl-2 to the pro-apoptotic Bax proteins (Kalantzi et al. 2004b). If low-dose treatments (for the experiments described herein, less than or equal to nanomolar concentrations of test agent in culture medium) induce genotoxic and/or intracellular modulations, then quantifying and modeling co-exposure effects at such environmentally relevant levels are of utmost importance. In this study we examined the effects of various binary mixtures consisting

This article is part of the monograph "Endocrine Disruptors—Exposure Assessment, Novel End Points, and Low-Dose and Mixture Effects."

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We gratefully acknowledge grant support from the North West Cancer Research Fund (R.H.) and Rosemere Cancer Foundation (F.L.M.). A.F. and P.J.L. were both recipients of a Fogarty Minority International Research Training Fellowship Program grant from the National Cancer Institute.

The authors declare they have no competing financial interests.

Received 22 May 2006; accepted 5 September 2006.

of a DNA-reactive procarcinogen (B[a]P or PhIP) and hormone-like agent (E₂ or lindane) to determine whether such *in vitro* exposures might markedly modulate genotoxicity and/or survival in damaged cells. Our goal was to determine whether low-dose exposure to such a binary mixture might give rise to a modulation both of genotoxicity and survival in target cells.

Materials and Methods

Chemicals and media. Unless otherwise stated, chemicals were obtained from Sigma Chemical Co. (Poole, UK), cell culture consumables from Invitrogen Life Technologies (Paisley, UK), and antibodies from Dako Cytomation (Ely, UK).

Cell culture. The estrogen-responsive breast carcinoma MCF-7 cell line was grown in Dulbecco's modified essential medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. MCF-7 cells were cultured routinely in 75-cm² flasks at 5% CO₂ in air and 37°C in a humidified atmosphere and subcultured (1:10, vol/vol) twice weekly. Before subculture or incorporation into experiments, cultured cells were disaggregated, with 0.05% trypsin/0.02% EDTA.

The cytokinesis-block micronucleus (CBMN) assay. MCF-7 cells were treated with B[a]P, PhIP, E₂, or lindane either individually or as a binary mixture, as indicated. Additions of test agent(s), in dimethylsulfoxide (DMSO), were made to a maximum concentration of 1% vol/vol.

After disaggregation and resuspension in complete medium, MCF-7 cells in 3-mL aliquots ($\approx 1 \times 10^4$ cells) were seeded into 30-mm petri dishes containing 20-mm diameter coverslips (Sarstedt, UK). Cells were allowed to attach for 24 hr before the addition of test agent(s). After 24-hr treatment, the medium was replaced with fresh medium containing 2 µg/mL cytochalasin-B. Cultured for a further 24 hr, cells were fixed with 70% ethanol (EtOH) after washing the coverslips with phosphate-buffered saline (PBS). Cells were then stained with 5% Giemsa (in dH₂O) before mounting the coverslips in DPX mountant (BDH Laboratory Supplies, Poole, UK) on microscope slides (Yared et al. 2002).

For each treatment condition, micronuclei (MNi) in 1,000 binucleate MCF-7 cells from a minimum of three experiments were objectively scored either as micronucleated binucleate cells (MN), as the total number of

micronuclei (TMN) or as the distribution of MNi in binucleate cells. Mitotic rate was assessed as percentage of binucleate cells (mean \pm SD, $n = 3$ counts of 500 cells). In 10 separate experiments, in the population of MCF-7 cells used in this study, $\leq 5\%$ fluctuation in the micronucleus-forming activity of 10⁻⁶ M B[a]P was observed.

The clonogenic assay. MCF-7 cells were disaggregated and resuspended in complete medium. Aliquots (5 mL) containing $\sim 1 \times 10^3$ cells were seeded into 25-cm² flasks with or without test agent(s) (B[a]P, PhIP, E₂, or lindane) either individually or as a binary mixture, as indicated. Cells were incubated in 5% CO₂ in air at 37°C in a humidified atmosphere for 24 hr. The medium was then replaced with fresh medium. Cells were cultured undisturbed for a further 7 days before removal of medium, washing with PBS and fixation with 70% EtOH. Colonies were then stained with 5% Giemsa and counted.

Immunohistochemical staining. Cells were disaggregated and resuspended in complete medium before seeding aliquots (5 mL; $\sim 1 \times 10^5$ cells) into 60-mm petri dishes containing 24-mm glass coverslips. After allowing 24 hr for attachment, cells were treated for 24 hr with test agents, as indicated. Medium was then removed and the cells washed with PBS before immediate fixation with CytoFix fixative (CellPath plc, Skelmersdale, UK). The following antisera in bovine serum albumin (0.2%) diluted with Tris-buffered saline (pH 7.6) (BSAT) were used: cyclin-dependent kinase inhibitor 1A (CDKN1A (P21^{Waf1/Cip1})) mouse monoclonal (SX118, Isotype: IgG1) in a 1:20 dilution; Bcl-2 mouse monoclonal (124, Isotype: IgG1) in a 1:100 dilution and Bax rabbit polyclonal in a 1:50 dilution. Fixative was removed by soaking coverslips in 95% industrial methylated spirits (IMS) for 30 min. After a 5-min wash with tap water, coverslips were incubated in 1:5 normal goat sera in Tris-buffered saline (TBS) (0.05 M, pH 7.6) for 15 min in a humidified environment. After removal of excess sera, the coverslips were incubated with primary antibody for 1 hr at room temperature. Using the StreptABCComplex duet kit (DakoCytomation), coverslips were then washed for 5 min with TBS, incubated for 30 min with secondary anti-sera (goat anti-mouse/rabbit) in BSAT and washed again for 5 min with TBS. Then coverslips were incubated with tertiary anti-sera (avidin-biotin complex) in BSAT for 30 min and washed with TBS for 5 min. 3,3'-Diaminobenzidine (DAB) chromogen in Tris/HCl buffer (0.05 M, pH 7.6) with hydrogen peroxide (0.1%) was applied to preparations for 15 min followed by another 5-min tap water wash. Finally, slides were transferred to a rack and stained (1 min) with hematoxylin (50%), rinsed with tap

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

GenBank accession no.	Gene symbol	Primer	Sequence (5'→3')
NM_078467	<i>P21^{WAF1/CIP1}</i>	<i>P21^{WAF1/CIP1}-F</i> <i>P21^{WAF1/CIP1}-R</i>	GAC CAG CAT GAC AGA TTT CTA CCA TTC CTG TGG GCG GAT TAG G
NM_000633	<i>BCL-2</i>	<i>BCL-2-F</i> <i>BCL-2-R</i>	GGC TGG GAT GCC TTT GTG GCC AAA CTG AGC AGA GTC TTC AG
AF007826	<i>BAX</i>	<i>BAX-F</i> <i>BAX-R</i>	CCT GGG TTC AAG CGA TTC AC GTG CAC AGG GCC TGT AAT CC
BC023019	<i>CYP1A1</i>	<i>CYP1A1-F</i> <i>CYP1A1-R</i>	ACT TCA TCC CTA TTC TTC GCT ACC T CGG ATG TGG CCC TTC TCA
NM_000761	<i>CYP1A2</i>	<i>CYP1A2-F</i> <i>CYP1A2-R</i>	GAC ATC TTT GGA GCA GGA TTT GA CTT CCT CTG TAT CTC AGG CTT GGT
NM_000104	<i>CYP1B1</i>	<i>CYP1B1-F</i> <i>CYP1B1-R</i>	GTA CCG GCC ACT ATC ACT GAC A CAC ATC AGG ATA CCT GGT GAA GAG
AK222925	β - <i>ACTIN</i>	β - <i>ACTIN-F</i> β - <i>ACTIN-R</i>	CCT GGC ACC CAG CAC AAT GCC GAT CCA CAC GGA GTA CT

Abbreviations: F, forward primer; R, reverse primer. Nucleotide sequences were obtained from GenBank (2006).

Table 2. Micronucleus formation in MCF-7 cells treated with B[a]P and/or lindane.

B[a]P (M)	Micronuclei/1,000 binucleate cells	No lindane co-addition	Lindane (γ -HCH) (M)		
			10 ⁻¹²	10 ⁻¹¹	10 ⁻¹⁰
Background	MN	21	67	64	78
	TMN	24	86	90	108
10 ⁻⁸	MN	28	86	77	123
	TMN	33	118	103	186
10 ⁻⁷	MN	45	120	115	159
	TMN	56	193	177	244
10 ⁻⁶	MN	91	167	135	139
	TMN	138	293	217	252
10 ⁻⁵	MN	110	170	132	200
	TMN	183	273	198	330

Abbreviations: MN, the number of micronucleated binucleate cells scored per treatment; TMN, the total number of micronuclei scored per treatment. MCF-7 cells were treated for 24 hr in the presence or absence of test agents, as indicated, before cytokinesis block with cytochalasin-B. After further 24-hr incubation, cells were fixed and stained with 5% Giemsa as described in "Materials and Methods."

water, blued in Scott's tap water for 15 sec and rinsed again. Preparations were stained for 1 min with eosin (0.1% in 0.1% calcium chloride), rinsed with tap water, and dehydrated with graded alcohol solutions through to xylene. Cell preparations were then mounted on microscope slides with Pertex mountant (CellPath plc, Newtown, UK). The percentage of positive cells was determined as the mean \pm SD of five separate counts.

Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). Routinely cultured cells were disaggregated and resuspended in complete medium before seeding aliquots (5 mL; $\sim 1 \times 10^5$ cells) into 60-mm petri dishes. After 24 hr, attached cells were treated for an additional 24 hr. Cells were then washed twice with PBS before lysis and total RNA extraction using the QIAGEN RNeasy Kit in combination with the QIAGEN RNase free DNase kit (QIAGEN Ltd, Crawley, UK). DNase was incorporated into the extraction procedure to remove residual DNA, for example, pseudogene. RNA quality was routinely assessed in a 1.2% formaldehyde agarose gel; yield and purity were checked using a BioPhotometer (Eppendorf, Hamburg, Germany). RNA (0.4 μ g) was reverse transcribed in a final volume of 20 μ L containing Taqman reverse transcription reagents (Applied Biosystems, Warrington, UK): 1 \times Taqman RT buffer; MgCl₂ (5.5 mM); oligo d(T)₁₆ (2.5 μ M); dNTP mix (dGTP, dCTP, dATP, and dTTP; each at a concentration of 500 μ M); RNase inhibitor (0.4 U/ μ L); reverse transcriptase (1.25 U/ μ L; Applied Biosystems, Warrington, UK); and RNase-free water. Reaction mixtures were then incubated at 25°C (10 min), 48°C (30 min), and 95°C (5 min).

cDNA samples were stored at -20°C before use. Primers (Table 1) for *P21^{WAF1/CIP1}*, *BCL-2*, *BAX*, *CYP1A1*, *CYP1A2*, *CYP1B1* and endogenous control or housekeeping gene cytoplasmic ACTB (β -ACTIN) were chosen using Primer Express software 2.0 (Applied Biosystems) and designed so that one primer spanned an exon boundary. Specificity was confirmed using the National Center for Biotechnology Information BLAST search tool (<http://www.ncbi.nlm.nih.gov/blast/index.shtml>). Quantitative real-time PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Reaction mixtures contained 1 \times SYBR Green PCR master mix (Applied Biosystems); forward and reverse primers (Invitrogen) at a concentration of 300 nM (*P21^{WAF1/CIP1}*, *BCL-2*, *BAX*, *CYP1A1*, *CYP1A2*, *CYP1B1* or β -ACTIN); for *P21^{WAF1/CIP1}*, *BCL-2*, *BAX*, *CYP1A1*, *CYP1A2*, or *CYP1B1* amplification 20 ng cDNA template cDNA template or for β -ACTIN amplification 5 ng cDNA template; made to a total volume of 25 μ L with sterile

H₂O. Thermal cycling parameters included activation at 95°C (1 min) followed by 40 cycles each of denaturation at 95°C (15 sec) and annealing/extending 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 nonspecific amplification, including primer dimers.

Results

Table 2 shows the micronucleus-forming activity of B[a]P, lindane, or a binary mixture of both test agents in MCF-7 cells. Dose-related increases in the number of MN and the TMN were observed following treatment with either B[a]P or lindane alone. Induction of MNI following B[a]P treatment at concentrations of 10^{-7} M, 10^{-6} M, or 10^{-5} M resulted in approximately 2-, 3-, and 5-fold increases in micronucleus-forming activity, respectively. Lindane-induced increases were also observed following treatment with 10^{-12} M (~ 3 -fold), 10^{-11} M (~ 3 -fold), or 10^{-10} M (~ 4 -fold). After

treatment with a binary mixture, a marked elevation in micronucleus formation was induced compared with the effect of a single-agent exposure. In the presence of 10^{-12} M or 10^{-11} M lindane, clear dose-related increases in micronucleus formation were observed in the presence of B[a]P up to a concentration of 10^{-6} M; in combination with 10^{-6} M or 10^{-5} M B[a]P, these increases appeared to plateau (Table 2; Figure 1). Figure 1 shows that the micronucleus-forming effects of B[a]P treatment, in the absence or presence of 10^{-12} M lindane, were a consequence both of elevations in MN and TMN, namely, the summation of MNI in binucleate cells. In combination with 10^{-10} M lindane, dose-related increases in micronucleus formation in the presence of all B[a]P concentrations tested occurred; in the presence of 10^{-5} M B[a]P, approximately 10-fold increases in MNI compared with background control levels were observed (Table 2). Of note was the observation that micronucleus formation following treatment with 10^{-11} M lindane, in the absence or presence of B[a]P, was less than that

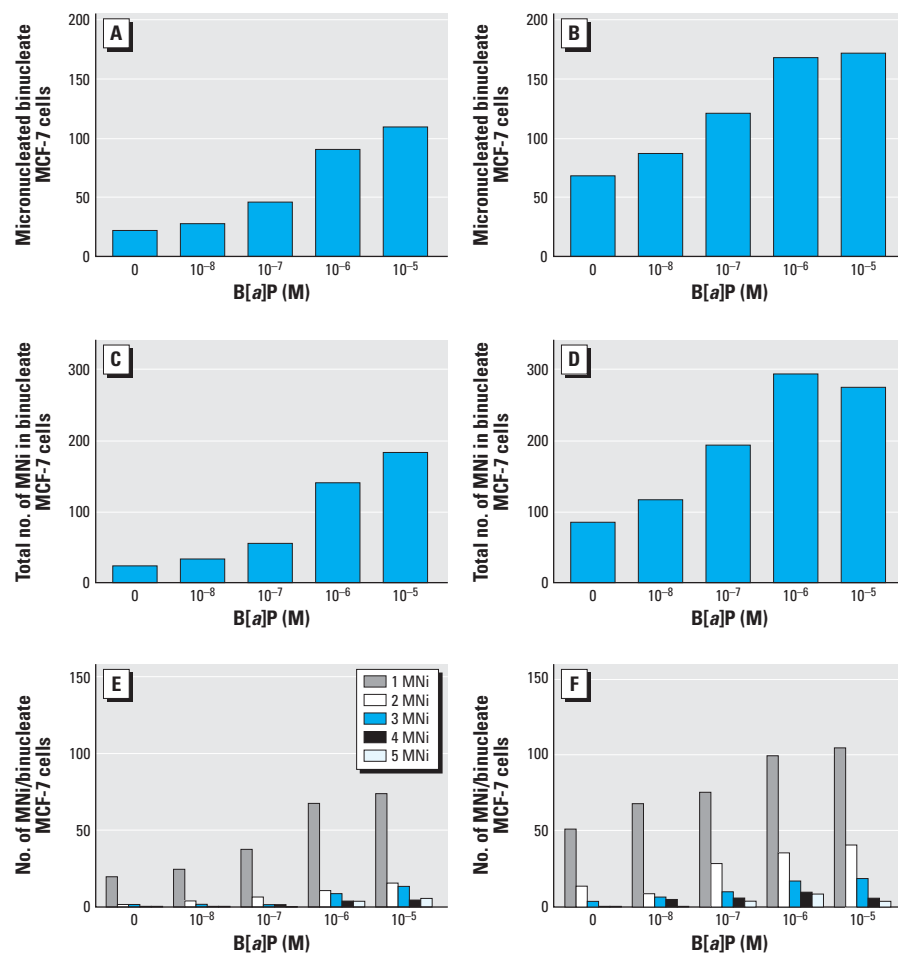


Figure 1. Micronucleus-forming activity of B[a]P with (A,C,E) or without lindane (B,D,F). Cell suspensions (3 mL, 1×10^4 cells) in 30-mm petri dishes were prepared and treated in the CBMN assay as described in "Materials and Methods." Micronucleus formation was scored in 1,000 binucleate cells. (E,F) Number of MNI in binucleate MCF-7 cells were scored from 1 to 5, with 5 representing the maximum score for this study.

observed with corresponding 10^{-12} M-induced effects; however, further increases in induced MNi were observed at the higher 10^{-10} M concentration (Table 2).

The effects of lindane on mitotic rate (percentage of binucleate cells) and clonogenic survival (percentage of plating efficiency) in B[a]P-treated cells are shown in Figure 2A,B. Treatment for 24 hr with B[a]P, in the absence of lindane, resulted in dose-related reductions in the percentage of binucleate cells from a background control level of $12.2\% \pm 0.7$ to $6.6\% \pm 0.5$ (10^{-5} M) (Figure 2A). In the presence of 10^{-12} M, 10^{-11} M, or 10^{-10} M lindane, concentration-dependent reductions in mitotic rate of approximately 20, 30, and 45%, respectively, were observed with or without B[a]P. Seven-day incubation, subsequent to a 24-hr treatment, resulted in B[a]P-induced reductions in the percentage of plating efficiency from a background control level of $35.4\% \pm 3.5$ to $32.8\% \pm 4.7$ (10^{-8} M), $28.1\% \pm 6.4$ (10^{-7} M), $14.9\% \pm 4.2$ (10^{-6} M), and 0 (10^{-5} M) (Figure 2B). Lindane, at the concentrations employed in this study, was not found to markedly alter clonogenic survival of MCF-7 cells in the absence or presence of B[a]P (Figure 2B).

Figure 3 shows the micronucleus-forming activities, in MCF-7 cells of 24-hr treatments with PhIP (0.5 μ M), lindane (10^{-12} M) or a binary mixture of both test agents. Both

agents individually induced 2- to 4-fold increases in MNi. However, treatment with a binary mixture resulted in levels of MN and TMN of 87 and 130 (a 5- to 8-fold increase) compared with a background control level of 15 and 16, respectively (Figure 3).

The effects of E_2 (10^{-9} M), lindane (10^{-12} M) or of a binary mixture of both test agents on the immunocytochemical detection of p53, p21^{Waf1/Cip1}, Bcl-2, and Bax proteins after 24-hr treatment are shown in Figure 4. E_2 induced a modest increase in p53- and p21^{Waf1/Cip1}-positive MCF-7 cells ($17.8\% \pm 3.3$ and $13.2\% \pm 1.5$ compared with background control levels of $8.4\% \pm 1.5$ and $8.2\% \pm 2.8$, respectively). Lindane appeared to reduce levels of p21^{Waf1/Cip1}-positive cells both in the absence ($7.2\% \pm 1.5$) or presence ($5.6\% \pm 1.5$) of E_2 . However, marked increases in Bcl-2-positive cells were induced by either E_2 or lindane; the binary mixture elevated the percentage of both Bcl-2- and Bax-positive cells (Figure 4). From background control levels of $33.8\% \pm 3.1$ (Bcl-2-positive) and $12.8\% \pm 1.9$ (Bax-positive) cells, E_2 , or lindane treatment gave rise to percentages of cells staining positive for Bcl-2 of $58.8\% \pm 6.5$ or $67.0\% \pm 4.3$ and for Bax of $9.8\% \pm 2.8$ or $6.4\% \pm 2.3$, respectively. A binary mixture gave rise to levels of $74.6\% \pm 5.6$ Bcl-2-positive cells and $55.0\% \pm 4.5$ Bax-positive cells (Figure 4).

The effects of different treatments on the quantitative expression of six genes (*P21^{WAF1/CIP1}*, *BCL-2*, *BAX*, *CYP1A1*, *CYP1A2*, and *CYP1B1*) were examined. To assess background fluctuation, we examined the variability in gene expression within control cell cultures ($n = 6$). Taking one control as the calibrator, marked intraexperimental variability was observed for relative levels of quantitative expression of *CYP1A1* (0.7–1.5), *CYP1A2* (0.5–1.7), and *CYP1B1* (0.6–1.5). In contrast, calibrator-controlled quantitative expression levels for *P21^{WAF1/CIP1}* (1.1–1.4), *BCL-2* (0.9–1.0), or *BAX* (0.9–1.3) appeared to be less variable.

In a panel of experiments (listed 1–5), the effects of a genotoxin (B[a]P or PhIP) in the absence or presence of 10^{-9} M E_2 or 10^{-11} M lindane, on the quantitative gene expression of *P21^{WAF1/CIP1}*, *BCL-2*, or *BAX* were examined (Table 3). Although 24-hr treatment with E_2 or lindane (with or without 10^{-8} M B[a]P or 0.5 μ M PhIP) was associated with a reduction in *P21^{WAF1/CIP1}* expression in experiments 1, 3, and 5, this was not apparent in experiments 2 and 4. In contrast, 24-hr treatment with 10^{-6} M B[a]P resulted in 36.1- and 56.3-fold elevations in *P21^{WAF1/CIP1}* expression in experiments 2 and 4, respectively; in the presence of E_2 or lindane, small reductions in these increases in gene expression were observed (Table 3). No marked alterations in *BCL-2* or *BAX* expression were observed following 24-hr treatment with 10^{-9} M E_2 or 10^{-11} M lindane. However, 10^{-6} M B[a]P was observed to reduce the ratio of *BCL-2*:*BAX* expression to approximately 0.1 as observed in experiments 2 and 4; effects that were not markedly altered in the presence of a binary mixture (Table 3).

Figure 5A,B shows the effects of 24-hr treatment with B[a]P (10^{-8} M or 10^{-6} M), in the absence or presence of E_2 (10^{-10} M or 10^{-9} M), on the immunocytochemical detection of p53, p21^{Waf1/Cip1}, Bcl-2, and Bax proteins in MCF-7 cells. Although treatment with B[a]P was associated with dose-related increases in the percentage of p53- and p21^{Waf1/Cip1}-positive cells, no such E_2 -induced alterations were observed. However, marked

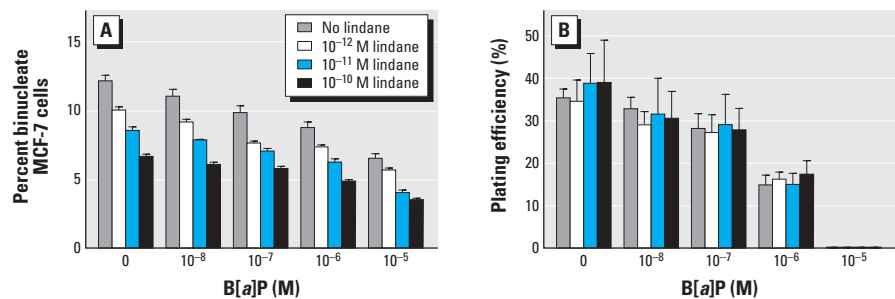


Figure 2. Effects of B[a]P, with or without lindane, on mitotic rate (A) and percentage of plating efficiency (B) in MCF-7 cells. After 24-hr treatment, cells were either blocked at cytokinesis before subsequent staining or cultured undisturbed for a further 7 days as described in “Materials and Methods.” Mitotic rate was estimated as percentage of binucleate cells from the mean \pm SD of three separate counts ($n = 500$ cells). Clonogenic survival was calculated by estimating the percentage of colonies counted over the number of cells initially seeded.

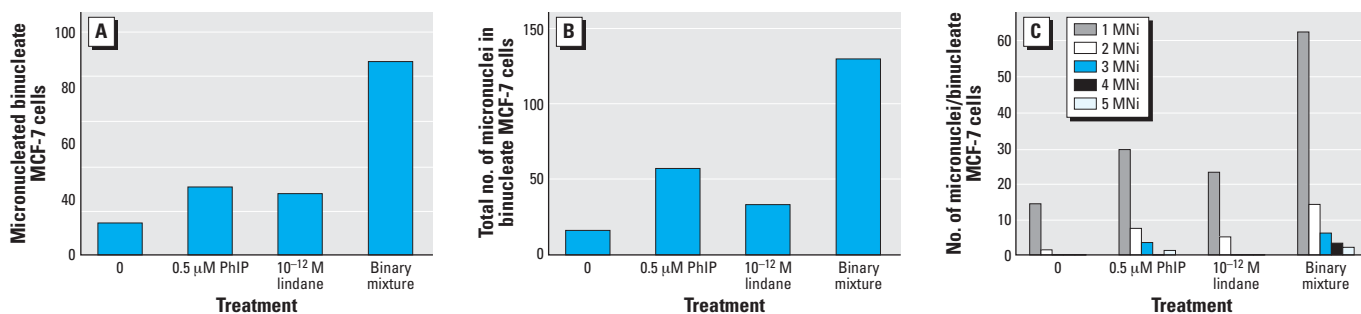


Figure 3. Micronucleus-forming activity of PhIP with or without lindane. Cell suspensions (3 mL, $\sim 1 \times 10^4$ cells) in 30-mm petri dishes were prepared and treated in the CBMN assay as described in “Materials and Methods.” Micronucleus formation was scored in 1,000 binucleate cells. (C) Number of MNi in binucleate MCF-7 cells were scored from 1 to 5, with 5 representing the maximum score for this study.

E_2 -induced alterations in the percentages of Bcl-2- and Bax-positive cells were observed. Treatment with 10^{-8} M B[a]P or 10^{-10} M E_2 resulted in marked elevations in the percentage of Bcl-2-positive cells ($52.4\% \pm 4.7$ and $72.0\% \pm 4.0$, respectively, compared with a background control level of $31.2\% \pm 3.7$); E_2 also reduced the percentage of Bax-positive cells from a background control level of $23.2\% \pm 4.1$ to $6.2\% \pm 1.5$ (Figure 5A). Similar modulations in the levels of Bcl-2- ($47.8\% \pm 7.9$) and Bax-positive ($7.4\% \pm 3.0$) cells were apparent after exposure to a binary mixture. The resulting ratios of Bcl-2-positive:Bax-positive cells were 1.3, 1.8, 11.6, and 6.5 in control, B[a]P-treated, E_2 -treated and binary mixture-treated cells, respectively (Figure 5A). In contrast, the ratio of Bcl-2-positive:Bax-positive cells following 24-hr treatment with 10^{-6} M B[a]P ($58.0\% \pm 3.0$ and $82.2\% \pm 1.9$ Bcl-2- and Bax-positive cells, respectively) was observed to be 0.7 compared with a background control level of 2.4 ($37.8\% \pm 5.1$ and $16.0\% \pm 2.3$ Bcl-2- and Bax-positive cells, respectively) (Figure 5B). The ratio of Bcl-2- positive:Bax-positive cells was again markedly elevated (8.4; from $78.6\% \pm 5.8$ and $9.4\% \pm 2.1$ Bcl-2- and Bax-positive cells, respectively) following 24-hr treatment with 10^{-9} M E_2 , whereas with a binary mixture (E_2 plus B[a]P), a ratio of 0.9 ($82.2\% \pm 2.8$ and $87.0\% \pm 2.9$ Bcl-2- and Bax-positive cells, respectively) was observed (Figure 5A).

Treatment with $0.5 \mu\text{M}$ PhIP and/or 10^{-9} M E_2 did not result in marked alterations in the levels of p53- or p21^{Waf1/Cip1}-positive MCF-7 cells (Figure 6). However, both agents induced marked modulations in the levels of Bcl-2- and Bax-positive cells when tested either individually or in a binary mixture. Treatment with PhIP or E_2 elevated the percentage of Bcl-2-positive cells ($73.0\% \pm 22.0$ and $78.6\% \pm 4.0$, respectively, compared with a background control level of $14.2\% \pm 4.9$);

PhIP also increased the percentage of Bax-positive cells from a background control level of $3.4\% \pm 1.7$ to $22.0\% \pm 4.8$. Exposure of MCF-7 cells to a binary mixture ($0.5 \mu\text{M}$ PhIP plus 10^{-9} M E_2) resulted in increases in both Bcl-2- ($55.0\% \pm 3.9$) and Bax-positive ($54.0\% \pm 4.6$) MCF-7 cells. The resulting ratios of Bcl-2-positive:Bax-positive cells were 4.2, 3.3, 19.7, and 1.0 in control, PhIP-treated, E_2 -treated, and binary mixture-treated cells, respectively (Figure 6).

Treatment with 10^{-8} M B[a]P, 10^{-12} M lindane, or $0.5 \mu\text{M}$ PhIP was not observed to alter levels of p53- or p21^{Waf1/Cip1}-positive cells; however, a binary mixture of lindane

plus PhIP induced marked increases in p53- ($41.4\% \pm 5.0$) and p21^{Waf1/Cip1}-positive ($33.0\% \pm 4.4$) cells compared with background control levels of $20.0\% \pm 1.9$ and $20.2\% \pm 2.6$, respectively (Figure 7A,B). Despite these observations, the low-dose treatments employed markedly altered levels of Bcl-2- and Bax-positive cells. B[a]P increased the levels of both Bcl-2- ($37.6\% \pm 3.6$) and Bax-positive ($13.8\% \pm 3.5$) cells giving rise to a ratio of 2.7 compared with a background control ratio of 7.5 ($16.6\% \pm 2.7$ and $2.2\% \pm 1.3$ Bcl-2- and Bax-positive cells, respectively) (Figure 7A). In contrast, lindane-induced alterations in the ratio of

Table 3. Relative gene expression measured by quantitative real-time RT-PCR.

Treatment	Relative expression levels in MCF-7 cells		
	P21 ^{WAF1/CIP1}	BCL-2	BAX
Experiment 1 (mean \pm SD, $n = 5$)			
Control	1 (c)	1 (c)	1 (c)
10^{-8} M B[a]P	1.2 ± 0.4	0.9 ± 0.3	1.1 ± 0.2
10^{-9} M E_2	0.6 ± 0.1	0.9 ± 0.3	1.2 ± 0.2
Binary mixture	0.9 ± 0.2	0.9 ± 0.3	0.6 ± 0.6
Experiment 2			
Control	1 (c)	1 (c)	1 (c)
10^{-6} M B[a]P	36.1	0.2	1.5
10^{-9} M E_2	1.0	1.3	1.3
Binary mixture	35.5	0.1	1.4
Experiment 3			
Control	1 (c)	1 (c)	1 (c)
10^{-8} M B[a]P	1.1	1.3	1.0
10^{-11} M lindane	0.7	1.2	1.0
Binary mixture	0.9	1.0	1.0
Experiment 4			
Control	1 (c)	1 (c)	1 (c)
10^{-6} M B[a]P	56.3	0.3	2.1
10^{-11} M lindane	1.9	2.0	2.9
Binary mixture	47.9	0.3	2.2
Experiment 5			
Control	1 (c)	1 (c)	1 (c)
$0.5 \mu\text{M}$ PhIP	1.4	1.2	1.4
10^{-9} M E_2	0.8	1.0	1.6
Binary mixture	0.6	0.7	1.1

(c), calibrator, which for the purposes of these experiments were untreated controls. Relative gene expression levels following 24-hr treatment in the presence or absence of test agents, as indicated. Reverse transcription of total RNA and subsequent 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 normalized to β -ACTIN values thus giving ΔC_T values. Alterations in gene expression were determined by comparing treatment groups with the calibrator, giving $\Delta\Delta C_T$ values. Finally, relative gene expression was calculated using the formula $2^{-\Delta\Delta C_T}$.

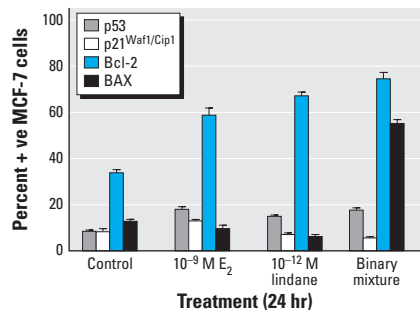


Figure 4. Immunocytochemical analysis of MCF-7 breast cells treated with E_2 and/or lindane. Cells were treated as indicated for 24 hr on coverslips, after which they were analyzed for protein expression as described in "Materials and Methods." The percentages of cells staining positive were determined after five separate counts of 100 cells and are presented as mean \pm SD.

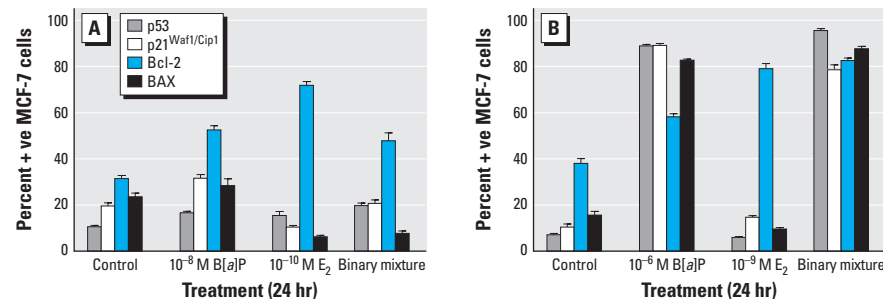


Figure 5. Immunocytochemical analysis of MCF-7 breast cells treated with B[a]P with or without E_2 . Cells were treated with 10^{-8} M B[a]P and/or 10^{-10} M E_2 (A) or 10^{-6} M B[a]P and/or 10^{-9} M E_2 (B) for 24 hr on coverslips, after which they were analyzed for protein expression as described in "Materials and Methods." The percentages of cells staining positive were determined after five separate counts of 100 cells and are presented as mean \pm SD.

Bcl-2-positive:Bax-positive cells were 13.4 (37.4% ± 2.9 and 2.8% ± 1.3 Bcl-2- and Bax-positive cells, respectively) (Figure 7A) and 6.1 (65.8% ± 2.8 and 10.8% ± 3.6 Bcl-2- and Bax-positive cells, respectively) (Figure 7B). The background control ratio of Bcl-2-positive:Bax-positive cells was 4.7 (40.8% ± 4.0 and 8.6% ± 1.8 Bcl-2- and Bax-positive cells, respectively) in Figure 7B. Exposure to a binary mixture resulted in ratios of Bcl-2-positive:Bax-positive cells of 4.2 (39.6% ± 3.8 and 9.4% ± 3.7 Bcl-2- and Bax-positive cells, respectively) (Figure 7A) and 0.6 (56.0% ± 4.9 and 89.6% ± 0.9 Bcl-2- and Bax-positive cells, respectively) (Figure 7B).

Table 4 shows the results of a panel of experiments (listed 1–4) examining the effects of B[a]P (10⁻⁸ M or 10⁻⁶ M), E₂ (10⁻¹⁰ M or 10⁻⁹ M), lindane (10⁻¹² M, 10⁻¹¹ M or 10⁻⁹ M), and various binary mixtures on the quantitative gene expression of *CYP1A1*, *CYP1A2* or *CYP1B1*. Interexperimental variations in the ability of low-dose treatments to alter *CYP* expression were observed; in experiment 1, 10⁻⁸ M B[a]P and/or 10⁻⁹ M E₂ did not induce the *CYP* isoenzymes examined, whereas in experiment 4, 3- to 4-fold increases in the *CYP1A1* expression were observed. Similarly, lindane-associated inter-experimental variation was noted; in experiment 3, 10⁻¹¹ M lindane treatment was associated with reductions in *CYP* expression, whereas in experiment 4, 24-hr treatment with either 10⁻¹² M or 10⁻⁹ M lindane induced a 2- to 4-fold increase in *CYP1A1*. Treatment with 10⁻⁶ M B[a]P consistently induced marked elevations in *CYP1A1*, *CYP1A2*, and *CYP1B1* expression in MCF-7 cells (Table 4).

Discussion

Humans may be exposed to different combinations of exogenous factors, including xenobiotics; evaluating only the toxicity of individual exposures might underestimate mixture effects

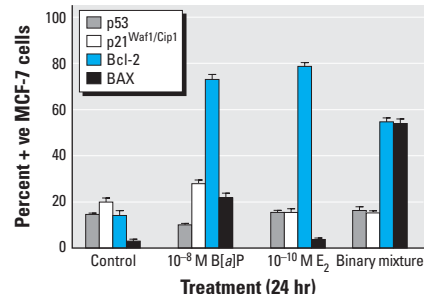


Figure 6. Immunocytochemical analysis of MCF-7 breast cells treated with PhIP and/or E₂. Cells were treated as indicated for 24 hr on coverslips, after which they were analyzed for protein expression as described in “Materials and Methods.” The percentages of cells staining positive were determined after five separate counts of 100 cells and are presented as mean ± SD.

(Culp et al. 2000; Maier et al. 2002; Marston et al. 2001; McLuckie et al. 2004). Apparently innocuous components of a mixture may significantly modulate underlying susceptibility of target cells to more toxic constituents (Slaga et al. 1979). Modulating effects may occur through ligand-activated, receptor-mediated mechanisms (Eltom et al. 1998) or interference with cell cycle control (Oikawa et al. 2001). Some xenobiotics may not only induce DNA damage but also may drive clonal expansion, often at low-dose levels not dissimilar to typical human exposures; such effects may explain the target-organ specificity of some procarcinogens (Lauber et al. 2004; Plíšková et al. 2004).

Endocrine-active properties of xenobiotics may play a role in the etiology of much human pathology (Safe 2004) in hormone-responsive tissues (Ragavan et al. 2004).

B[a]P, PhIP, or lindane individually induced 2- to 5-fold increases in MNi; treatment with a binary mixture gave rise to approximately 10-fold increases (Table 2, Figures 1 and 3). Consistent with previous findings (Kalantzi et al. 2004b) that lindane (γ-HCH) induces a cell cycle arrest in MCF-7 cells, dose-related reductions in mitotic rate were observed following treatment with this agent (Figure 2A). However, in a binary mixture lindane was not observed to alter

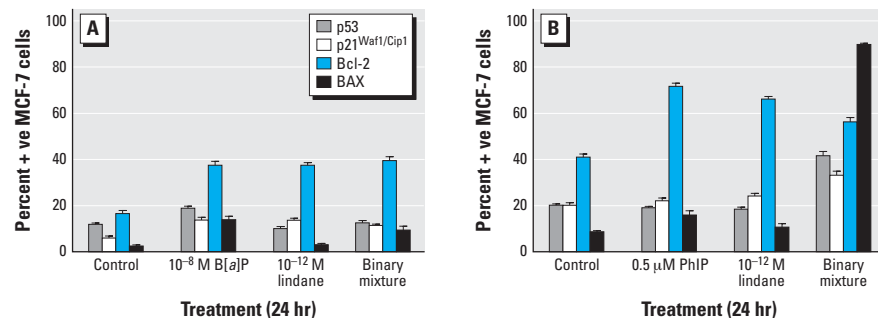


Figure 7. Immunocytochemical analysis of MCF-7 breast cells treated with (A) B[a]P and/or lindane or (B) PhIP and/or lindane. Cells were treated as indicated for 24 hr on coverslips, after which they were analyzed for protein expression as described in “Materials and Methods.” The percentages of cells staining positive were determined after five separate counts of 100 cells and are presented as the mean ± SD.

Table 4. Relative gene expression measured by quantitative real-time RT-PCR.

Treatment	Relative expression levels in MCF-7 cells		
	<i>CYP1A1</i>	<i>CYP1A2</i>	<i>CYP1B1</i>
Experiment 1			
Control	1 (c)	1 (c)	1 (c)
10 ⁻⁸ M B[a]P	0.8	1.0	0.9
10 ⁻⁹ M E ₂	0.7	0.8	1.5
Binary mixture	0.9	0.5	1.8
Experiment 2			
Control	1 (c)	1 (c)	1 (c)
10 ⁻¹⁰ M E ₂	1.4	0.3	0.9
10 ⁻⁸ M B[a]P	1.6	1.2	1.4
Binary mixture1	2.5	1.0	1.8
10 ⁻⁶ M B[a]P	462.7	5.6	28.6
Binary mixture 2	430.5	3.7	26.5
Experiment 3			
Control	1 (c)	1 (c)	1 (c)
10 ⁻⁶ M B[a]P	370.9	15.4	41.6
10 ⁻¹¹ M lindane	0.6	0.4	0.6
Binary mixture	183.9	12.0	23.3
Experiment 4			
Control	1 (c)	1 (c)	1 (c)
10 ⁻⁹ M E ₂	3.6	0.9	1.6
10 ⁻⁸ M B[a]P	3.6	1.4	1.3
10 ⁻⁶ M B[a]P	457.7	9.4	23.9
10 ⁻¹² M lindane	2.0	0.8	1.0
10 ⁻⁹ M lindane	3.6	1.3	1.4

(c), calibrator, which for the purposes of these experiments were untreated controls. Where indicated binary mixture 1 = 10⁻¹⁰ M E₂ + 10⁻⁸ M B[a]P; binary mixture 2 = 10⁻¹⁰ M E₂ + 10⁻⁶ M B[a]P. Relative gene expression levels after 24-hr treatment in the presence or absence of test agents, as indicated. Reverse transcription of total RNA and subsequent 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 normalized to β-ACTIN values, thus giving ΔC_T values. Alterations in gene expression were determined by comparing treatment groups with the calibrator, giving ΔΔC_T values. Finally, relative gene expression was calculated using the formula 2^{-ΔΔC_T}.

B[a]P-induced reductions in clonogenic survival (Figure 2B). At micromolar concentrations, B[a]P markedly altered gene expression by inducing up-regulation of *P21^{WAF1/CIP1}* and *CYP* isoenzymes and down-regulation of *BCL-2* (Tables 4 and 5). However, with 24-hr treatment, no marked modulations in gene expression were apparent following low-dose treatments (less than or equal to nanomolar range) with either individual-agent or binary-mixture treatments (Tables 4 and 5). This was despite observations that 10^{-9} M *E₂* or lindane did induce a 3-fold elevation in *CYP1A1* expression in one experiment (Table 4). Such observations suggest that modulations in expression of the candidate genes examined may be transient following low-dose treatment or that other factors are responsible for the effects observed, for example, oxidative damage (Jiao et al. 2007; Rajapakse et al. 2005).

Pleiotropic induction of unrelated phenotypic effects appears to be a feature of oestrogens, endocrine disruptors and PAHs (Jeffy et al. 2002; Moggs and Orphanides 2001; Mueller et al. 2004). Endogenous hormones facilitate cell growth/proliferation, differentiation, behavior, and activity in many tissues. In responsive cells, stimulatory effects are observable at less than or equal to nanomolar concentrations (Yared et al. 2002). Different xenobiotics may also induce effects through receptor-mediated processes (Kalantzi et al. 2004b; Lauber et al. 2004), for example, *E₂* appears to increase the percentage of Bcl-2-positive cells (Figures 4–6). A binary mixture of *E₂* and lindane induced a marked reduction in the ratio of Bcl-2-positive to Bax-positive cells (Figure 4); this was due to an apparently induced increase in Bax-positive cells while the percentage of Bcl-2-positive cells remained unchanged. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin is cytostatic in MCF-7 cells through an ability to function as an antiestrogen and down-regulate a battery of *E₂*-induced proliferative responses (Döhr et al. 1995; Wang et al. 1998); whether lindane may induce a similar effect through a reduction in Bcl-2:Bax ratio remains to be determined.

In a dose-related fashion, B[a]P elevated the percentage of p53-positive cells (Kaspin and Baird 1996) and the downstream cyclin-dependent kinase inhibitor, p21^{Waf1/Cip1} (Figure 5). PhIP (0.5 μM) apparently failed to increase the percentage of p53-positive or p21^{Waf1/Cip1}-positive MCF-7 cells (Figures 6 and 7); at higher concentrations and after metabolic activation, PhIP has been shown to elevate these intracellular factors in MCF10A cells (Creton et al. 2005). However, PhIP markedly elevated the percentage of Bcl-2-positive cells while the levels of Bax-positive cells remained relatively low; although B[a]P

also increased levels of Bcl-2-positive cells, marked increases in Bax-positive cells were also apparent (Figures 5–7). In a binary mixture with *E₂* or lindane, a reversal in B[a]P-induced reductions in the ratio of Bcl-2-positive to Bax-positive cells was observed (Figures 5 and 7). However, *E₂* or lindane in combination with PhIP appeared to reverse potential hormone-driven survival characteristics observed as marked reductions in the ratio of Bcl-2-positive to Bax-positive cells (Figures 6 and 7).

In this study we investigated whether binary mixtures of DNA-reactive procarcinogens and hormonelike compounds at environmentally relevant low-dose concentrations give rise to markedly elevated DNA damage in target cells while also modulating survival. Our results suggest that this might indeed be the case.

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