**Examination of the Estrogenicity of 2,4,6,2',6'-Pentachlorobiphenyl (PCB 104), Its Hydroxylated Metabolite 2,4,6,2',6'-Pentachloro-4-Biphenylolethanol (HO-PCB 104), and a Further Chlorinated Derivative, 2,4,6,2',4',6'-Hexachlorobiphenyl (PCB 155)**

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Several studies have reported that polychlorinated biphenyls (PCBs) exhibit estrogenic activity; however, it is not clear if these responses are associated with the polychlorinated hydrocarbon or its hydroxylated metabolite. In order to further test this hypothesis, a battery of in vitro and in vivo assays were used to investigate the estrogenic and antiestrogenic activities of 2,4,6,2',6'-pentachlorobiphenyl (PCB 104), its para-hydroxylated derivative 2,4,6,2',6'-pentachloro-4-biphenylolethanol (HO-PCB 104), and its para-chlorinated derivative 2,4,6,2',4',6'-hexachlorobiphenyl (PCB 155). PCB 104 was found to 1) compete with tritiated 17β-estradiol (E2) for binding to the mouse uterine estrogen receptor (ER); 2) induce gene expression in MCF-7 human breast cancer cells transiently transfected with the Gal4-human ER chimeric construct (Gal4-HEGO) and the Gal4-regulated luciferase reporter gene (17m5-G-Luc); and 3) increase MCF-7 cell proliferation in a dose-dependent manner. HO-PCB 104 exhibited greater estrogenic activity than PCB 104 in the in vitro assays examined. However, gas chromatographic-mass spectrophotometric analysis of extracts prepared from MCF-7 cells incubated with PCB 104 failed to detect the presence of the expected major metabolite HO-PCB 104. The estrogenic activity of the para-chlorinated derivative, PCB 155, was minimal compared to PCB 104 and HO-PCB 104, but it did exhibit significant antiestrogenic activity following co-treatment with 1 nM E2. Co-treatment of PCB 104 with 1 nM E2 had no effect on reporter gene expression compared to E2 alone, while 10 μM HO-PCB 104 exhibited additive with 1 nM E2. At a dose of 202 μg/kg, PCB 104 increased uterine wet weight in ovariectomized CD-1 mice and induced vaginal epithelial cell cornification at 202, 16, and 1.7 mg/kg in a dose-dependent manner. These studies demonstrate that in addition to the hydroxylated metabolites, selected parent PCB congeners may also exhibit estrogenic and antiestrogenic activities.

Key words: environmental estrogen, estrogenicity, estrogen receptor, hydroxylation, in vitro, in vivo, polychlorinated biphenyls.

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Estrogens influence the growth, differentiation, development, and function of several target tissues involved in reproduction, cardiovascular performance, bone maintenance, homeostasis, and behavior. Many of these processes are modulated as a result of estrogen receptor (ER)-mediated expression of estrogen responsive genes. The binding of estrogens to the ligand binding domain of the ER causes dissociation of associated heat shock proteins and subsequent dimerization of ligand-occupied ERs. The homodimer complex then interacts with specific DNA sequences referred to as estrogen response elements (ERE) located in the regulatory region of estrogen-inducible genes. ER complexes that are bound to an ERE recruit additional transcription factors, leading to increases in gene transcription (1,2). In addition to their known normal physiological functions, estrogens have also been implicated in the development of hormone-dependent cancers of the breast, ovaries, endometrium, and prostate (3,4). Studies suggest that estrogens promote the growth and invasiveness of hormone-dependent tumors by inducing genes such as growth factors, growth factor receptors, protooncogenes, and proteases, which contribute to cell proliferation, invasion, and metastasis (4–8).

Estrogenic chemicals or mixtures have been defined as substances whose effects are mediated through the estrogen receptor, initiating a cascade of cellular/tissue effects similar to those initiated by 17β-estradiol (E2). In contrast, chemicals or complex mixtures whose effects resemble those of estrogen but are not mediated through the estrogen receptor are referred to as estrogenlike (9). Recently, xenobiotics capable of eliciting estrogenic activities have also been implicated as contributing factors in the development of hormone-dependent cancers, as well as compromising the reproductive fitness of humans and wildlife (10–12). These chemicals, commonly referred to as environmental estrogens, xenoestrogens, or exoestrogens, encompass a wide range of compounds including natural products, environmental pollutants, pharmaceuticals, and industrial chemicals. Many xenoestrogens do not share any structural similarity to the prototypical estrogen, E2, the preeminent female sex steroid (13). However, results from in vitro studies have demonstrated that xenoestrogens can interact with the ER and induce estrogenic responses (13–15). Nevertheless, in vivo studies are required to definitively demonstrate that a chemical possesses sufficient endocrine disrupting activities to adversely affect an organism (14,15).

Polychlorinated biphenyls (PCBs) are a class of synthetic, persistent, lipophilic, halo- genated aromatics that, despite their discontinued use, are still found throughout the environment as complex mixtures (16,17). PCBs induce a broad spectrum of toxic and biochemical responses in a number of in vitro and in vivo models. Many of the responses elicited by coplanar PCBs correlate with their binding affinity to the aryl hydrocarbon receptor (AhR), which is believed to mediate several of the effects induced by these compounds (18). However, noncoplanar PCBs that exhibit low or negligible binding affinity for the AhR have also been shown to evoke a number of responses including immunotoxicity and neurotoxicity (19–23). Recent studies suggest that some of these effects may be due to interactions with calcium-dependent pathways (19,21,23). In addition, hydroxylated metabolites of PCBs have been found to interact with uteroglobin (24) and transthyretin (25). Interactions with these proteins have been implicated in the diverse spectrum of reproductive and endocrine-modulating activities reported in laboratory animals as well as in humans (16,18,26–28).

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Using three-dimensional quantitative structure–activity relationships (QSAR), Wailer and co-workers (29) have suggested that structural similarities exist between E$_2$ and PCBs. The model is based on hydroxylated chlorobiphenyl binding affinities for the ER (30) and the alignment of the para-substituted ring of the test molecule to the hydroxylated A ring of E$_2$. It suggests that biphenyls possessing both ortho- and para-substituents are capable of competing with E$_2$ for binding to the ER. Furthermore, conformational restriction about the twist bond of the biphenyl molecule due to ortho-substitution is also predicted to be an important factor. In vitro and in vivo studies have shown that selected PCBs and their mixtures are capable of mimicking some of the biological activities of estrogens. In vitro, selected PCB congeners as well as commercial Aroclor mixtures have been reported to compete with E$_2$ for binding to the ER, promote MCF-7 human breast cancer cell proliferation, and induce gene expression (31–35). Rodents treated with PCBs and commercial Aroclors exhibited increased levels of estrogenic effects including precocious puberty, disrupted estrus, altered temperature-sensitive sex determination, and increases in the wet weight, water imbibition, and glycogen content of the uterus, as well as the induction of estrogen-responsive enzyme activities (36–42). Based on their chemical structure and estrogenic activities, it has been suggested that only those PCB congeners that possess ortho-substituents are capable of eliciting an estrogenic response following hydroxylation at a vacant para position (30). Results obtained by Soto et al. (34) support this hypothesis because 2,5-dichlorobiphenyl was found to be inactive in the MCF-7 cell proliferation assay (E-Screen), whereas ortho-, meta-, and para-hydroxylation on the unsubstituted ring significantly activated activity, with para-hydroxylation conferring the greatest effect. Similarly, it has been proposed that methoxychlor and benzo[a]anthracene also require metabolic activation to hydroxylated metabolites to elicit estrogenic effects (43,44). Numerous other experimental paradigms have been used to demonstrate that hydroxylated PCBs elicit ER-mediated responses (30,39,45–47).

Taken together, these findings bolstered preliminary epidemiological reports that found higher levels of PCBs in women with breast cancer (48,49), leading some to suggest that exposure to estrogenic PCBs may be a contributing factor in the increased incidence of hormone-dependent diseases and compromised reproductive fitness in humans and wildlife (10,11,50,51). However, subsequent epidemiological studies have failed to demonstrate an association between PCB body burdens and increased risk of breast cancer (52–56). Furthermore, the antiestrogenic activities of selected PCB congeners and mass balance calculations indicate the estrogenic burden contributed by PCBs is minimal relative to other sources of estrogen (12,57,58).

To further investigate the alleged estrogenic activities of PCBs and their metabolites, we examined the ER-mediated effects of 2,4,6,2',6'-pentachlorobiphenyl (PCB 104), 2,4,6,2',6'-pentachloro-4-biphenylol (HO-PCB 104), and 2,4,6,2',4',6'-hexachlorobiphenyl (PCB 155) (Fig. 1). Although these compounds are not environmentally relevant, they were chosen because 1) PCB 104 exhibited significant estrogenic activity in preliminary studies; 2) PCB 104 should be preferentially hydroxylated at the vacant para position to produce HO-PCB 104, and 3) PCB 155 represents the hexachloro-derivative of PCB 104, with the chlorine substituent occupying the vacant para position in PCB 104. Therefore, these three derivatives provide an excellent model to investigate the effect of para-hydroxylation and para-chlorination on the ER-mediated activities of an alleged estrogenic PCB. In vitro assays were used to determine the activities of PCB 104, HO-PCB 104, and PCB 155 to compete with E$_2$ for binding to the mouse uterine ER, promote MCF-7 human breast cancer cell proliferation, and induce gene expression using a recombinant receptor/reporter gene assay. In addition, gas chromatography-mass spectrophotometry (GC-MS) was used to analyze media collected from PCB 104-induced gene expression assays performed in transiently transduced MCF-7 cells to determine the presence of the major expected metabolite HO-PCB 104. Uterotrophic and vaginal cell cornification assays were also performed to examine the in vivo estrogenic effects of PCB 104. Collectively, these results indicate that all three compounds, PCB 104, HO-PCB 104, and PCB 155, were capable of eliciting ER-mediated activities.

Materials and Methods

Chemicals and consumables. PCB 104, PCB 155, and HO-PCB 104 were synthesized and chemically analyzed as previously described and were >98% pure as determined by gas chromatographic analysis (59,60). E$_2$ was purchased from Sigma Chemical Company (St. Louis, MO). Serial dilutions of the PCBs and E$_2$ were prepared in dimethyl sulfoxide (DMSO), which was purchased from BDH (Toronto, Ontario, Canada). Phenol red-free Dulbecco’s modified eagle’s medium (MEM) powder and media supplements were purchased from Gibco/BRL (Burlington, Ontario, Canada). Fetal bovine serum (FBS) was obtained from CanSera (Rexdale, Ontario, Canada). d-Luciferin was purchased from Molecular Probes (Eugene, OR). Sesame oil was obtained from Aldon Corporation (Avon, NY). All other chemicals and biochemicals were of the highest quality available from commercial sources.

Standards of PCB 104, 3,4,5,3',4',5'-hexachloro[13C12]biphenyl (MBP126), and HO-PCB 104 were obtained from Wellington Laboratories (Guelph, Ontario, Canada). All solvents used for GC-MS analyses were of glass-distilled quality and were obtained from Caledon Laboratories (Georgetown, Ontario, Canada). Iodo-methane and tetramethylammonium hydroxide (1.0 M in methanol) were obtained from Fisher Scientific Limited (Ottawa, Ontario, Canada).

Animals. CD-1 mice were obtained from Charles River Breeding Laboratories (Montreal, Quebec, Canada) and housed in a controlled environment with a 12 hr light–dark cycle, ambient air temperature was controlled at 20–22°C, and the relative humidity was maintained at 40–60%. Purina RMH 3000 chow (Purina, New Hamburg, Ontario, Canada) and tap water were provided ad libitum. Animals were euthanized by CO$_2$ asphyxiation 24 hr following the last treatment.

Ligand binding assay. Shortly after weaning, uterine tissue was collected, weighed, and homogenized on ice in 1 ml of ice-cold TEGD (10 mM tris base, 1.5 mM EDTA, 10% glycerol, 1.0 mM dithiothreitol, pH 7.6) per 50 mg uterine tissue with three 20-sec bursts using a Brinkman Polytron tissue homogenizer (Brinkman, Mississauga, Ontario, Canada) at 50% output. Samples were centrifuged at 3,000 x g for 10 min at

Figure 1. Structure of 17β-estradiol, the polychlorinated biphenyls, and the hydroxylated metabolite.
4°C. The supernatant was centrifuged at 105,000 × g for 1 hr at 4°C. The resulting supernatant, constituting the cytosol, was carefully decanted, and the protein concentration was adjusted to 2.0 mg/ml. The cytosol was stored at −80°C for use within 4 weeks.

Specific binding of [3H]-E2 was measured as previously described by Laws et al. (67). Briefly, aliquots of cytosol (240µl) were incubated with 30 µl 1 nM [3H]-E2 and 30 µl competitor. The final concentration of competitor was either 0.1–1,000 nM unlabeled E2, 0.001–1,000 µM PCB, or solvent. Incubations were carried out at 30°C for 30 min and then cooled to 4°C. Each sample was divided into two 130-µl aliquots, and bound ligand was separated from free ligand by the addition of 125 µl 60% (v/v) hydroxylapatite (HAP) suspension in TEGD buffer. The mixture was washed three times with ice-cold TEGD buffer containing 1% Triton X-100, and the test tubes were inverted and allowed to dry. The ER was denatured by the addition of 1 ml absolute ethanol and bound [3H]-E2 was measured using a scintillation counter. Each treatment was performed in duplicate, and two samples were counted from each test tube. Results are expressed as percent specific binding of [3H]-E2 versus log of the competitor concentration.

Cell culture. MCF-7 cells are ER-positive estrogen responsive human breast cancer cells (provided by L. Murphy, University of Manitoba, Manitoba, Canada). MCF-7 cells were grown in phenol red-free MEM supplemented with 10% FBS and 24 mM sodium bicarbonate, 1 mM glucose, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesul- fonic acid (HEPES), 1× nonessential amino acids, 1× vitamin supplement solution, 10 mM sodium pyruvate, 2 mg/ml lipoic acid, 1.38 mg/ml vitamin B12, 0.5 mM zinc sulfate, 2 mM glutamine, 50 µg/ml gentamycin, 100 IU penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B. The cells were grown at 37°C in a 5% CO2 humidified environment.

Transfections and reporter gene assays. Transient transfections and gene transcription assays were performed essentially as previously described (47,62–64). Briefly, MCF-7 cells were seeded at approximately 50% confluency in 6-well tissue culture plates in medium supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) and allowed to attach for 6 hr. Cells were then transiently transfected by the calcium phosphate co-precipitation method (65) with 2.5 µg 17m5-G-Luc (provided by P. Chambon, IGBMC, Illkirch, France), 0.5 µg Gal4-human ER chimera (Gal4-HEGO) (66), along with 0.01 µg pCMV-lacZ (β-galactosidase expression vector, provided by G. DiMattia, London Regional Cancer Centre, London, Ontario, Canada) or 1.5 µg pCH1110 (Pharmacia, Baie d’Urfe, Quebec, Canada). Cells were washed 16 hr later with sterile phosphate-buffered saline (PBS) and fresh medium was added to each well.

Transfected MCF-7 cells were exposed to the following final concentrations: 0.1, 1, and 10 µM of test compound; 1 pM–10 nM E2, or DMSO (solvent) alone. Final concentrations were obtained by adding 2.5 µl of test chemical to 2.5 ml of medium. Following incubation with the test compound for 24 hr, cells were harvested and assayed for luciferase activity according to the method described by Brasier et al. (67).

The reference placmid, pCMV-lacZ or pCH1110, was co-transfected as an internal control to correct for differences in transfection efficiencies and sample extractions. β-Galactosidase activity was measured according to standard protocols (65). Transiently transfected MCF-7 cells were treated in duplicate and two samples were taken from each replicate. Therefore, the means and standard deviations were calculated from four measurements. Each experiment was repeated three times. Values were reported as a percentage relative to the maximum induction observed with E2.

Cell proliferation assay. MCF-7 cells (obtained from ATCC, Rockville, MD) were grown and the assay performed as previously described (63). The cells were treated with 0.01–10 µM PCP 104, HO-PCP 104, or PCB 155 alone or in the presence of 1 nM E2. Each treatment was performed in triplicate, and the assay was performed two times.

Mouse uterotrophic and vaginal cornification assay. Ovariectomized (OVX) CD-1 mice were received at approximately 12 weeks of age with a mean weight of 41 g (Charles River, Montreal, Quebec, Canada). Following 6 days of acclimatization, the mice were orally gavaged daily for 4 consecutive days with 0.1 ml of sesame oil containing 17β-ethynyl estradiol (EE) or PCB 104 at the doses indicated in Table 1. Doses were calculated based on the average weight of the animal groups on day 1 of treatment. On day 5, mice were euthanized by CO2 asphyxiation 24 hr following the last treatment, the animals were weighed, and the uteri were then removed, trimmed of adhering fat and connective tissue, blotted to remove water, and weighed. Results were expressed as the mean ± standard deviation (SD) of the uterine wet weight to body weight ratio.

Vaginal smears were obtained using physiological saline on day 5 following euthanization. The smears were evaluated by two independent evaluators who were blind to the treatment protocol and were scored as the percentage of cornified cells relative to the population of all cell types present, as described by Terenius (68). Interscorer reliability was greater than 90%. Smears were also evaluated as positive or negative, depending on the presence or absence of cornified cells.

GC-MS analysis. The medium from treated MCF-7 cells was initially extracted with chloroform and evaporated to dryness for shipping. The extracts were dissolved in dichloromethane (DCM; 3 ml) and exactly

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**Table 1. In vivo effects of 17α-ethynyl estradiol and 2,4,6,2′,6′-pentachlorobiphenyl (PCB 104) on mature ovariectomized CD-1 mice**

| Treatmenta | Daily dose | Uterine wet weight (mg/g)b | Number of positive smearsc | Cornification of smears (%)d |
|------------|------------|---------------------------|---------------------------|---------------------------|
| Sesame oil (vehicle) | 0.1 ml | 1.1 ± 0.1 | 0/5 | 0 |
| 17α-Ethynyl estradiol | 1 mg/kg | 4.6 ± 1.2c | 5/5 | 02 ± 20 |
| PCB 104 | 202 mg/kg | 1.8 ± 0.1c | 5/5 | 44 ± 26 |
| PCB 104 | 16 mg/kg | 1.3 ± 0.3 | 4/5 | 20 ± 34 |
| PCB 104 | 1.7 mg/kg | 1.1 ± 0.2 | 4/5 | 8 ± 6 |

*aAnimals were orally gavaged for 4 consecutive days with the indicated doses.

bThe mean ± standard deviation were results obtained from five mice.

cA positive smear is defined as a vaginal smear that contained cornified cells as determined by histologi
cal examination.

dSmears were scored by two investigators blind to the treatments as the percentage of cornified cells relative to the population of all other cell types present in the smear, as described by Terenius (68).

*Denotes increase in uterine wet weight (p < 0.0002) relative to the sesame oil control.
one-third was taken for cleanup, methylation, and analysis. The GC-MS internal standard, 3,4,5,3',4',5'-hexachloro[13C5]biphenyl (MBP 126, 1,000 ng) was added to each sample and the extracts were blown down to dryness with nitrogen. Sulfuric acid (2 ml of 0.5 M) was then added to each extract and the mixtures were shaken. These solutions were then extracted twice with pentane/diethyl ether (1:1, v/v). The pentane/diethyl ether extracts were combined and blown down to dryness, and 3 ml of acetone was then added to each extract.

The procedure used for the methylation of the chlorinated biphenyls (if present) followed the methodology developed by Hopper (69) and modified by Sandau and Norstrom (70). After methylation, the extracts were concentrated with solvent exchange into toluene (500 μl) for GC-MS analysis.

Analyses of the final extracts were performed using a Hewlett Packard 5890 capillary GC (Hewlett Packard, Mississauga, Ontario, Canada) coupled to a Hewlett Packard 5970 series mass selective detector (GC-MSD). The GC was filled with a 30 m DB-5 capillary column (0.25 mm inner diameter, 0.25 mm film thickness; J & W Scientific, Brockville, Ontario, Canada). The injector and detector temperatures were 250°C and 300°C, respectively. The GC oven had the following temperature program: initial temperature of 90°C, hold time, 7.5 min; temperature program, 10°C/min to 320°C; hold time, 10 min. Splitless injections of 2 ml of each extract were made with a purge valve time of 1.5 min. The GC-MSD was operated in the SIM (selected ion monitoring) mode. The ions monitored were 324, 326, and 328 for PCB 104; 336, 338, and 340 for the GC/MS internal standard MBP126; and 354, 356, and 358 for HO-PCB 104. Four calibration standard solutions were prepared by accurately mixing stock solutions of PCB 104, MBP126, and HO-PCB 104 in various proportions and diluting these mixtures with acetone. Aliquots of these solutions were tested using the same procedures that were used for the extracts.

Statistics. Statistical analysis was by Duncan's new multiple range test (one-way analysis of variance [ANOVA]) at the p<0.05 significance level. If significant differences were found, subsequent Student's t-tests were calculated. Significant differences were defined when p<0.05, unless otherwise indicated.

Results
In vitro competitive ligand-binding assay. Competitive ligand-binding assays with [3H]-E2 were used to determine the binding affinities of PCB 104, HO-PCB 104, and PCB 155 to the mouse uterine ER (Fig. 2). The concentration of PCB 104 needed to inhibit binding of 50% of the labeled ligand (IC50) was 1.7 μM, which is approximately 110-fold less effective than E2 (IC50 = 15 nM). para-Hydroxylation of PCB 104 to HO-PCB 104 increased the binding affinity for the ER by 24-fold. The IC50 value for HO-PCB 104 was 70 nM, approximately 4.5-fold less than the IC50 value for E2. PCB 155 was the least effective competitor, with an IC50 value of 5.6 μM, which is 362-fold less affinity for the ER than E2.

Estrogen receptor-mediated agonist and antagonist activities using recombinant receptor/reporter gene assays. The ER-mediated estrogenic and antiestrogenic activities of PCB 104, HO-PCB 104, and PCB 155 were assessed using a recombinant receptor/reporter gene assay. This assay measures luciferase activity in MCF-7 cells transiently transfected with a Gal4-HEGO chimeric receptor, which consists of the yeast Gal4 DNA binding domain linked to the ligand binding domain of the human ER, and a Gal4 response element (17mer)-regulated reporter gene (17mer-G-Luc), which contains five tandem 17mer response elements upstream of the rabbit β-globin promoter linked to the firefly luciferase reporter gene. Because expression of luciferase in this system can only be induced through activation of the Gal4-HEGO chimeric receptor, induction of 17mer-G-Luc activity provides definitive evidence of ER-mediated activity. This assay has been previously used to identify and assess the ER-mediated activities of a number of alleged exoestrogens and complex mixtures (46,47,62–64,71–73).

E2 induced a dose-dependent increase in luciferase activity, reaching a 100% response (69-fold induction) at 50 nM E2 (Fig. 3). PCB 104 induced luciferase activity in a dose-dependent manner with a relative activity of 45% (31-fold) at a concentration of 10 μM, the highest concentration tested, when compared to the 100% response observed with E2. The para-hydroxylated derivative HO-PCB 104 produced a modest increase in estrogenicity at 0.1 and 1.0 μM, reaching a maximum of 15-fold induction. However, the observed induction was only 9-fold at 10 μM. Visual inspection of the cells and measurement of β-galactosidase activity did not indicate that the low level of 17mer-G-Luc activity was due to HO-PCB 104 toxicity (data not shown). PCB 155 showed minimal estrogenic activity (Fig. 3). Induction by E2, PCB 104, and HO-PCB 104 was completely inhibited by the pure antiestrogen ICI 164,384 (100 nM), thus confirming the role of the ER in mediating the response (Fig. 4).
In the absence of Gal4-HEGO, E$_2$, PCB 104, and HO-PCB 104 failed to induced luciferase activity (data not shown).

To determine the optimal incubation time for the induction of luciferase activity, transiently transfected MCF-7 cells were treated for 8, 16, 24, 36, and 48 hr with increasing concentrations of E$_2$. PCB 104, or PCB 155. Maximum luciferase activity was induced by 5 nM E$_2$, the highest concentration tested, following incubation of the cells for approximately 16 hr (Fig. 5A). Luciferase activity decreased only twofold after 24 hr and remained induced over a period of 36–48 hr. Similarly, 10 µM PCB 104 induced maximum reporter gene activity after approximately 16 hr of exposure (Fig. 5B). However, the activity induced by 1 and 10 µM PCB 104 decreased sixfold after 24 hr and remained low over the 36 and 48 hr incubation periods (Fig. 5B). This dramatic decline in luciferase activity cannot be attributed to toxicity because the activity of the constitutively expressed β-galactosidase expression vector (i.e., pCH110) did not decrease, but remained constant over the same time period (data not shown).

PCB 155 did not significantly induce luciferase at any concentration tested at any time point (data not shown). Although maximum induction was observed following a 16-hr incubation period, 24-hr incubation periods were used in order for comparison to be consistent with previous studies.

In order to investigate the effects of E$_2$ plus PCBs on gene expression, MCF-7 cells transiently transfected with Gal4-HEGO and 17m5-G-Luc were treated with 1 nM E$_2$ and increasing concentrations of PCB 104, HO-PCB 104, and PCB 155 (0.1, 1, and 10 µM). As shown in Figure 4, co-treatment with E$_2$ plus PCB 104 had little effect when compared to E$_2$ alone. However, HO-PCB 104 exhibited additive effects at 10 µM in the presence of E$_2$, whereas lower concentrations of HO-PCB 104 showed little effect when compared to E$_2$ alone. In contrast, PCB 155 significantly decreased E$_2$-induced luciferase activity in a dose-dependent manner.

Metabolic hydroxylation of PCBs is preferentially targeted to the para position (74,75). Therefore, media from transiently transfected MCF-7 cells treated with PCB 104 were collected following incubation with PCB 104 at 8, 24, and 48 hr and were specifically analyzed for the presence of the para-hydroxylated product, HO-PCB 104. Selective ion monitoring (SIM) GC-MS analysis failed to detect the presence of HO-PCB 104 or any other hydroxylated isomers of this product in the media extracts. HO-PCB 104 was detected in two spiked samples, and the efficiency of recovery from the media was calculated to be 66 and 83%. A consistent level of PCB 104 was detected in all of the extracts. Recoveries of the internal standard (MBP126) were essentially quantitative. The average percent recovery was 118%, and the values ranged from 95% to 133% for the 10 extracts and 2 spiked extracts.

**Effect on MCF-7 cell proliferation.** The effects of PCB 104, HO-PCB 104, and PCB 155 treatments on the estrogen-dependent proliferation of MCF-7 cells were also investigated (Fig. 6A). Cell proliferation was induced twofold following treatment with 1 µM PCB 104, whereas at 10 µM, PCB 104 dramatically decreased cell numbers to below control levels. HO-PCB 104 (0.1 µM) was found to be more potent, inducing a fourfold increase in cell proliferation. Maximum induction (fivefold) of cell proliferation was observed with 1 µM HO-PCB 104. Cell numbers dramatically decreased to below control levels when incubated with 10 µM HO-PCB 104. PCB 155 induced small but significant increases in cell growth; however, the response did not appear to be dose dependent.

Co-treatment of MCF-7 cells with 1 nM E$_2$ plus 1 µM PCB 104 or 1 µM HO-PCB 104 resulted in significant increases in cell growth compared to cells treated with E$_2$ alone (Fig. 6B). However, dramatic decreases in cell numbers were once again observed after treatment of the cells with 10 µM PCB 104 or HO-PCB 104 (Fig. 6B). Co-treatment with 1 nM E$_2$ plus PCB 155 at 0.1, 0.1, and 1.0 µM increased cell numbers when compared to E$_2$ alone, but not in a dose-dependent manner. In contrast, 10 µM PCB 155 significantly inhibited E$_2$-induced cell proliferation when compared to 1 nM E$_2$ alone.

**Uterotrophic and vaginal cell cornification effects of PCB 104.** Because in vitro assays do not accurately reproduce the myriad pharmacodynamic and pharmacokinetic interactions that may occur in vivo, the effects of PCB 104 on uterine wet weight and vaginal epithelial cell cornification were examined. PCB 104 was administered by oral gavage to more accurately reproduce a more probable route of exposure.

Table 1 summarizes the results obtained from the uterotrophic and vaginal cell cornification assays. Mature, OVX mice treated daily with 1 mg/kg EE for 4 days exhibited approximately a fourfold increase in uterine wet weight. PCB 104 produced a modest dose–response increase in uterine wet weight, though only the highest dose of 202 mg/kg was significantly greater (p < 0.0002) than the vehicle control (sesame oil) alone. Treatment of the animals with 202, 16, and 1.7 mg/kg PCB 104 did not produce significant changes in body weight throughout the duration of the experiment (data not shown).

The occurrence of vaginal cell cornification was also assessed in the same mice.
Administration of EE and PCB 104 induced cornification of vaginal epithelial cells at all doses examined. Using a semiquantitative evaluation method described by Terenius (68), 1 mg/kg EE was assigned a score of 82% efficacy in inducing the cornification of vaginal cells. Treatment of mice with PCB 104 resulted in a dose-dependent increase in vaginal cell cornification, even at the lowest dose (1.7 mg/kg) of PCB 104.

Discussion

It has been suggested that selected PCBs may be rendered estrogenically active following metabolic hydroxylation at vacant para positions (30). Earlier studies examining exoestrogens have demonstrated that the degree of chlorination, as well as the substitution pattern, can significantly influence the estrogenic activities of a compound (13,76). Similar findings have also been reported for PCBs and their hydroxylated metabolites (30,31,39,46,47). However, few studies have systematically investigated the in vitro and in vivo ER-mediated activities of PCB hydrocarbons (31). The results in this report confirm the importance of para-hydroxylation and para-chlorination on the estrogenic activities of PCBs and demonstrate that nonhydroxylated PCBs are capable of eliciting ER-mediated responses.

PCB 104, HO-PCB 104, and PCB 155 all exhibited significant ER-mediated activities in the in vitro assays used in this study. All three compounds effectively competed with \([^3H]E_2\) for binding to the mouse uterine ER. Hydroxylation of PCB 104 at the vacant para position significantly increased the binding affinity of HO-PCB 104. The median effective concentration (EC$_{50}$) values obtained for these compounds are comparable to the EC$_{50}$ values reported for other hydroxylated PCBs as well as other exoestrogens (15). Although these results demonstrate that PCB 104, HO-PCB 104, and PCB 155 can interact with the mouse uterine ER, they do not provide sufficient evidence to conclude that these compounds are estrogenic. In addition, results obtained from competitive binding assays do not provide information regarding the agonist or antagonist activities of a chemical.

Recombinant receptor/reporter gene expression and MCF-7 cell proliferation assays were also used to investigate the agonist and antagonist activities of the three aforementioned compounds. PCB 104 and HO-PCB 104 exhibited significant agonist activity in the gene expression assay. GC-MS analysis confirmed that the induction of 17m5-G-Luc activity was due to PCB 104 because neither the expected HO-PCB 104 metabolite nor any isomers of this product were detected in the media. In addition, GC-MS analysis also confirmed that there were no measurable changes in PCB 104 levels in the media. Luciferase activity induced by PCB 104 and HO-PCB 104 was inhibited by the pure antiestrogen ICI 164,384 and was not observed in the absence of Gal4-HEGO (data not shown), thus, further verifying that the observed responses were mediated by the ER. Co-treatment of transiently transfected MCF-7 cells with E$_2$ plus PCB 104 or HO-PCB 104 did not induce a synergistic response, although additivity was observed following co-treatment with E$_2$ and 10 μM HO-PCB.
In contrast, PCB 155 exhibited little agonist activity, but significantly inhibited E2-induced 17β-G-Luc activity in a dose-dependent manner. Comparable agonist and antagonist activities were observed in MCF-7 cell proliferation assays; however, some significant differences were observed at the higher concentrations and in co-treatment studies. Previous studies have demonstrated that the activity of an exoestrogen is species, tissue, cell, and response specific (15, 46, 47). Consequently, differences in potency and efficacy may also result from variations in the culture conditions as well as differences in MCF-7 cell lines (15, 77, 78).

The decrease in PCB 104-induced luciferase activity following longer periods of incubation is somewhat perplexing. MCF-7 cells have been reported to possess a number of metabolic capabilities (15), suggesting that PCB 104 may have been biotransformed into nonestrogenic metabolites. PCBs are also metabolized into dihydriodiol and catechols as well as phenolic, glutathione, and methylsulfonyl conjugates (27), although none these forms has been investigated for estrogenic activity. The decrease in observed activity could also be attributed to the weak estrogenicity of PCB 104. The sustained output model suggests that, in addition to ER binding, the estrogenic potency of a compound is dependent on the binding affinity and the ability of the ligand to maintain nuclear ER residency to initiate a cascade of events that culminate in a response (79–82). Whether this phenomenon is an artifact of the experiment or a genuine observation requires further investigation.

Induction of uterine wet weight and the cornification of vaginal epithelial cells has been accepted as the benchmark to determine if a compound or complex mixture is estrogenic (83–85). Although in vitro assays can more definitively demonstrate that a compound can interact with the ER and elicit a response, these bioassays cannot replicate the myriad of pharmacokinetic and pharmacodynamic interactions that may influence the estrogenic activity of an alleged exoestrogen. For example, it has been reported that select phthalate esters elicit estrogenic responses in vitro, but have failed to induce uterine wet weight or vaginal cell cornification (72). Therefore, the effect of PCB 104 on uterine wet weight and vaginal cell cornification assays were conducted to determine its estrogenic activity in an in vivo model.

Due to the limited availability of PCB 104, mature OVX CD-1 mice were used to examine the effects on uterine wet weight and vaginal epithelial cell cornification in the same experiment. Daily treatment of OVX mice with 202 mg/kg PCB 104 for 4 consecutive days induced a modest but significant increase in uterine wet weight. The sensitivity of this assay may have been somewhat compromised by using mature animals, which has been reported to be less sensitive to the effects of estrogenic substances (84, 86, 87). Moreover, a semiquantitative procedure, which compares the disappearance of leukocytes and the appearance of cornified epithelial cells (68), showed that PCB 104 induced a dose-dependent increase in vaginal epithelial cell cornification. This assay confirmed the in vivo estrogenicity of PCB 104 since the cornification response was more specific for estrogens than the uterotrophic response (85) and demonstrated that a cornification response could be induced using 1.7 μg/kg.
PCB 104, the lowest dose examined. These results suggest that, with selected classes of exoeostrogens, vaginal epithelial cell cornification may be a more sensitive indicator to exposure to an estrogenic chemical than the uterotropic response. However, it is not possible to definitively conclude that the uterotropic and vaginal epithelial cell cornification responses were due to PCB 104 because it is conceivable that the compound was metabolized in vivo to HO-PCB 104 and/or related isomers.

Although the PCB congeners used in this study are not detected at appreciable levels in the environment, they are useful probes to investigate the importance of para-hydroxylation and para-chlorination on the estrogenic activity of noncoplanar PCBs, which represent the majority of relevant congeners found in environmental and biological matrices (17). The estrogenic activities of PCB 104 and PCB 155 support the theoretical structural requirements predicted for PCB estrogenicity. Both congeners are conformationally restricted about the twist bond due to ortho-substitution and both also possess para-substituents (29). In addition, these structural features confer differential cytochrome P450 induction patterns. For example, coplanar PCBs are primarily CYP1A inducers, noncoplanar PCBs are primarily CYP2B inducers, and mono-ortho coplanar congeners can be classified as mixed inducers (18). Metabolism of these three classes of PCBs is similar with preferential hydroxylation on the vacant para and meta positions. However, they differ in their ability to act as substrates for P4501A or 2B. P4502B from phenobarbital-treated rats has been shown to primarily hydroxylate noncoplanar, di-ortho-substituted dichlorobiphenyls, while coplanar dichlorobiphenyl congeners containing meta- and para-substitution, are primarily hydroxylated following incubation with purified P4501A obtained from β-naphthoflavone-treated rats (75). Mono-ortho-substituted dichlorobiphenyl congeners are metabolized to a similar extent by both isozymes. Therefore, substitution patterns can influence P450 isozyme induction, thus affecting the extent and type of their own metabolism. Biphenyls have been shown to be preferentially hydroxylated on the para position in vivo (88) and in vivo (89). 2- and 3-Hydroxybiphenyl metabolites were also detected to a lesser degree with species-specific differences (89).

Moreover, a single chlorine atom has been shown to direct metabolism exclusively to the unchlorinated ring, yielding a single major metabolite that is hydroxylated on the para position (90,91). Although metabolism can be restricted when both rings are chlorinated (92), the existence of adjacent unchlorinated carbon atoms, preferably at the 3,4-position (93,94) greatly facilitates hydroxylation on the para position via arene oxide intermediates (91). Taken together, these results strongly suggest that multi-ortho-substituted PCB congeners with vacant lateral positions are amenable to para-hydroxylation, thus conforming to the predicted structural requirements necessary for estrogenicity (29). The increased estrogenic activity observed in this study for PCB 104 following hydroxylation on the para position (i.e., HO-PCB 104) provides experimental support for the theoretical requirements for PCB estrogenicity as suggested by Waller et al. (29). Therefore, environmentally relevant noncoplanar ortho-substituted PCBs that have vacant para positions with unsubstituted carbon atoms (i.e., 2,5,2'-trichlorobiphenyl (PCB 18); 2,3,2',5',3'-tetrachlorobiphenyl (PCB 44); 2,4,2',5'-tetrachlorobiphenyl (PCB 49); 2,5,2',5'-tetrachlorobiphenyl (PCB 52); 2,3',4'5'-tetrachlorobiphenyl (PCB 70); 2,4,5,2'-pentachlorobiphenyl (PCB 101); and 2,3,5,6,2',5'-hexachlorobiphenyl (PCB 151) may also be susceptible to para-hydroxylation and therefore conform to the structural requirements necessary for ER binding.

The ER is a member of the nuclear receptor superfamily, which can be divided into six domains labeled A–F (2). Although the activities of estrogen and the ER are highly conserved between species, the amino acid sequence of the region responsible for ligand binding and ligand-dependent gene expression (i.e., domains D, E, and F) are not as well conserved (Table 2). Examination of the amino acid sequence of the ER ligand binding domain (i.e., domain E) indicates that significant differences in similarity exist between species (Fig. 7). For example, the hormone binding domain (i.e., domain E) of the human ER (hER) shares 93% identity with the chicken ER, 82% identity with the Xenopus laevis ER (xER), but only 60% similarity with the rainbow trout ER (rER). The percent identity between species is even less when the D, E, and F domains are considered as a functional unit (Table 2 and Fig. 7) because all three domains contribute to ligand binding and gene expression (105). This leads to serious doubts regarding the viability of using one surrogate species to accurately predict responses in other species, especially when investigating structurally diverse exoeostrogens. For example: 1) the rER has a 10-fold lower binding affinity for E2 than hER (110); 2) the rER exhibits a fivefold lower affinity for diethylstilbestrol (DES) when compared to its affinity for E2, while DES has a greater affinity for the hER than E2 (111); 3) HO-PCBs exhibit a significantly different rank order binding affinity for rat ER compared to mouse ER (40; 4) atrazine and symazine, two chloro-S-triazine-derived compounds, do not bind to rodent uterine ERs and have failed to induce reporter gene activity and cell proliferation in MCF-7 human breast cancer cells (34,63,112) while atrazine and another related chloro-S-triazine-derived compound, cyanazine, have recently been shown to competitively displace [3H]-E2 from American alligator ER (35); 5) the hER exhibits a greater binding affinity for dibutyl and butylbenzyl phthalate ester than the rER (72,113); 6) the pig ER exhibited a slightly greater binding affinity for t-cleearanol than the rat ER, but a significantly greater affinity than the chicken ER (114); and 7) human ERα and the recently cloned rat ERβ have been shown to exhibit significantly different binding preferences and binding affinities for selected exoeostrogens (115). A comparison of their ligand binding domains (i.e., domains D, E, and F) indicates that they share approximately 55% similarity in this region. In contrast, the rER

### Table 2. Comparison of the percent amino acid similarity within the estrogen receptor D, E, and F domains between species

| Animal | Source | Species | Accession No. | Percent similarity* | References |
|--------|--------|---------|---------------|----------------------|------------|
| Human | MCF-7 cells | Homo sapiens | M11457 | 100 | (95,96) |
| Pig |  | Sus scrofa | M11457 | 99 | (89) |
| Sheep |  | Ovis aries | M11457 | 89 | (90,100) |
| Rat | Sprague Dawley | Rattus norvegicus | M11457 | 89 | (89,100) |
| Mouse | | Mus musculus | M11457 | 89 | (100) |
| Bird | Chicken | Gallus gallus | M11457 | 89 | (100) |
| Zebra finch |  | Poephila guttata | M11457 | 89 | (100) |
| Frog | African clawed frog | Xenopus laevis | M11457 | 89 | (100) |
| Fish | Rainbow trout | ONCHOMYKES MYKISS | M11457 | 89 | (100) |
| Killifish |  | GRYLLACUS sp. | M11457 | 89 | (100) |
| Japanese eel | | Anguilla japonica | M11457 | 89 | (100) |
| ERβ | Leucocytes | Homo sapiens | M11457 | 89 | (100) |

* Amino acid similarities were calculated using MacVector and were based on a comparison to the D, E, and F domains of the human estrogen receptor α.

** Boundaries for the human estrogen receptor β D, E, and F domains were determined based on amino acid sequence similarity to the Japanese eel estrogen receptor.
and hER ligand binding domains (i.e., domains D, E, and F) share only 40% identity, yet it is assumed that the two receptors exhibit comparable binding affinities and ligand preferences.

Although these examples clearly illustrate that species-specific ligand binding preferences and affinities exist, these differences may also be attributed to the use of different competitive binding assay protocols and/or differences within ER containing cytosolic preparations. Nevertheless, these examples demonstrate that ligand preferences and affinities for ERs may differ significantly between species; therefore, it may not be possible to extrapolate results from a single surrogate species-based assay to other species. Consequently, further research is needed to investigate the potential species-specific estrogenic activity of other exoenstogens including parent PCBs and their hydroxylated metabolites.

To summarize, results from this study have demonstrated that selected PCBs may not require oxidation to a hydroxylated metabolite to elicit ER-mediated activities. Hydroxylation of the parent PCB was found to enhance its binding affinity and potency, although the latter affect was endpoint specific. Further studies are required in order to identify other estrogenic PCBs and also to examine the role of variable ER ligand binding domain sequences in determining species-specific sensitivities to exoenstogens.

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