2,2',6,6'-Tetrachlorobiphenyl Is Estrogenic In Vitro and In Vivo

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Abstract   Polychlorinated biphenyls (PCBs) are ubiquitous environmental contaminants whose effects on biological systems depend on the number of and the positions of the chlorine substitutions. In the present study we examined the estrogenicity of the fully *ortho*-substituted PCB, 2,2',6,6'-tetrachlorobiphenyl (2,2',6,6'-TeCB). This PCB was chosen as the prototypical *ortho*-substituted PCB to test the hypothesis that *ortho*-substitution of a PCB with no *para*- or *meta*-chlorine-substitutions results in enhanced estrogenic activity. The results indicate that 2,2',6,6'-TeCB is estrogenic both in vitro, in the MCF-7 cell focus assay, and in vivo, in the rat uterotropic assay. The estrogenic activity elicited by the addition of 5 μM 2,2',6,6'-TeCB to the medium of MCF-7 cultures was inhibited by the estrogen receptor (ER) antagonist, LY156758, suggesting that 2,2',6,6'-TeCB or a metabolite is acting through an ER-dependent mechanism. Results from competitive binding assays using recombinant human (rh) ER indicate that 2,2',6,6'-TeCB does not bind rhERα or rhERβ. A metabolite of 2,2',6,6'-TeCB, 2,2',6,6'-tetrachloro-4-biphenylol (4-OH-2,6,2',6'-TCB), does bind rhERα and rhERβ and is also 10-fold more estrogenic than 2,2',6,6'-TeCB in the MCF-7 focus assay; however, this metabolite is not detected in the medium of MCF-7 cultures exposed to 2,2',6,6'-TeCB. Taken together, the results suggest that the estrogenicity observed in human breast cancer cells and the rat uterus may be due to 1) an undetected metabolite of 2,2',6,6'-TeCB binding to the ER, 2) 2,2',6,6'-TeCB binding directly to a novel form of the ER, or 3) an unknown mechanism involving the ER. J. Cell. Biochem. 72:94–102, 1999.   © 1999 Wiley-Liss, Inc.

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Chronic exposure to industrial chemicals and other environmental contaminants that produce alterations in endocrine function is a major public health concern. Studies suggest that the toxicity associated with these exposures reflects the pleopotent nature of the endocrine system. Implications for the etiology of estrogen-dependent breast cancer, reproductive and gestational difficulties, and developmental problems associated with sexual dysfunction, neurological dysfunction, and immunotoxicity due to chronic or acute exposure to pollutants have been discussed extensively [Birnbaum, 1994; Colborn et al., 1993; Falck et al., 1992; Davidson and Yager, 1997; Silkworth and Brown, 1996; Hunter et al., 1997; Sharpe and Skakkebaek, 1993; Seegal, 1996; Hess et al., 1997].

Polychlorinated biphenyls (PCBs) are persistent in the environment even though they are no longer produced or used in the United States. There are 209 theoretically possible PCB congeners, although commercial mixtures (Aroclors) that were manufactured with varying percentages of chlorine contained only a subset of the possible congeners. These compositions have been further altered in the environment as a result of differing stability and solubility characteristics of individual PCBs in aqueous and lipid environments and by various rates of bioaccumulation, degradation and metabolism, resulting in mixtures with abundances of the individual congeners that are very different from those of the original Aroclor formulations [Bright et al., 1995; Rhee and Sokol, 1994; Sokol et al., 1994].

Estrogenic activity associated with the lower-chlorinated Aroclor formulations and *ortho*- sub-

stituted PCB congeners was reported over 25 years ago [Bitman and Cecil, 1970]. The contribution of *para*-hydroxylation of PCBs to estrogenicity was demonstrated by Korach et al. [1987] using in vitro ER binding studies and induction of uterine weight increases. This study suggests that *ortho*-chlorine substitution and *para*-hydroxylation may be structural requirements for estrogenic activity. Since *ortho*-substitution inhibits attainment of a planar configuration, these congeners do not exhibit high-affinity binding to the aryl hydrocarbon (Ah) receptor. In contrast, some *para*- and *meta*-chlorinated congeners exhibit Ah receptor agonist activity, which can result in an inhibition of estrogenic activity, possibly by the depletion of cellular estrogen through enhanced cytochrome P450-catalyzed metabolism [Spink et al., 1990; Gierthy et al., 1996] and/or interaction of the ligand-bound Ah receptor at inhibitory xenobiotic response elements of specific genes [Krishnan et al., 1995].

In the present study, effects of the prototypical *ortho*-substituted PCB congener, 2,2′,6,6′-tetrachlorobiphenyl (2,2′,6,6′-TeCB), were investigated to determine the role of tetra-*ortho*-substitution on estrogenic activity. The chlorine-substitution pattern of this congener results in the highest degree of nonplanarity [Erickson, 1997], and the data regarding estrogenic activity provide a basis for comparing other chlorinated congeners as well as *meta-* and/or *para-* hydroxylated metabolites of this congener. Although 2,2′,6,6′-TeCB is a very minor component of environmental samples, it, and compounds from which it can be derived by reductive dechlorination, were present in Aroclors released into the environment. Results from the present study indicate that 2,2′,6,6′-TeCB exhibits significant estrogenic activity both in vitro, in the MCF-7 focus assay, and in vivo, in the rat uterotropic assay. Tetra-*ortho*-substitution is important for the observed estrogenicity, as evidenced by the reduced estrogenicity in the MCF-7 focus assay of the tri-*ortho*-substituted congener, 2,2′,6-trichlorobiphenyl (2,2′,6-TrCB). 2,2′,6,6′-TeCB did not bind recombinant human estrogen receptor-α (rhERα) or rhERβ. Although the metabolite 2,2′,6,6′-tetrachloro-4-biphenylol (4-OH-2,2′,6,6′-TeCB) did bind rhERα and rhERβ, it was not detected in cells treated with 2,2′,6,6′-TeCB.

MATERIALS AND METHODS
Chemicals

2,2′,6,6′-TeCB, (purity ≥99%) was purchased from Ultra Scientific (North Kingston, RI), and synthesized (purity ≥99%) at the Wadsworth Laboratories [Yi, 1998]. 2,2′,6-TrCB, 2,2′,3- trichlorobiphenyl (2,2′,3-TrCB) and 2,3′,6-trichlorobiphenyl (2,3′,6-TrCB; purity ≥99%) were purchased from Ultra Scientific. 4-OH-2,2′,6,6′-TeCB was kindly provided by Dr. Stephen Safe (Texas A&M University, College Station, TX). 17β-estradiol, phenobarbital, metyrapone (2-Methyl-1,2-di-3-pyridyl-1-propanone) and β-glucuronidase/sulfatase type H-2 were purchased from Sigma Chemical Company (St. Louis, MO). LY156758 was kindly provided by Lilly Research Laboratories (Indianapolis, IN). [2,4,6,7,16,17-³H]-17β-estradiol (specific activity = 140 to 150 Ci/mmol), [2,4,6,7,-³H]-17β-estradiol (specific activity = 72 Ci/mmol) and [2,-³H]-17β-estradiol (specific activity = 15 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from Cambridge Isotope Laboratories (Woburn, MA). Pentafluorbenzylbromide was purchased from Lancaster Synthesis Inc. (Windham, NH).

MCF-7 Focus Assay

In the MCF-7 focus assay, estrogen-dependent, postconfluent changes in cell growth resulting in multicellular structures, referred to as nodules or foci, on a confluent monolayer background are quantified. The assay was conducted as previously described [Gierthy et al., 1991]. Briefly, MCF-7 cells were treated with trypsin (0.25%) and suspended in medium (DC$_5$) consisting of Dulbecco's Modified Eagle Medium (without phenol red) supplemented with 5% bovine calf serum and other components as previously described [Gierthy et al., 1991]. Cells (1×10^5) in 1 ml of DC$_5$ were seeded into each well of 24-well (2 cm² per well) plastic tissue culture plates and placed in a 37°C humidified incubator with 5% CO$_2$. Cultures were refed at 24 h and every 3–4 days thereafter with 2 ml of DC$_5$ containing various concentrations of the experimental compounds in DMSO (the final concentration of DMSO was ≤0.1%). Cells in the 24-well plates were visually inspected for evidence of cytotoxic or cytostatic effects of the test compound. Cytotoxic effects were indicated by changes in cell morphology, e.g., pycnosis,

lysis, or detachment; cytostatic effects were indicated by a delay in or inhibition of attainment of confluence as compared to the control cultures. After 14 days, the cultures were fixed with formalin and stained with 1% Rhodamine B. The stained foci were then counted using a New Brunswick automated colony counter (Edison, NJ). All experiments were done as four replicates and repeated three times. The Sigma-Plot® software (Jandel Scientific Software, San Rafael, CA) was used to analyze the data and perform a linear regression from which the EC_{50} values were calculated from each curve.

Uterotropic Assay

Precisely-timed, pregnant Sprague-Dawley rats were obtained from Zivic-Miller Labs (Zelienople, PA) and housed in the animal facility at the Wadsworth Laboratories. Litters were culled to five female and five male pups at birth. On postnatal day (PND) 21 and 22, female offspring were randomly assigned to one of six groups and received an intraperitoneal injection of either 20 µg/kg E_2, 3, 10, 20, or 30 mg/kg 2,2',6,6'-TeCB, or corn oil as the control. E_2 and 2,2',6,6'-TeCB were made up in corn oil and injected at 200 µl per 100 g body weight. On PND 23 animals were weighed and sacrificed; the uterus was quickly removed and weighed after dissecting away and trimming the outer sheath. Data are expressed as a ratio of uterine wet weight in mg to body weight in g × 100. The experiment was conducted in three replications of 10 litters each and the experimenter was blind with respect to the animal's treatment.

Radiometric Analysis of E_2 Metabolism

Radiometric analysis of E_2 metabolism by enzymes induced by 2,2',6,6'-TeCB was conducted with MCF-7 cells seeded in 24-well plates at 1×10^6 cells/ml. After 24 h, cells were refed with either DC_5, 0.001 µM TCDD, or 5 µM 2,2',6,6'-TeCB. After 3 days of incubation with the various test compounds, the 2.0 ml of medium from each well was removed and mixed with 0.2 ml of 10 nM [³H]E_2 and then returned to the appropriate well. Media were collected after 24 h, and the [³H]E_2 was separated from the media by adsorption on charcoal. The tritium recovered in the media (present as [³H]$_2$O) was then counted in a Beckman LS2800 liquid scintillation counter (Irvine, CA). The amount of dissociated tritium is an indication of the degree of E_2 metabolism at any or all of the [³H]-substituted positions. The radiometric analysis was conducted with both [2,4,6,7,16,17-³H]E_2 and [2-³H]E_2 and was also run with the human hepatoma cells, HepG2.

Competitive ER Binding Assay

Competitive binding of ERα and ERβ by 2,2',6,6'-TeCB and 4-OH-2,2',6,6'-TeCB was examined using rhER as previously described [Arcaro et al., 1998]. Briefly, rhERα or rhERβ (1.2 nM) was incubated for four hours at room temperature with differing concentrations of E_2, 2,2',6,6'-TeCB, 4-OH-2,2',6,6'-TeCB or LY156758 in the presence of [2,4,6,7-³H]E_2 (2.5 nM) in a total reaction volume of 100 µl. After incubation, 100 µl of 50% hydroxyapatite (HAP) slurry was added to the reaction mixture. After 15 min of incubation, 3 ml of wash buffer was added, and the HAP-bound receptor-[2,4,6,7-³H]E_2 complex was separated by centrifugation at $200g$ for 20 min. Radioactivity of the pellet was counted in a Beckman liquid scintillation counter. The amount of receptor-bound [³H]E_2 in the presence or absence of the test compounds was calculated after correcting for nonspecific binding in the presence of a 200-fold excess of E_2. The nonspecific binding ranged from 5 to 7% of the total bound [³H]E_2 in the absence of a competitor. The specific binding in the absence of a competitor ranged from 25 to 30% of the total [³H]E_2 added to the cells. The samples were tested in triplicate, and each compound was evaluated in at least three separate experiments. SigmaPlot® software was used to plot the data and derive a linear regression line from which the IC_{50} values were calculated for each curve.

Gas Chromatography/Mass Spectrometry (GC/MS) of PCB Metabolites

To evaluate possible metabolism of 2,2',6,6'-TeCB in MCF-7 cells, confluent cultures were exposed to the solvent vehicle or 5 µM 2,2',6,6'-TeCB for 4 days. To 3.5 ml portions of media from these cultures 50 ng of 4'-OH-2,3,4,5-TeCB was added as internal standard. These fortified media were subjected to solid-phase extraction on Extrelut QE columns (EM Science, Cherry Hill, NJ), which were eluted with methylene chloride. These extracts were evaporated to dryness, and 100 µl of 0.1 M triethylamine in benzene and 10 µl of pentafluorobenzylbromide were added and the mixture was heated as described [Halket, 1993] at 100°C for
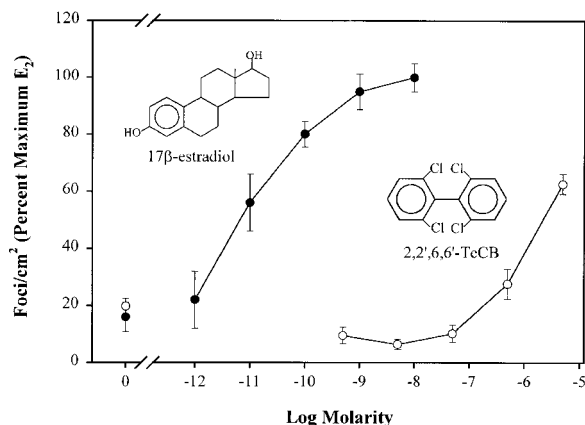
Fig. 1. Induction of foci in MCF-7 cells by E_2 (●) and 2,6,2′,6′-TeCB (○). Results from a representative experiment are expressed as percent of response observed with 10 nM E_2; n = 4 for each concentration, means, and standard deviations are shown.

15 min. The derivitization mixtures were diluted in EtOAc for analysis by gas chromatography-negative ion chemical ionization mass spectrometry (GC-NCI/MS). In 4-OH-2,2′,6,6′-TeCB recovery experiments, 10 ng of 4-OH-2,2′,6,6′-TeCB was added and samples were processed in the usual manner. The derivatized extracts were analyzed by GC/MS in the negative-ion chemical ionization mode with a Micromass Quattro instrument (Manchester, UK). The instrument was operated in the scanning (m/z 50:550) and selected-ion-monitoring (100 msec dwell, six ions per cycle) modes.

RESULTS

Estrogenicity and Anti-Estrogenicity in the MCF-7 Focus Assay

Initial experiments were conducted to determine whether 2,2′,6,6′-TeCB was estrogenic in human breast cancer cells. Results from the MCF-7 focus assay demonstrated that 2,2′,6,6′-TeCB induced the formation of foci in post-confluent cultures in a dose-dependent manner (Fig. 1). At 50 μM, 2,2′,6,6′-TeCB slowed preconfluent cell growth (data not shown), indicating toxicity at this concentration; therefore, in all subsequent experiments 5 μM was the highest concentration tested. The EC_{50} values for 2,2′,6,6′-TeCB and E_2 were 2.0 μM and 0.01 nM respectively, based on the maximal response of E_2. 2,2′,6,6′-TeCB was therefore about 200,000 times less potent than E_2, and 2,2′,6,6′-TeCB produced 60% of the maximal E_2 response. The estrogenic activity induced by 2,2′,6,6′-TeCB or

its metabolite(s) appears to occur through an ER-mediated mechanism, since the foci induced by 5 μM 2,2′,6,6′-TeCB were inhibited by LY156758 (IC_{50} = 3.6 nM), a specific ER antagonist (Fig. 2).

To investigate whether 2,2′,6,6′-TeCB acts as an anti-estrogen, we examined the response of MCF-7 cells to increasing concentrations of 2,2′,6,6′-TeCB in the presence of 1.0 nM E_2, a concentration which approaches the upper limit of the linear dose-response. As can be seen in Figure 3, 2,2′,6,6′-TeCB at concentrations up to 5 μM did not inhibit the focus formation elicited by 1.0 nM E_2. In contrast, known antiestrogens, LY156758 and TCDD, completely inhibited the focus formation elicited by 1.0 nM E_2 (Fig. 3) with IC_{50} values of 1.73 nM and 0.76 nM, respectively.

Position and Number of Cl Substitution and Para Hydroxylation

To examine the necessity of full *ortho*-chlorine-substitution in the estrogenic response induced by 2,2′,6,6′-TeCB, we tested three trichlorobiphenyls (the tri *ortho*- and two di *ortho*-substituted congeners) in the MCF-7 focus assay. Results showed that both the number of and ring position of the *ortho*-chlorine substitutions are important in inducing the estrogenic response. Both the di-*ortho*-(2,2′,3-TrCB) and tri-*ortho*- (2,2′,6-TrCB)-substituted congeners with at least one chlorine on each ring were estrogenic in the MCF-7 focus assay (Fig. 4). In contrast, the di-*ortho* congener with both *ortho*
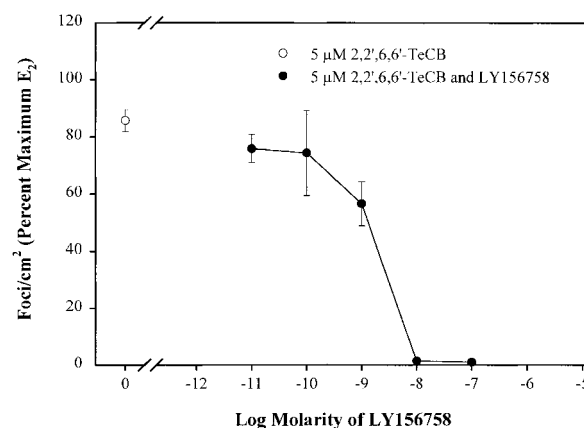

Fig. 2. Inhibition by LY156758 of foci induced in MCF-7 cells by 5 μM 2,6,2′,6′-TeCB. Results from a representative experiment are expressed as percent of response observed with 10 nM E_2; n = 4 for each concentration, means, and standard deviations are shown.
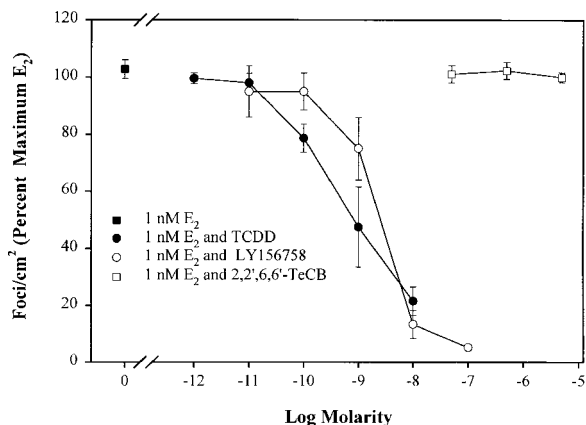
Fig. 3. Foci induced in MCF-7 cells by 1 nM E_2 are inhibited by 10 nM TCDD and 10 nM LY156758 but not by 2,2′,6,6′-TeCB. Results from a representative experiment are expressed as response observed with 10 nM E_2; n = 4 for each concentration, means, and standard deviations are shown.
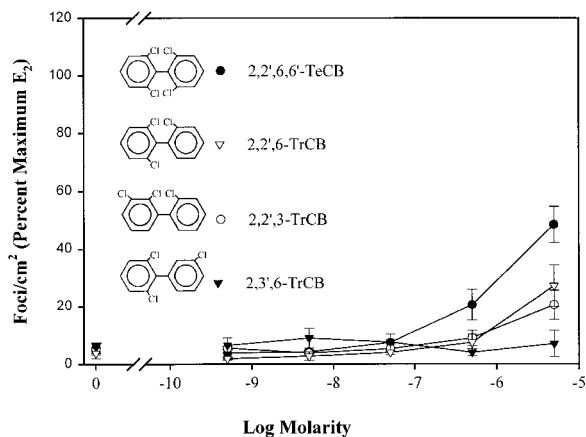

Fig. 4. Comparison of foci induced in MCF-7 cells by 2,2′,6,6′-TeCB, 2,2′,6-TCB, 2,2′,3-TCB, and 2,3′,6-TCB. Results from a representative experiment are expressed as percent of foci observed with 10 nM E_2; n = 4 for each concentration, means, and standard deviations are shown.

chlorines on the same ring (2,3′,6-TrCB) was not estrogenic (Fig. 4). None of the trichlorobiphenyls produced an estrogenic response equal to or greater than the maximal response observed with 2,2′,6,6′-TeCB at any of the concentrations tested.

If 2,2′,6,6′-TeCB produces its estrogenic response through conversion to a hydroxy-PCB metabolite, it is probable that the metabolite would be significantly more potent than 2,2′,6,6′-TeCB. We tested one available potential metabolite of 2,2′,6,6′-TeCB, 4-OH-2,2′,6,6′-TeCB, in the MCF-7 focus assay. As can be seen in Figure 5, the hydroxy-PCB metabolite was 10 times more potent than the 2,2′,6,6′-TeCB (EC_{50}s =
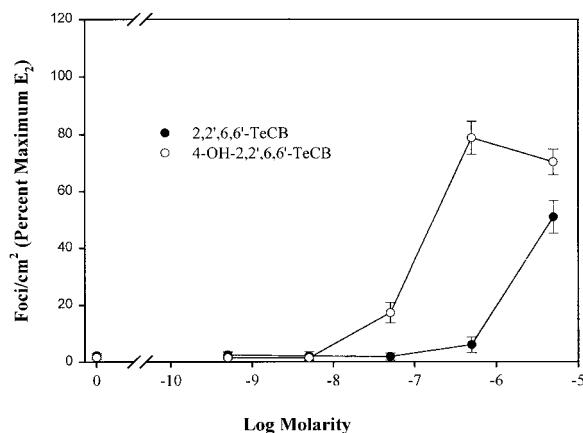

Fig. 5. Comparison of foci induced in MCF-7 cells by 2,2′,6,6′-TeCB and 4-OH-2,2′,6,6′-TeCB. Results from a representative experiment are expressed as response observed with 10 nM E_2; n = 4 for each concentration, means, and standard deviations are shown.

0.3 μM and 2.9 μM, respectively based on the maximal E_2 response).

Uterotropic Assay

To determine whether 2,2′,6,6′-TeCB could induce an estrogenic response in vivo, we measured the uterotropic response in immature female rats given intraperitoneal injections of various concentrations of 2,2′,6,6′-TeCB, E_2, or corn-oil as a control on PND 21 and 22. 2,2′,6,6′-TeCB produced a significant increase in uterine wet weight as compared with corn-oil controls (F = 38.32, d.f. = 4, 107, $P \leq 0.001$; Fig. 6). Three of the four doses (10, 20, and 30 mg/kg/day) significantly increased uterine wet weight (t-tests with Bonferoni-corrections for each comparison, $P \leq 0.001$). The lowest effective total dose was greater than 6 mg/kg (3 mg/kg/day) and less than 20 mg/kg. 2,2′,6,6′-TeCB produced essentially the same increase in uterine wet weight between 40 and 60 mg/kg total dose, and there was no indication of antagonism at the higher concentration. The increase in uterine wet weight produced by 2,2′,6,6′-TeCB was less than half that produced by 20 μg/kg/day of E_2. Treatment with either 2,2′,6,6′-TeCB or E_2 did not significantly alter body weight.

Radiometric Assay for E_2 Metabolism

It is possible that the estrogenicity observed in the MCF-7 focus assay in the presence of 5 μM 2,2′,6,6′-TeCB is due to inhibition of metabolism of the low levels of E_2 in DC_5 (>0.25 pg/ml).
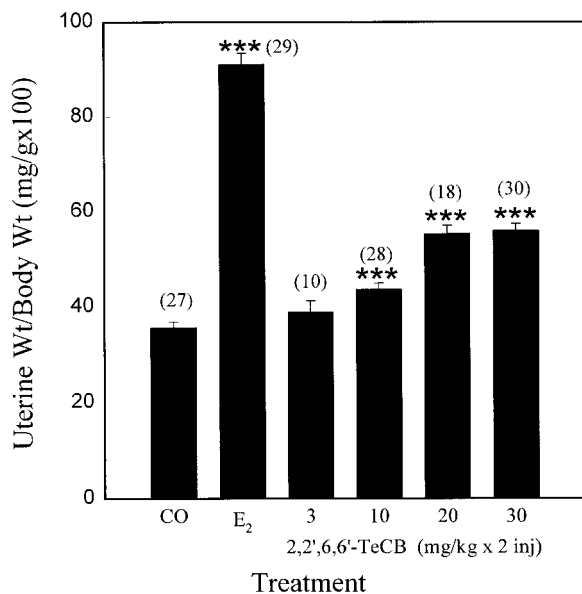
Fig. 6. Uterine wet weight in immature rats exposed to corn oil control (CO), 20 μg/kg E_2, or increasing concentrations (3, 10, 20, or 30 mg/kg) of 2,2',6,6'-TeCB. Data were analyzed with Oneway ANOVA (E_2: F = 442.7; df = 1, 54; p ≤ 0.001 and 2,2',6,6'-TeCB: F = 38.3; df = 4, 107; P ≤ 0.001) and post hoc comparisons were made with t-tests, number in parentheses = n for each group. ***P ≤0.001 after a Bonferroni-correction for t-tests comparing each treatment group with the control.

To determine whether 2,2',6,6'-TeCB inhibits enzymes which metabolize E_2 and thereby cause the low levels of E_2 to persist in the cell for a longer period of time and induce the formation of foci, we examined the ability of 2,2',6,6'-TeCB to alter the metabolism of E_2 in MCF-7 cells. The presence of 5 μM 2,2',6,6'-TeCB did not decrease or increase the metabolism of E_2 above that of the control level (data not shown). In comparison, 1 nM TCDD significantly increased the rate of metabolism of E_2 (eight-fold over background). We repeated the metabolism assay with HepG2 human liver-derived cells to determine whether 2,2',6,6'-TeCB altered the metabolism of E_2 in cells in which CYP1A1 is induced to a higher level. TCDD significantly increased the metabolism of E_2 (14-fold over background) whereas 2,2',6,6'-TeCB neither increased nor decreased it. Further evidence supporting the contention that 2,2',6,6'-TeCB does not produce its estrogenic effect in MCF-7 cells by inhibiting E_2 metabolism comes from a study using 5% charcoal-stripped calf serum. When stripped serum was used in the MCF-7 focus assay, 5 μM 2,2',6,6'-TeCB still induced the formation of foci (data not shown).

Competitive Binding Assays

To determine whether 2,2',6,6'-TeCB binds the human ER we ran competitive binding assays using rhERα and rhERβ. 2,2',6,6'-TeCB did not bind rhERα or rhERβ in this assay, whereas the metabolite 4-OH-2,2',6,6'-TeCB, did bind rhERα and rhERβ (Fig. 7). The metabolite is about 1,000 times less potent than E_2 (IC_{50} = 0.5 nM).

Assay of 2,2',6,6'-TeCB Hydroxylation

Since *para*-hydroxylation of *ortho*-substituted PCBs is thought to give rise to estrogenic metabolites, we investigated whether 4-OH-2,2',6,6'-TeCB was formed in MCF-7 cultures. Using a highly sensitive GC-NCI/MS technique, we were unable to detect 4-OH-2,2',6,6'-TeCB in the medium of MCF-7 cultures exposed to 2,2',6,6'-TeCB (method detection limit = 3 nM) even though the 4-OH-2,2',6,6'-TeCB added to the medium (spent DC_5) could be efficiently recovered by extraction (130 ± 7%). In addition, treating the media with β-glucuronidase/sulfatase (6,000 units β-glucuronidase, 200 units sulfatase) for 18 h at 37°C failed to result in detectable 4-OH- 2,2',6,6'-TeCB as a result of hydrolysis of putative conjugate of the metabolite.

DISCUSSION

Korach and colleagues [1988] examined the ER binding affinities of 12 hydroxylated PCBs
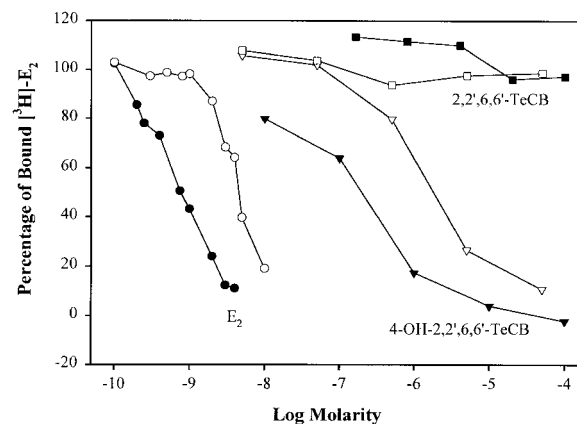

Fig. 7. Displacement of [3H] E_2 by unlabeled E_2 (○ ●), 2,2',6,6'-TeCB (□, ■), and 4-OH- 2,2',6,6'-TeCB (△, ▲) in ER competitive binding assays using rhERα (filled symbols) and rhERβ (hollow symbols). Data are from two representative experiments. Variation within an assay was ≤10% and variation between assays was ≤15%.

and hypothesized that the conformational restriction conferred by *ortho*-Cl-substitution was correlated with the observed estrogenicity. However, they restricted their analysis to hydroxylated PCBs with two, one or no *ortho*-Cl-substitutions. In the present report we examine the fully *ortho*-substituted, non-coplanar PCB congener, 2,2',6,6'-TeCB, in MCF-7 focus, uterotropic, and ER-binding assays. Our results demonstrate that exposure of MCF-7 cells and immature female rats to 2,2',6,6'-TeCB elicits estrogenic activity. In addition, we have investigated the possible mechanisms for this activity.

The low potency of 2,2',6,6'-TeCB demonstrated in the MCF-7 focus assay compared to E_2 suggests that, although the effect is reproducible, this congener is a weak estrogen. Saturation of the *ortho* positions by Cl enhances the estrogenic action, as shown by the examination of other less chlorinated congeners in the MCF-7 focus assay. Of the three trichlorobiphenyls examined, only 2,6,2'-TrCB and 2,2',3-TrCB showed significant estrogenicity; however, the maximum response of 2,6,2'-TrCB was still significantly less than that of the tetra-*ortho*-chlorinated congener, 2,2',6,6'-TeCB. 2,3',6-TrCB, a congener which has both *ortho* substitutions on the same ring, shows no activity, suggesting a requirement for *ortho* substitution on each of the biphenyl rings and the resulting noncoplanar configuration.

There are a number of possible mechanisms for the estrogenic response elicited by 2,2',6,6'-TeCB. The congener may inhibit metabolism of the low levels (< 0.5 pg/ml) of E_2 in the DC_5 culture media [Gierthy et al., 1991] and the immature female rat, thereby resulting in the persistence of these low levels of E_2 and subsequent E_2-mediated activity. The classic example of this is the apparent estrogenic response caused by carbon tetrachloride in the uterotropic model, which is through an indirect, non-ER-mediated mechanism. In this example, enhanced estrogenic effects are thought to result from hepatotoxicity and increased E_2 persistence due to suppressed metabolism [Welch et al., 1969]. Alternatively, 2,2',6,6'-TeCB could be a weak ER agonist, acting directly through binding to ER. A third possibility is that 2,2',6,6'-TeCB is being metabolized to a hydroxylated PCB by constitutive or induced cytochromes P450, resulting in an ER agonist and a direct mimic of E_2. Examination of these possible mechanisms was undertaken using the

MCF-7 focus assay, ER binding assays, and GC/MS analysis.

Studies on the effects of 2,2',6,6'-TeCB on the metabolism of E_2 demonstrated that 2,2',6,6'-TeCB neither suppresses nor increases constitutive E_2 metabolism. Further evidence supporting the conclusion that 2,2',6,6'-TeCB is not acting by suppression of endogenous E_2 metabolism comes from the finding that 2,2',6,6'-TCB still elicits the formation of foci when the focus assay is conducted in the absence of E_2 (with stripped serum).

The suppression of 2,2',6,6'-TeCB-induced-foci by the antiestrogen LY156758, which acts by competing with E_2 for the ER, indicates involvement of the ER in the induction of foci. However, ER competitive-binding studies using rhERα and rhERβ showed no evidence of direct binding of 2,2',6,6'-TeCB to the ER. These results suggest that 2,2',6,6'-TeCB is converted to a form which binds the ER and induces the estrogenic response. The demonstration of a 10-fold increase by 4-OH-2,2',6,6'-TeCB over 2,2',6,6'-TeCB in the estrogenic response in the MCF-7 focus assay, and ER binding of 4-OH-2,2',6,6'-TeCB suggests that the observed estrogenic activity of 2,2',6,6'-TeCB may be due to 4-OH-2,2',6,6'-TeCB working through the classical ER-mediated mechanism. However, GC/MS analysis of culture media from cells exposed to 2,2',6,6'-TeCB revealed no 4-OH-2,2',6,6'-TeCB. At this point the mechanism resulting in the observed estrogenicity remains unknown, and further studies need to be conducted.

A number of studies have reported estrogenic activity induced by both PCBs and hydroxylated PCBs [Bergeron et al., 1994; Bitman and Cecil, 1970; Fielden et al., 1997; Gierthy et al., 1997; Jansen et al., 1993; Korach et al., 1987; Li and Hansen, 1995; Nesaretnam et al., 1996; Nesaretnam and Darbre, 1997; Ramamoorthy et al., 1997; Soto et al., 1995]. Although results from these studies suggest that hydroxylation is important for estrogenic activity, weak estrogenicity of a PCB was observed in a number of studies [Fielden et al., 1997; Gierthy et al., 1997; Nesaretnam et al., 1996; Nesaretnam and Darbre, 1997; Soto et al., 1995]. It is possible that in some cases the PCB was metabolized in the test system to a hydroxylated PCB. Metabolism may play a role even in ER binding studies that were conducted with cell cytosol

and incubated at 30°C as was done in a number of studies [Fielden et al., 1997; Nesaretnam et al., 1997].

As with hydroxylation, some of the studies mentioned above support the hypothesis that *ortho*-Cl-substitution is important for estrogenic activity [Korach et al., 1988; Gierthy et al., 1997], but it is clearly not required for the effect [Fielden et al., 1997; Nesaretnam et al., 1996; Nesaretnam and Darbre, 1997]. Nesaretnam and Darbre [1997] detected no estrogenicity with the di-*ortho*-substituted congener, 2,2′,5,5′-TeCB but found 3,3′,4,4′-TeCB and 3,3′5,5′-TeCB to exhibit estrogenic activity in a number of estrogen-sensitive assays.

Antiestrogenic activity of hydroxylated PCBs is associated generally with higher chlorinated congeners (tetra, penta, and hepta substituted) and includes chlorine substitutions in *meta* and *para* as well as *ortho* positions. Moore and colleagues [1997] examined seven hydroxylated PCBs detected in human serum and determined that none of the compounds were estrogenic nor did they significantly bind the ER. However, all seven hydroxylated PCBs were antiestrogenic in at least one estrogen-sensitive assay. Kramer et al. [1997] reported that 11 of the 13 hydroxylated PCBs they tested were antiestrogenic in an MCF-7 cell line stably transfected with a luciferase reporter gene linked to estrogen-responsive elements. Although only two of the compounds bound the ER, the antiestrogenic effects could be blocked with physiologic levels of E_2 for all but one of the hydroxylated PCBs.

The significance of the weak estrogenic activity observed for the fully *ortho*-substituted TeCB to public health lies in the concern of some that ingestion of PCB-contaminated fish or inhalation of volatile PCBs may produce a health hazard [Chiarenzelli et al., 1998]. While 2,2′,6,6′-TeCB is a very minor component of environmental samples, mono-, di-, and tri-*ortho*-substituted PCBs are more common [McFarland and Clarke, 1989]. Although the original Aroclor formulations contained small percentages of compounds substituted with chlorine in only *ortho* positions, microbial anaerobic dechlorination in the sedimentary layer results in *meta* and *para* dechlorination and enrichment of *ortho*-chlorinated compounds [Abramowicz, 1995; Rhee and Sokol, 1994; Sokol et al., 1994] which, due at least partially to their higher water solubility, bioaccumulate in fish [Bright et al.,

1995; Clarkson, 1995]. Certain species of fish lack the metabolic enzymes necessary for clearance of some of the *ortho*-substituted PCBs such as 2,5,2′,5′-TeCB [Bright et al., 1995]; this lack of metabolism and increased accumulation raises the likelihood of human exposure through ingestion. In addition, the higher water solubility relative to the non-*ortho*-substituted PCBs can result in increased distribution of these compounds through the water column. A recent study demonstrated that these lower-chlorinated PCBs are more volatile than the more highly chlorinated congeners and evaporate into the atmosphere [Chiarenzelli et al., 1997]. This provides a second source of exposure to *ortho*-chlorinated PCBs, and there is concern that hydroxylation of this particular class of PCBs may result in the production of estrogenic metabolites.

In summary, the fully *ortho*-substituted PCB, 2,2′,6,6′-TeCB, is estrogenic in both an in vitro human estrogen-responsive breast-cancer cell assay and the in vivo rat uterotropic assay. However, the potency of this activity in the MCF-7 focus assay is two hundred thousand fold less than that of the natural estrogen, E_2. Any proposed health effects due to volitization and inhalation of *ortho*-chlorinated PCBs must take into account the mechanism of toxicity as well as the potency and levels of exposure in order for human health risks to be placed in an appropriate public health perspective.

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