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In Vitro Antiestrogenic Effects of Aryl Methyl Sulfone Metabolites of Polychlorinated Biphenyls and 2,2-Bis(4-chlorophenyl)-1,1-dichloroethene on 17β-Estradiol-Induced Gene Expression in Several Bioassay Systems

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Methylsulfonyl ($MeSO_2$) metabolites of polychlorinated biphenyls (PCBs) and 2,2-bis(4-chlorophenyl)-1,1-dichloroethene (4,4′-DDE), itself a metabolite of the insecticide 4,4′-DDT, are emerging as a major class of contaminants in the tissues of wildlife and humans. We investigated the antiestrogenic capacity and potencies of 3′- and 4′-$MeSO_2$-2,2′,4,5,5′-pentachlorobiphenyl (CB101) and -2,2′,4,5′-tetrachlorobiphenyl (CB49), which are among the most environmentally persistent $MeSO_2$-PCBs, and 3-$MeSO_2$-4,4′-DDE on estrogen receptor (ER)-dependent gene expression in four cell-based bioassay systems. Congener- and concentration-dependent antagonism of 17β-estradiol (E2)-induced gene expression, rather than induction of ER-dependent gene expression, was observed for the $MeSO_2$-PCBs on lucifierase activity in stably transfected human breast adenocarcinoma T47D cells (ER-CALUX) and vitellogenin (vtg) production in primary hepatocytes from male carp fish (*Cyprinus carpio*) (CARP-HEP/vtg). 4′-$MeSO_2$-CB101 and -CB49 had the highest antagonistic potency (i.e., maximum inhibition of about 70%, LOECs of 1.0 μM and 2.5 μM), whereas 3′-$MeSO_2$-CB101 and -CB49 were less antagonistic; the precursor CB101 and $MeSO_2$-PCB analog $MeSO_2$-2,5-dichlorobenzene had no effect. Relative to the 4-$MeSO_2$-PCBs, tamoxifen (IC_{50}, 0.06 μM and 0.7 μM) was about 40 and 7 times more potent in the ER-CALUX and CARP-HEP/vtg assays, respectively. Congener- and concentration-dependent effects on aryl hydrocarbon receptor-mediated induction of EROD activity (carp hepatocytes), luciferase expression (H4IIE rat hepatoma [H4IIE.luc] cell line), or cell viability were not observed. 3-$MeSO_2$-4,4′-DDE was neither estrogenic nor antiestrogenic in either of the bioassays. Inhibitory trends for the $MeSO_2$-PCBs in a bioassay based on stably transfected human embryonic kidney cell (HEK293-ERα-ERE) were similar to the ER-CALUX and CARP-HEP/vtg bioassays, whereas the antagonism was weaker in a related HEK293-ERβ-ERE bioassay. Our findings suggest that the 4′-$MeSO_2$-PCBs are antiestrogenic *in vitro* via a reversible or surmountable interaction with fish or human ER, and that the interaction with human ERα is apparently favored over ERβ. $MeSO_2$-PCB metabolites are persistent and bioaccumulative contaminants, and therefore, could be potentially active as environmental antiestrogens in wildlife and humans.

Key Words: **polychlorinated biphenyls; methylsulfone metabolites; estrogen-responsive cells; *in vitro* bioassays; antiestrogenicity; gene expression.**

The endocrine-related activity of polychlorinated biphenyls (PCBs) has been implicated in adverse affects on reproduction, sexual differentiation, and development; and, thus, the general population fitness in exposed wildlife species and humans (Brouwer *et al*., 1999; Colborn *et al*., 1993; Li and Hansen, 1997; Safe, 1994). PCBs are among a growing number of environmental contaminants, industrial chemicals, and pharmaceuticals classified as estrogenic endocrine disruptors, or xenoestrogens. Exposure to PCBs has been associated with testicular cancer and decreased sperm counts in human males (Sharpe and Shakkebaek, 1993; Shakkebaek *et al.*, 1998), and may play a role in the development of breast cancer in women (Aronson *et al*., 2000; Horwitz and McGuire, 1978; Safe, 1994). PCBs have exhibited antiestrogenic as well as estrogenic activity *in vitro* and *in vivo*, depending on the congener studied (Brouwer *et al*., 1999; Safe, 1994).

A growing number of xenobiotics that possess antiestrogenic activities have been identified, i.e., compounds that antagonize estrogen-dependent processes in target tissues. Antiestrogenic substances may interfere at several sites with estrogen-regulated cellular processes, both by estrogen receptor (ER)-dependent (α or β) and -independent mechanisms (Navas et al., 1998). Major examples of synthetic antiestrogens that are active via ER-independent and AhR-dependent mechanisms

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are polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs), and dioxin-like non- and mono-ortho PCBs (Gierthy *et al.*, 1993; Jansen *et al.*, 1993; Safe, 1994). Tamoxifen was specifically designed to function via competitive interaction with the ER, and is used clinically as an antiestrogenic drug in the treatment of estrogen-dependent human breast cancer (Webb *et al.*, 1995).

Recent studies have reported that the endocrine-related activities associated with PCBs are mediated, in part, through the formation of hydroxylated (OH) and methylsulfonyl ($MeSO_2$) PCB metabolites. OH-PCB metabolites possessing an OH group mainly in the *para* position, and chlorinated on the OH-containing phenyl ring have been identified almost exclusively in the blood of wildlife and humans (Bergman *et al*., 1994; Letcher *et al*., 2000a; Sandau *et al*., 2000). The estrogenic activity of retained, and thus environmentally relevant, OH-PCB congeners via interaction with the ER appears not to be very significant. Retained OH-PCBs have been shown *in vitro* to weakly bind to ERα or ERβ (Kuiper *et al*., 1998), and weakly induce gene expression in estrogen-responsive cell systems (Kramer *et al*., 1997; Matthews and Zacharewski, 2000; Moore *et al*., 1997).

PCBs with 2,5-dichloro- or 2,5,6-trichloro-substitution, and thus *meta-para* hydrogens on at least one phenyl ring are biotransformed into persistent and bioaccumulative 3′- and 4′-$MeSO_2$-PCB metabolites (Bakke *et al*., 1999). With respect to endocrine disruption-related effects, persistent $MeSO_2$-PCBs are known to cause chronic and reproductive toxicity in exposed mink (Lund *et al*., 1999), bind to mouse and human glucocorticoid receptors (Johansson *et al*., 1998b), are cytotoxic to human placental JEG-3 and JAR choriocarcinoma cells (Letcher *et al*., 1999), are thyroidogenic in rats (Kato *et al*., 1999), competitively inhibit CYP11B1 activity in mouse Y1 adenocortical cells (Johansson *et al*., 1998a), and inhibit CYP11β activity in the adrenal of Baltic grey seal (Lund, 1994). In humans and some wildlife species 2,2-(4-chlorophenyl)-1,1-dichloroethene (4,4′-DDE), a metabolite of the insecticide 2,2-(4-chlorophenyl)-1,1,1-trichloroethane (4,4′-DDT) is biotransformed to a persistent 3-$MeSO_2$-4,4′-DDE metabolite (Letcher *et al*., 2000a,b; Weistrand and Norén, 1997). To our knowledge, no previous studies have been conducted of ER-binding affinities or antiestrogenic activity *in vitro* or *in vivo* via agonism or antagonism of estrogen-responsive gene expression by $MeSO_2$-PCBs or 3-$MeSO_2$-4,4′-DDE.

In vitro assays developed for the routine screening of potential xenoestrogens include proliferation of MCF-7 human breast cancer cells, competitive ER binding assays, and induction of reporter gene expression in transiently or stably infected cell lines (Ankley *et al*., 1998; Reel *et al*., 1996). The present study investigated the estrogenic and antiestrogenic effects of the highly persistent and bioaccumulative 3′- and 4′-$MeSO_2$-CB49 and -CB101, and 3-$MeSO_2$-4,4′-DDE on estrogen-responsive gene expression in four *in vitro* assays. The effects of the precursors CB101 and 4,4′-DDE, the $MeSO_2$-PCB analog $MeSO_2$-2,5-dichlorobenzene, and the known ER antagonists, tamoxifen and ICI 182,780 were also examined. Agonistic and antagonistic effects on 17β-estradiol- (E2)-induced luciferase activity were determined in three different ER-CALUX (estrogen-responsive, chemically activated luciferase expression) assays; recombinant human breast ER-CALUX adenocarcinoma cells (Legler *et al*., 1999), and recombinant human embryonic kidney HEK293-ERα-ERE.luc and HEK293-ERβ-ERE.luc cells (Lemmen *et al*., submitted). Agonist/antagonist effects on E2-induced vitellogenin (vtg) production were assessed in an assay using hepatocytes from male carp fish (*Cyprinus carpio*) (Smeets *et al*., 1999).

MATERIALS AND METHODS

Chemicals. The four PCB metabolites, 3′- and 4′-$MeSO_2$-2,2′,4,5′-tetrachlorobiphenyl (3′- and 4′-$MeSO_2$-CB49), 3′- and 4′-$MeSO_2$-2,2′,4,5,5′-pentachlorobiphenyl (3′- and 4′-$MeSO_2$-CB101), and the DDT metabolite, 3-$MeSO_2$-4,4′-DDE (purity > 99%; Fig. 1) were prepared in-house (Bergman and Wachtmeister, 1977, 1978). 2,2′,4,5,5′-Pentachlorobiphenyl (CB101) was purchased from Dr. Ehrenstorfer GmbH (>99%, Augsburg, Germany), and $MeSO_2$-2,5-dichloro-benzene ($MeSO_2$-Cl_2Bz, >99%) was kindly supplied by Dr. Yoshita Kato (University of Shizuoka, Shizuoka, Japan). The purity of the test compounds was assessed by gas chromatography/mass spectrometry. 17β-Estradiol (E2, >99%), tamoxifen (>99%) and dimethyl sulfoxide (DMSO, 99.9%; Janssen Chimica, Geel, Belgium) were purchased from Sigma Chemical Co. (St Louis, MO). ICI 182,780 was a kind gift from Dr. A. Wakeling (Zeneca Pharmaceuticals, U.K.). Each concentration of the test chemicals and E2 were prepared in DMSO as 1000-fold stock solutions for screening in the *in vitro* bioassays.

ER-CALUX assays. The stable transfections of the human ER-CALUX breast-cancer cell line, and human embryonic kidney cells (HEK293-ERα-ERE and HEK293-ERβ-ERE) have been described in detail elsewhere (Legler *et al*., 1999; Lemmen *et al*., submitted). Briefly, HEK293 cells (ATCC, American Type Culture Collection, Rockville, MD) were stably transfected with the luciferase reporter gene construct pEREtata-Luc, and cotransfected with an antibiotic resistance gene. This cell line was subsequently transfected with a recombinant human estrogen receptor (ERα or ERβ) cDNA and a different antibiotic resistance gene. T47D cells were stably transfected with the pEREtata-Luc construct. The ER-CALUX cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's medium (DMEM) and Ham's F12 (DF) medium (Gibco Brl, Life Technologies, Breda, The Netherlands) supplemented with sodium bicarbonate, non-essential amino acids, sodium pyruvate, and 7.5% fetal calf serum at a temperature of 37°C and 7.5% CO_2. 293-ERα-ERE and 293-ERβ-ERE cells were cultured in a 1:1 mixture of DMEM and DF medium.

The three assays using stably transfected cell lines were performed as previously described for T47D cells (Legler *et al*., 1999). Cells were trypsinized, resuspended in assay media, and seeded in 96-well plates (Packard, Meriden, CT) at a density of 5000 cells per well in 100 ml of HEK293-ERα-ERE.luc, and HEK293-ERβ-ERE.luc cells were seeded in 96-well plates (NUNC) at a density of 15,000 cells per well in 200 ml of assay medium. The assay media were steroid-free by using phenol red-free DF and fetal calf serum treated with 5% dextran-coated charcoal (DCC-FCS; Horwitz and McGuire, 1978). After 24 h, the ER-CALUX cells were approximately 50% confluent, and the assay medium was renewed. HEK293-ERα-ERE.luc, and HEK293-ERβ-ERE.luc cells were cultured without medium renewal for 48 h, after which time 50–60% confluency was reached.

For the cells of the ER-CALUX assays, the assay medium was replaced by incubation medium 48 h after initial cell plating. Prior to the cell incubations with the compounds, the DMSO stock solutions were diluted 1000-fold in assay medium. The nominal concentration of the compounds in the assay

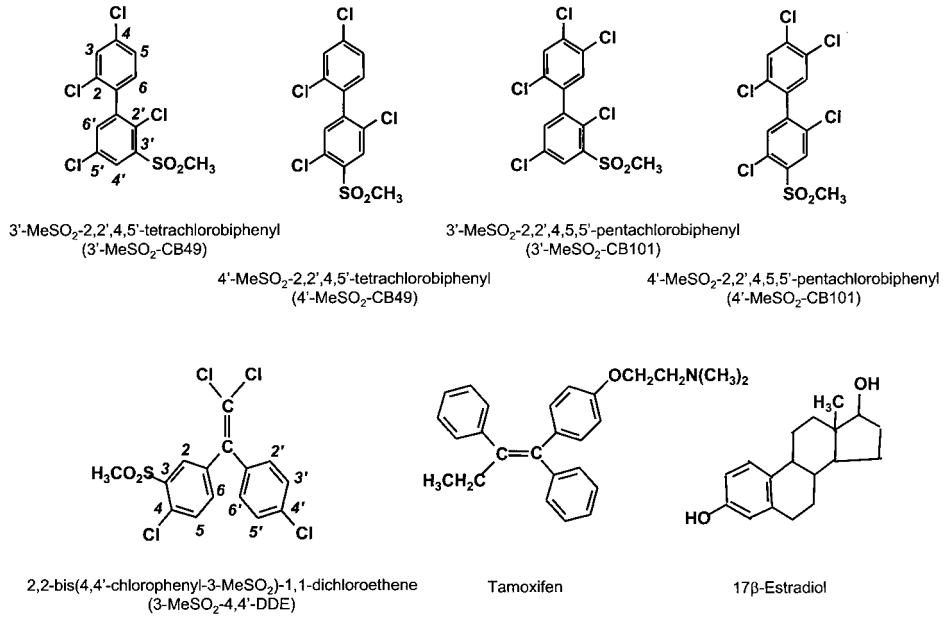

FIG. 1. Chemical structures of the aryl methyl sulfones, tamoxifen and 17β-estradiol (E2). The abbreviated chemical names for the aryl sulfones are indicated in brackets, based on an extension of the numbering system applied to PCB congeners (Guitart *et al*., 1993). The hydrogen atoms are omitted from the aromatic rings for clarity.

medium ranged from 0.05 to 20 μM. For each set of experimental plates a complete E2 standard curve (1 to 100 pM) was included. In addition, The EC_{50} concentration (10 pM) of E2 was included in every 96-well plate. For all three ER-CALUX assays, all compound concentrations were tested in two sets of three replicates. DMSO concentrations in the assay medium did not exceed 0.2% (v/v).

After an incubation period of 24 h at 37°C in an atmosphere of 7.5% CO_2, ER-CALUX plates were transferred to ice, and the assay medium was removed. To each well, 50 ml of lysis buffer was added, containing 1% (v/v) Triton X-100, 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EDTA (pH 7.8), and 1 mM DTT. After vigorous shaking of the plates at 4°C for at least 10 min, 25 ml of the lysate suspension was transferred to a 96-well view plate (Packard). To each well, 25 ml of LucLite solution was added. The Luclite luciferase reporter gene assay system (Packard BioScience B.V., Groningen, The Netherlands) was used to measure luciferase activity, according to the manufacturer's instructions. Alternatively, cells were washed twice with 100 ml of phosphate-buffered saline (PBS), and subsequently lysed in 30 ml low-salt (LS) buffer containing 10 mM Tris (pH 7.8), 2 mM dithiotreitol (DTT), and 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid. Following a 10-min incubation on ice, the 96-well plates were frozen at –80°C for a minimum of 30 min and maximum of 1 day to lyse the cells. The plates were thawed on ice and shaken for 5 min at room temperature. Luciferase activity was measured in a luminometer (Labsystems Luminoscan RS) with automatic injection of 100 ml flash mix (pH 7.8) per well containing 470 mM luciferin, 20 mM trycine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 5 mM ATP, and 2 mM DTT (pH 7.8).

CARP-HEP/vtg assay. The common carp (*Cyprinus carpio*) used in the carp hepatocyte/vitellogenin (CARP-HEP/vtg) assay were genetically uniform, all male (XY), F1 hybrid progenies. Further details of the carp, the maintenance of the fish prior to use in the assay, and the perfusion procedure are described in detail in Smeets *et al.* (1999). Briefly, carp hepatocytes were freshly perfused by a 2-step retrograde technique, isolated, and cultured. The liver was first perfused with Ca^{2+}- and Mg^{2+}-free buffer containing EDTA (0.145 M NaCl; 5.4 mM KCl; 5 mM EDTA; 1.1 mM KH_2PO_4; 12 mM $NaHCO_3$; 3 mM NaH_2PO_4; 100 mM HEPES; pH: 7.5) and followed by a step with the same buffer containing no EDTA and 0.26 mg/ml collagenase D (Boehringer, Mannheim, Germany). The perfused liver sections were filtered through nylon mesh. After removal, mincing, sieving, and washing, the hepatocytes were resuspended in culture medium. The cell viability was >90% as assessed with trypan-blue staining.

The isolated hepatocytes were cultured in phenol red-free DMEM/F12 medium (D2906, Sigma Chemical Co., St. Louis, MO) supplemented with 14.3 mM $NaHCO_3$, HEPES (final concentration: 20 μM), 50 mg/l gentamycin, 1 μM insulin, 10 μM hydrocortisone, 2% v/v Ultroser SF (steroid-free) serum (Jones Chromatography, Mid Glamorgan, UK), and 2 mg/l of the protease-inhibitor aprotinin (Fluka, Buchs, Switzerland) at pH 7.4. The concentration of the cell suspension was 1.0×10^6 cells/ml, and 0.18 ml (or 180,000 cells) was added to each well of 96-well tissue plates (Greiner, Alphen a/d Rijn, the Netherlands). The plates were maintained at 24°C for a period of 36 h to acclimatize the cells. The proportion of erythrocytes relative to hepatocytes did not exceed 10%.

After the 36-h acclimatization period, the cell confluence was about 70% and was ready for dosing. All DMSO stock solutions of compounds and concentrations used in the ER-CALUX were diluted 1000-fold in assay medium and used in the CARP-HEP/vtg assay. The culture medium (90%) was replaced by assay medium (164 μM) containing 10% higher compound concentrations to obtain the desired 1000-fold dilution. After two days, the assay medium (90%) was refreshed with new assay medium containing nominal compound and E2 concentrations. After a further twodays, the medium was transferred to new 96-well plates, and frozen at –70°C until further use. The remaining hepatocytes were used to determine EROD activity, cell viability, or protein content. An indirect competitive ELISA was used to quantify the vtg present in the assay medium. The ELISA procedure, as well as calculations to quantify vtg, have been thoroughly described (Smeets *et al*., 1999). For each experiment set, an E2 dose-response curve plate was included with concentrations ranging from 0.6 to 6000 nM. An E2 positive control (EC_{50}, 100 nM) was included on all other plates. All E2 and compound concentrations were tested in two separate sets of 6-fold replicates. The DMSO concentration in the assay medium did not exceed 0.2% (v/v).

Antiestrogenic effects. Antiestrogenic activity of the test compounds was assessed in the *in vitro* assays using the same nominal concentrations, procedures, and number of replicates used in the estrogenicity screening. The ER-CALUX, HEK293-ERα-ERE.luc and HEK293-ERβ-ERE.luc cells were co-administered with 10 pM (EC_{50}) or 100 pM E2 in separate co-incubations with the different test compounds and concentrations. In the CARP-HEP/vtg assays, the hepatocytes were co-administered with 100 nM (EC_{50}) or 20 nM

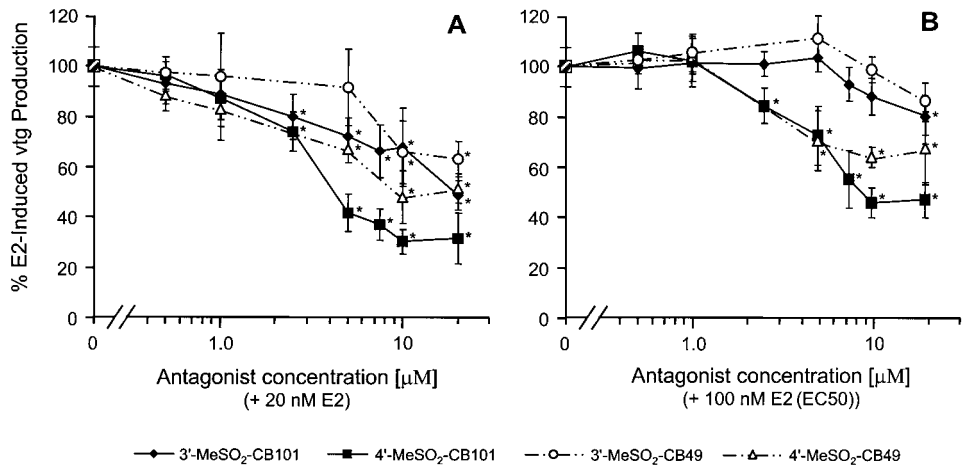

FIG. 2. The antiestrogenic effect of $MeSO_2$-PCBs on 17β-estradiol (E2)-induced vitellogenin (vtg) production in the hepatocytes of male carp. The hepatocytes were treated with different concentrations of the individual $MeSO_2$-PCB congeners in the presence of either (A) 20 nM of E2 or (B) 100 nM of E2 (EC_{50}). Vtg was measured after four days of treatment as described under Materials and Methods. Vtg production is plotted as a percentage of the controls, i.e., induction by E2 alone (square with cross-hatched lines). Results for the E2 control and $MeSO_2$-PCB + E2 treatments are expressed as means ± SD of two sets of $n = 6$ replicate determinations. The significance of $MeSO_2$-PCB inhibition relative to the E2 controls is indicated (*$p < 0.05$).

E2. For both the CALUX and CARP-HEP/vtg assays, E2 concentrations differing from the EC_{50} by 10 times and 5 times, respectively, were used to assess the competitive antagonism of the test compounds with E2-induced gene expression. An E2 amount 5 times lower than the EC_{50} concentration, rather than 10 times higher as in the CALUX assays, was used in the CARP-HEP/vtg assays because 3′-$MeSO_2$-CB49 antagonized E2-induced vtg production at 20 nM (Fig. 2A), but not at 100 nM (Fig. 2B). If 1000 nM E2 (10 times the EC_{50}) had been used, no competitive reduction in the 3′-$MeSO_2$-CB49 antagonism would have been observed. DMSO present in the compound incubations did not exceed 0.2% (v/v). In all four *in vitro* assays, 0.1, 1.0, and 10 μM of the known competitive ER antagonist tamoxifen were included as positive controls for antiestrogenicity. In addition, 0.25, 2.5, and 10 nM concentrations of the ER antagonist ICI 182,780 were also included as positive controls in the ER-CALUX and 293-ERα-ERE assays. Tamoxifen and ICI 182,780 inhibited >90% of the E2-induced (10 pM) gene expression at 10 μM and 10 nM, respectively.

An antiestrogenic effect was defined by the capacity of an antagonist to inhibit the luciferase activity or vitellogenin production induced by E2. The percentage of the remaining, E2-induced response (%RR) is calculated according to Equation 1:

$$\%RR = [(A_{compound+E2} - A_{control})/(A_{E2} - A_{control}]) \times 100\% \quad (1)$$

where $A_{compound+E2}$, $A_{control}$, and A_{E2} are the average activity of the test wells, control wells, and wells incubated with E2 alone, respectively. A compound that is not antagonistic will elicit a %RR of 100%, i.e., the response of the co-administered concentration of E2 will be the same as the E2 concentration administered alone.

Cell viability. A possible cytotoxic effect evoked by the compounds in the ER-CALUX cells and carp hepatocytes was assessed by changes in the mitochondrial dehydrogenase-mediated metabolism of 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), or the MTT test (Denizot and Lang, 1986). The procedure for the ER-CALUX cells were similar to Meerts *et al.* (2001). After vtg harvesting, the remaining hepatocytes were incubated with MTT for 1 h, similar to Smeets *et al*. (1999) with minor modifications.

AhR-CALUX assay, EROD activity in carp hepatocytes and protein content. The $MeSO_2$-PCBs, or more likely, dioxin-like impurities in the pure compound samples, were assessed for AhR-mediated activity. After vtg harvesting, carp hepatocytes were frozen or used immediately for the determination of CYP1A enzyme activity by measurement of ethoxyresorufin-*O*-deethylase (EROD) activity (Burke and Mayer, 1974; Smeets *et al.*, 1999). A recombinant H4IIE rat hepatoma (H4IIE.luc) cell line, or AhR-CALUX, containing a luciferase reporter gene under the control of dioxin-responsive enhancers, also was used to assess the possible dioxin-like activity and was performed as described elsewhere (Sanderson *et al.*, 1996). TCDD concentrations of 0.3, 1.0, 10, 100 and 1000 pM were measured as positive controls.

Protein content was determined for dosed and control carp hepatocytes according to the procedure of Lowry *et al.* (1951) and Rutten *et al.* (1987), with some modifications. After vtg harvesting, the hepatocytes were washed twice with 200 ml of phosphate-buffered saline (PBS). An aqueous solution of 0.5% Triton X-100 (v/v, 200 ml) was then added. The plates were frozen at –70°C for at least 2 h. After thawing to room temperature, the protein content in each well was diluted, if necessary, for protein determination by comparison relative to a BSA standard curve.

Dose-response curves, calculations and statistics. Dose-response relations were described by the sigmoidal function $y = a_0 + a_1/(1 + \exp([a_2 + x)/a_3])$ (SlideWrite Plus, 4.0, Advanced Graphics Software, Carlsbad, CA), in which y is the induction of or remaining (%RR, equation 1) luciferase activity or vtg production, and x is the logarithm of the dose. For estrogenic effects, vtg or luciferase levels were normalized to the maximum levels induced by E2, and expressed as a percent of the maximum level. For antiestrogenic effects, the levels were normalized to the vtg or luciferase induced by the co-administered E2 concentration alone. The statistical significance of the differences between compound-treated and control cells were determined using a 2-way ANOVA ($p < 0.05$).

RESULTS

E2-Induced gene expression, in vitro *bioassay.* With reference to previous studies, a comparison was made as to the consistency of the responsiveness and potency of E2 treatment and the antagonism of E2-induced activity in the three human cell-based ER-CALUX assays and the fish cell-based CARP-HEP/vtg assay. The LOEC (0.5 pM) and EC_{50} (3.7 pM) concentrations of E2-induced luciferase activity in the T47D.luc cells was similar to that reported by Legler *et al.* (1999), with a maximum-fold induction relative to DMSO of about 50-fold. Tamoxifen did not induce a luciferase response up to treatment levels of 10 μM. The antagonistic potency of tamoxifen on E2-induced activity (10 pM, EC_{50}) was about 90% at concentrations > 0.1 μM (Table 1). ICI 182,780 inhibited 100% of the E2-induced luciferase activity at concentrations > 1.0 nM.

TABLE 1

Antagonistic Effects of $MeSO_2$-PCBs on 17β-Estradiol-Induced (EC_{50} Concentrations) Luciferase Activity in Transfected Human ER-CALUX Breast Adenocarcinoma T47D.luc Cells and Vitellogenin Production in Hepatocytes of Male Carp

In vitro assay and compound[a]	Inhibitory LOEC[b] (μM)	Relative potency (LOEC)[c]	IC_{50} (μM)[d]
ER-CALUX			
Tamoxifen	0.010	1	0.06
3′-$MeSO_2$-CB49	>10	<0.0010	N/A
4′-$MeSO_2$-CB49	1.0	0.010	2.9
3′-$MeSO_2$-CB101	2.5	0.0040	N/A
4′-$MeSO_2$-CB101	1.0	0.0010	2.4
CARP-HEP/vtg			
Tamoxifen	0.3	1	0.7
3′-$MeSO_2$-CB49	>20	<0.015	N/A
4′-$MeSO_2$-CB49	~2.5	~0.12	4.7
3′-$MeSO_2$-CB101	20	0.015	N/A
4′-$MeSO_2$-CB101	2.5	0.12	5.3

[a]The structures of the $MeSO_2$-PCBs are illustrated in Figure 1.

[b]The lowest-effect concentration (LOEC) where a significant ($p < 0.05$) inhibition of E2-induced (EC_{50}, 10 pM) luciferase activity or vitellogenin production (EC_{50}, 100 nM) was observed (see Figs. 2 and 3).

[c]Ratio of LOEC of significant inhibition ($p < 0.05$) of E2-induced response of tamoxifen over $MeSO_2$-PCB.

[d]The IC_{50} is the concentration for 50% inhibition of E2-induced luciferase activity (EC_{50}, 10 pM) or vitellogenin production (EC_{50}, 100 nM; see Figs. 2 and 3). For the $MeSO_2$-PCBs, the IC_{50} concentrations are somewhat overestimated or the concept was not considered applicable (N/A). In some cases, a clear level of maximum inhibition was not achieved up to a treatment concentration of 10 or 20 μM.

Relative to DMSO controls, the efficacy of E2-induced luciferase activity in the HEK293-ERα-ERE.luc cells (35-fold) was greater than in the HEK293-ERβ-ERE.luc cells (16-fold). The LOEC concentrations were 2.0 and 30 pM, and the EC_{50} values 5.2 pM and 110 pM, for the HEK293-ERα-ERE.luc and HEK293-ERβ-ERE.luc, respectively. The EC_{50} values for E2 are consistent with data from earlier studies (Seinen *et al*., 1999). The responsiveness and potency of the HEK293-ERα-ERE.luc cells towards E2 were only slightly less than in the T47D.luc cells. Much like the T47D.luc cells, tamoxifen comparably antagonized E2-induced activity in both the HEK293-ERα-ERE.luc and HEK293-ERβ-ERE.luc cells, where the LOEC and IC_{50} concentrations were 0.01 μM and 0.04 to 0.05 μM, respectively. Similar to the T47D.luc cells, up to a maximum of ~80% inhibition by tamoxifen occurred at concentrations >0.1 μM. ICI 182,780 completely inhibited luciferase activity in the HEK293-ERα-ERE.luc cells at concentrations >1.0 nM.

The LOEC (4.1 nM) and EC_{50} (~100 nM) concentrations of E2 for vtg production in the CARP-HEP assay were similar to the findings by Smeets *et al.* (1999). These potencies were 3 to 4 orders of magnitude lower than in the three ER-CALUX assays. Variability in the sensitivity to E2 among carp hepatocytes cultures was not observed in our study, which was a similar finding to E2-induced vtg production in hepatocytes from the same genetically uniform strain of male carp used in Smeets *et al.* (1999). However, the efficacy (>1000-fold) of vtg production was 2 orders of magnitude higher than that described in Smeets *et al.* (1999). Pelissero *et al*. (1993) reported variability in E2 sensitivity for vtg expression among different cultures for rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Pelissero *et al.* (1993) suggested that, considering the ER itself is induced by exposure to E2, ER and vtg gene transcription, mRNA stability for vtg, ER turnover, E2 metabolism in culture, and the rate of exocytosis of vtg, all factor into the rate of vtg secretion into the surrounding medium. We also recently demonstrated that exposure of carp hepatocytes to low or background levels of estrogens and xenoestrogens (e.g., methoxychlor and bisphenol A) can increase the sensitivity for vtg production from exposure to endogenous estrogens (Rankouhi *et al*., 2002). Therefore, it is necessary to compare xenoestrogen responses to an E2 dose-response curve obtained in the same hepatocytes culture, as was done in the present study.

E2-induced vtg production (EC_{50}) was inhibited essentially 100% at tamoxifen concentrations approaching 10 μM. Using the CARP-HEP/vtg assay, the LOEC and IC_{50} concentrations (Table 1) were calculated from dose-response curves with 100 nM E2 (EC_{50}) and 0.01, 0.03, 0.1, 0.3, 0.7, 1.0, 3.0, or 10 μM of tamoxifen (not shown). For the ER-CALUX the LOEC and IC_{50} concentrations (Table 1) were with 10 pM E2 (EC_{50}) and 0.00005, 0.0005, 0.01, 0.1, 0.5, 1.0, or 10 μM of tamoxifen (not shown). The IC_{50} and LOEC concentrations were 1 and 3 orders of magnitude greater, respectively, than for tamoxifen inhibition of luciferase activity in the T47D.luc cells (Table 1). Tamoxifen alone did not induce vtg production up to 10 μM.

In vitro *antiestrogenic activity of the aryl methyl sulfones and their precursors.* The $MeSO_2$-PCBs, CB101 and $MeSO_2$-2,5-Cl_2Bz (Fig. 1) were examined for agonist and antagonist activity in the four estrogen-responsive *in vitro* assays. $MeSO_2$-2,5-Cl_2Bz has the same functional group substitution as the $MeSO_2$-containing phenyl ring of the 4-$MeSO_2$-PCBs (Fig. 1). This permitted an evaluation of the biphenyl backbone in any observed estrogenic or antiestrogenic activity of the $MeSO_2$-PCBs. 4,4′-DDE (10 μM)-induced luciferase activity about 17-fold relative to the DMSO control, whereas E2-induced activity was not significantly ($p < 0.05$) antagonized (103 ± 11% of EC_{50}). 3-$MeSO_2$-4,4′-DDE (10 μM) did not significantly ($p < 0.05$) induce luciferase activity (1.3-fold relative to control) or antagonize (88 ± 9% of EC_{50}) E2-induced luciferase activity. 4,4′-DDE and 3-$MeSO_2$-4,4′-DDE were not screened in the remaining assays.

The 3′- and 4′-$MeSO_2$-CB49 and -CB101 congeners, CB101 and $MeSO_2$-2,5-Cl_2Bz did not significantly ($p < 0.05$) induce ER-mediated gene expression in any of the four *in vitro* assays up to maximum concentrations of 10 or 20 μM. Con-

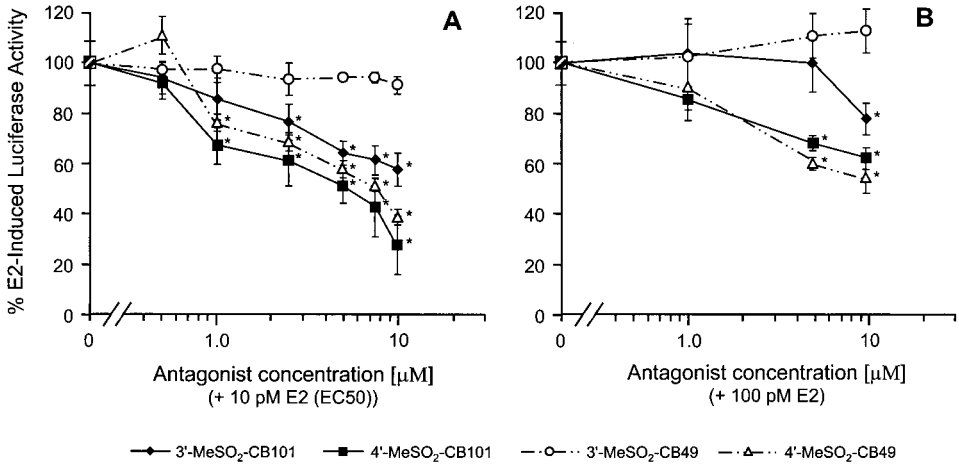

FIG. 3. The antiestrogenic effect of $MeSO_2$-PCBs on 17β-estradiol- (E2-) induced luciferase activity in stably transfected ER-CALUX human breast T47D.luc adenocarcinoma cells. ER-CALUX cells were treated with different concentrations of the individual $MeSO_2$-PCB congeners in the presence of either (A) 10 pM (EC_{50}) of E2 or (B) 100 pM of E2. Luciferase activity was measured after 24 h of treatment as described under Materials and Methods. Luciferase activity is plotted as a percentage of the controls, i.e., induction by E2 alone (square with cross-hatched lines). Results for the E2 control and $MeSO_2$-PCB + E2 treatments are expressed as means ± SD of three sets of $n = 3$ replicate determinations. The significance of $MeSO_2$-PCB inhibition relative to the E2 controls is indicated (* $p < 0.05$).

centrations up to 10 μM of CB101 and $MeSO_2$-2,5-Cl_2Bz, and in the presence of an EC_{50} concentration of E2, did not significantly ($p < 0.05$) antagonize E2-induced responses in the assays.

With the exception of 3′-$MeSO_2$-CB49, the $MeSO_2$-PCBs consistently antagonized the E2-induced response (EC_{50} concentration) in a concentration-dependent manner in all four *in vitro* assays (Table 1, Figs. 2 to 5). Based on LOECs and IC_{50} concentrations (Table 1), the antiestrogenic effect was in the order of 4′-$MeSO_2$-CB101 > 4′-$MeSO_2$-CB49 > 3′-$MeSO_2$-CB101 >> 3′-$MeSO_2$-CB49. Thus, the 4′-$MeSO_2$-PCBs were generally more effective anti-estrogens relative to the 3′-$MeSO_2$-PCB congeners. The E2-induced response (EC_{50}) in the presence of 4′-$MeSO_2$-CB101 (41 ± 7%) and -CB49 (64 ± 4%) reached an apparent maximum in the CARP-HEP/vtg assay (Fig. 2B). IC_{50} concentrations were about 5.0 μM. In the ER-CALUX assay, the remaining E2-induced activity (EC_{50}) for 4′-$MeSO_2$-CB101 (28 ± 4%) and -CB49 (39 ± 3%) were lower, but did not appear minimized at the maximum treatment concentration of 10 μM (Fig. 3A). In the ER-CALUX cells, the estimated IC_{50} concentrations for 4′-$MeSO_2$-CB101 and -CB49 were about 2 orders of magnitude lower when compared to the CARP-HEP/vtg assay (Table 1).

Relative to tamoxifen, in the ER-CALUX (T47D.luc) the antiestrogenic potencies for the $MeSO_2$-PCBs, and based on the LOECs, were about 2 to 3 orders higher, while the CARP-HEP were about 1 to 2 orders of magnitude higher (Table 1). Thus, the relative potencies as ER antagonists were 100- to 1000-fold lower than that of tamoxifen.

Mechanism of the $MeSO_2$-PCB-mediated, antiestrogenic effect. In the ER-CALUX assay, the antagonistic effect of 3′- and 4′-$MeSO_2$-CB101 and 4′-$MeSO_2$-CB49 were largely reduced by exposure to E2 at 10 times (100 pM) the EC_{50} concentration (Fig. 3B). In the CARP-HEP/vtg assay, an E2

TABLE 2

Induction of EROD Activity in Carp Hepatocytes and Luciferase Activity in Stably Transfected (Aryl Hydrocarbon Receptor, AhR) H4IIE Rat Hepatoma (H4IIE.luc) Cells After Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and Polychlorinated Biphenyl Methyl Sulfones ($MeSO_2$-PCBs)

		% Max response induced by TCDD[b]	
Compound[a]	Concentration (μM)	AhR-CALUX	CARP-HEP (EROD)
TCDD	3.0×10^{-7}	0.7 ± 0.1	<0.5[c]
	1.0×10^{-6}	2.3 ± 0.3	0.5 ± 0.1
	1.0×10^{-5}	22.6 ± 3.6	61.9 ± 8.6
	1.0×10^{-4}	70.2 ± 0.8	80.7 ± 3.2
3′-$MeSO_2$-CB49	0.1	—	<0.5[c]
	1.0	—	<0.5[c]
	10	<0.7[c]	<0.5[c]
4′-$MeSO_2$-CB49	0.1	—	<0.5[c]
	1.0	—	<0.5[c]
	10	<0.7[c]	<0.5[c]
3′-$MeSO_2$-CB101	0.1	—	<0.5[c]
	1.0	—	13.2 ± 4.4
	10	30.8 ± 2.3	38.3 ± 6.4
4′-$MeSO_2$-CB101	0.1	—	<0.5[c]
	1.0	—	<0.5[c]
	10	1.9 ± 0.5	<0.5[c]

[a]Figure 1 illustrates the structures of the $MeSO_2$-PCBs.

[b]The responses were corrected for cells exposed to DMSO alone. Prior to calculating the percent response relative to the maximum response at 1000 pM (1.0×10^{-3} μM) TCDD, the EROD activity was corrected for the protein content per well (pmol/mg protein/min), whereas the luciferase activity was not. A TCDD concentration of 1000 pM maximally induced both EROD activity in the carp hepatocytes and luciferase activity in the HII4E.luc cells. A dash indicates that the effect of the concentration was not measured.

[c]The limit of detection in the AhR-CALUX and for EROD activity are calculated as 0.7% (equivalent to 0.3 pM of TCDD) and 0.5% (equivalent to 1.0 pM of TCDD) of the maximum TCDD-induced.

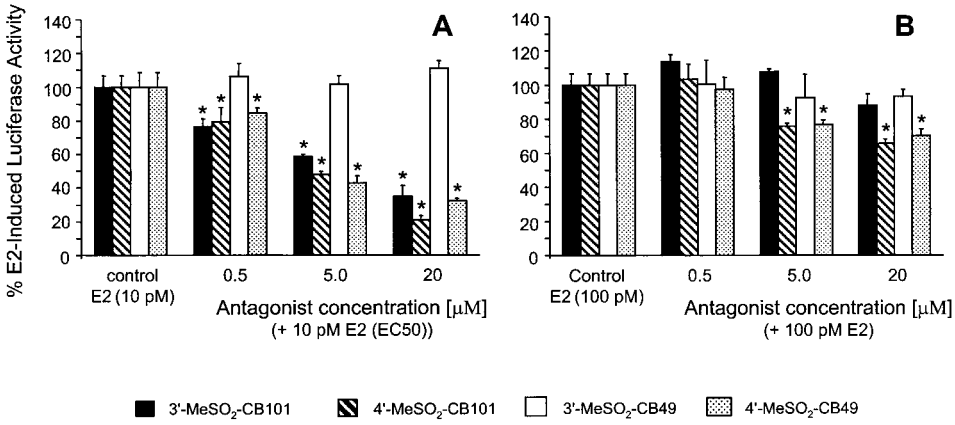

FIG. 4. The antiestrogenic effect of $MeSO_2$-PCBs on 17β-estradiol (E2)-induced luciferase activity in human embryonic kidney (HEK) 293 cells stably transfected with a luciferase reporter gene construct and recombinant human estrogen receptor alpha (HEK293-ERα-ERE.luc). The cells were treated with different concentrations of the individual $MeSO_2$-PCB congeners in the presence of either (A) 10 pM (approximate EC_{50}) of E2 or (B) 100 pM of E2. Luciferase activity was measured after 24 h of treatment as described under Materials and Methods. Luciferase activity is plotted as a percentage of the controls, i.e.. induction by E2 alone. Results for the E2 control and $MeSO_2$-PCB + E2 treatments are expressed as means ± SD (3 sets, $n = 3$ replicates). The significance of $MeSO_2$-PCB inhibition relative to the E2 controls is indicated (*$p < 0.05$).

concentration 5 times lower (20 nM) than the EC_{50} facilitated an increase in the inhibitory effects of 4′-$MeSO_2$-CB101 and -CB49 (Fig. 2A). Antagonistic effects on E2-induced activity were observed for 3′-$MeSO_2$-CB49 at concentrations of 10 and 20 μM, where >60% of the E2-induced activity was maintained.

In the ER-CALUX and CARP-HEP/vtg assays, for any compound at any of the concentrations tested (up to 10 μM), the $MeSO_2$-PCBs, tamoxifen or ICI 182,780 did not influence cell viability as indicated by a lack of significant ($p < 0.05$) change in the MTT activity relative to the DMSO or blank controls. AhR-induced effects of the TCDD controls were observed on E2-induced (EC_{50}) vtg production and EROD activity in the CARP-HEP assay, whereas only 3′-$MeSO_2$-CB101 (at 10 μM) demonstrated any effect (Table 2). In the presence of E2 (EC_{50}), 10 and 100 pM TCDD inhibited the production of vtg by 13 ± 5% and 64 ± 6%, respectively (not shown). With the exception of 3′-$MeSO_2$-CB101, the $MeSO_2$-PCBs were not AhR-active up to 10 μM in the AhR-CALUX assay (Table 2). The same TCDD concentrations as used for CARP-HEP/EROD activity inhibited E2-induced luciferase activity (not shown), similar to previous studies (Sanderson *et al.*, 1996).

ERα and ERβ. The $MeSO_2$-PCBs were tested in two assays based on stably transfected HEK293-ERα-ERE.luc and HEK293-ERβ-ERE.luc cells to determine if $MeSO_2$-PCB interaction with the ER, and subsequent gene transcription, discriminated between the two ER subtypes. None of the four $MeSO_2$-PCBs were found to significantly ($p < 0.05$) induce

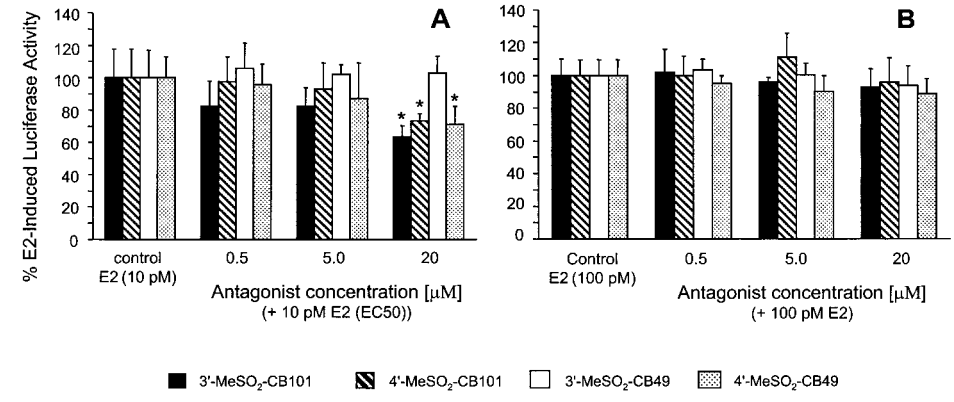

FIG. 5. The antiestrogenic effect of $MeSO_2$-PCBs on 17β-estradiol (E2)-induced luciferase activity in human embryonic kidney (HEK) 293 cells stably transfected with a luciferase reporter gene construct and recombinant human estrogen receptor beta (HEK293-ERβ-ERE.luc). The cells were treated with different concentrations of the individual $MeSO_2$-PCB congeners in the presence of either (A) 10 pM of E2 or (B) 100 pM (approximate EC_{50}) of E2. Luciferase activity was measured after 24 h of treatment as described under Materials and Methods. Luciferase activity is plotted as a percentage of the controls, i.e., induction by E2 alone. Results for the E2 control and $MeSO_2$-PCB + E2 treatments are expressed as means ± SD (3 sets, $n = 3$ replicates). The significance of $MeSO_2$-PCB inhibition relative to the E2 controls is indicated (*$p < 0.05$).

luciferase activity in either cell line (not shown). The dose-dependent trends found for the $MeSO_2$-PCB-mediated antagonism of E2-induced responsiveness in the ER-CALUX assay and CARP-HEP/vtg assays were similar in the HEK293-ERα-ERE.luc assay (Fig. 4), and to a lesser extent in the HEK293-ERβ-ERE.luc assay (Fig. 5). The remaining E2-induced luciferase (approximate EC_{50} concentration) activity resulting from co-administration with 3′- and 4′-$MeSO_2$-CB101 and 4′-$MeSO_2$-CB49 (20 μM) was 60% or higher in the HEK293-ERβ-ERE.luc assay than in the HEK293-ERα-ERE.luc assay (Fig. 5A). The remaining E2-induced activity for the same 3 $MeSO_2$-PCBs in the HEK293-ERα-ERE.luc assay (Fig. 4A) was 30% or lower, and similar to the inhibitory effect found in the ER-CALUX assay (Fig. 3A). In the presence of 100 pM of E2, which is 10 times higher than the approximate EC_{50} concentration of E2 (10 pM), the antagonistic effects of 3′- and 4′-$MeSO_2$-CB101 and 4′-$MeSO_2$-CB49 were reduced or eliminated in the HEK293-ERα-ERE.luc assay (Fig. 4B). In contrast, the antagonistic effect of the sulfones was greater with about 10 times lower concentrations (10 pM) of the approximate EC_{50} of E2 (100 pM) in the HEK293-ERβ-ERE.luc assay (Fig. 5B). As in the ER-CALUX cells, 3′-$MeSO_2$-CB49 was not antagonistic towards E2-induced luciferase at either 10 pM (EC_{50}) or 100 pM of E2.

DISCUSSION

Several studies have demonstrated that environmentally persistent $MeSO_2$-PCBs, including 3′- and 4′-$MeSO_2$-CB49 and -CB101, are capable of endocrine-related activity and effects via interaction with steroid hormone receptors, by inhibiting enzymatic activity associated with steroid metabolism, or by interaction with steroid hormone binding proteins. Examples include competitive binding affinity with human and mouse GR (Johansson *et al*., 1998a), antagonism of dexamethasone-induced gene expression in Chinese hamster ovary cells that were stably transfected with a human GR reporter construct, inhibition of CYB11B1-dependent corticosterone synthesis (11β-hydroxylase) in mouse adrenocortical Y1 cells (Johansson *et al*., 1998b), and thyroidogenic activity in rats (Kato *et al*., 1999). To our knowledge, however, persistent $MeSO_2$-PCB metabolites have not been investigated *in vitro* or *in vivo* to assess estrogenic or antiestrogenic potential (Letcher *et al*., 2000a).

The present study demonstrated that persistent 3′- and 4′-$MeSO_2$-CB49 and -CB101 metabolites do not agonize transactivation of estrogen-responsive reporter genes in *in vitro* assays based on recombinant human cells or primary carp hepatocytes. However, $MeSO_2$-PCBs, especially 4-$MeSO_2$-PCBs, are capable of inhibiting estrogen-responsive gene expression in a concentration- and structure-dependent manner by interaction with human or fish ER. Higher E2 concentrations partially reversed the inhibitory effects of the $MeSO_2$-PCBs in all four assay systems, which demonstrates that the antagonist interaction with substrate binding site on the ER is at least reversible (or surmountable).

$MeSO_2$-PCB antiestrogenicity was dependent on the presence and position of the $MeSO_2$-functional group and the chlorinated (substitution pattern) biphenyl backbone. 4′-$MeSO_2$-substitution, rather than 3′-$MeSO_2$-substitution, resulted in more effective $MeSO_2$-PCB antiestrogenicity. CB101 is the non-$MeSO_2$-containing base structure of 3′- and 4′-$MeSO_2$-CB101, yet the parent compound itself was not estrogenic or antiestrogenic. Thus, our study shows that the intact $MeSO_2$-PCB structure appears to be essential for antagonistic interaction with both the carp and human ERs, and is optimized by 4′-$MeSO_2$-substitution. The increased inhibitive effect of increasing $MeSO_2$-PCB concentrations in competition with E2 concentrations, similar to tamoxifen, further exemplified that the antagonism of E2-induced activity proceeds, most likely, via direct interaction with the human and carp ERs. The structural diversity of potential ER antagonists is also shown by tamoxifen, which lacks the phenolic A-ring of E2 (Fig. 1), yet competitively binds to the ER. Tamoxifen can be agonistic as well as antagonistic, depending on the target tissue and the species (Nimrod *et al*., 1997).

Non-ER-mediated mechanisms are an unlikely explanation of the observed $MeSO_2$-PCB antiestrogenic effects. The antiestrogenicity of $MeSO_2$-PCBs was not mediated via concentration-dependent cytotoxic effects, as shown by the negative effects on MTT activity. CYP1A1/1B1 activity has been reported in wild-type T47D and the ER-CALUX cells derived from the T47D cells (Legler *et al*., 1999; Spink *et al*., 1998), and carp hepatocytes possess inducible CYP1A1 activity (Smeets *et al*., 1999). However, *in situ* transformation of $MeSO_2$-PCBs to active, antiestrogenic metabolites was unlikely in these cell systems because 3′- and 4′-$MeSO_2$-CB101 have no adjacent, hydrogen-bound carbons on the biphenyl structure, which is a necessary substrate feature for Phase I, CYP enzyme-mediated PCB biotransformation (McFarland and Clarke, 1989). The hydrogen substitution patterns of 3′- and 4′-$MeSO_2$-CB101 are analogous to 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB153), which has a biological half-life in humans estimated to be 338 days (Bühler *et al*., 1988). Furthermore, in species where $MeSO_2$-PCB metabolites have been shown to be formed, 3′- and 4′-$MeSO_2$-CB49 and –CB101 are increasingly dominant residues relative to other $MeSO_2$-PCB congeners, which indicates the greater resistance of these metabolite structures to further metabolism (Letcher *et al*., 2000a, 1998).

Compounds may interfere with E2-regulated cellular processes by ER-dependent mechanisms, but also ER-independent mechanisms, so as to decrease the effectiveness of the E2-mediated response (Navas *et al*., 1998). For example, AhR-mediated induction of CYP1A enzymes can evoke dose-dependent hydroxylation of E2, and thus decrease the level of E2 that is present, and therefore bioavailable to the cell. The antiestrogenic activity of AhR agonists such as TCDD and

related dioxins and furans, PCBs, and PAHs in cell systems is often a direct result of their binding affinity for the AhR and the subsequent induction of CYP1A activity (Hoivik *et al*., 1998). TCDD and non-ortho (co-planar) PCBs are antiestrogens that can also function via an AhR-mediated mechanism of action involving "cross talk" between ER and AhR signaling pathways (Safe *et al*., 1998). These AhR-dependent pathways are improbable mechanisms for the observed $MeSO_2$-PCB antiestrogenic activity in our bioassay systems since interaction with the AhR and subsequently mediated activities was minimal if existing at all.

Our findings indicate that an AhR-mediated mechanism similar, e.g., to TCDD (Safe *et al*., 1998) is not an explanation for the observed $MeSO_2$-PCB antiestrogenicity. With the exception of 3′-$MeSO_2$-CB101, the $MeSO_2$-PCBs were not AhR-active in the AhR-CALUX and CARP-HEP/EROD assays. Direct AhR-interaction by 3′-$MeSO_2$-CB101 is unlikely since persistent $MeSO_2$-PCBs are not expected to have affinity for the AhR. 3′- and 4′-$MeSO_2$-CB49 and –CB101 contain 2 *ortho* chlorines. By analogy to di-*ortho* PCBs, persistent $MeSO_2$-PCBs cannot attain the obligatory co-planarity of the phenyl rings necessary for a dioxin-like structure (Safe, 1994) and thus are expected to have a low binding affinity for the AhR. In a human lymphoblastoid cell line (Kiyohara *et al*., 1990), 10 of the 11 $MeSO_2$-PCB congeners tested (at about 2 μM) did not induce CYP1A-mediated, benzo[*a*]pyrene hydroxylase (AHH) activity. The AhR-mediated EROD and luciferase activity of 3′-$MeSO_2$-CB101 in this study can perhaps be explained by the presence of a dioxin-like impurity formed during the chemical preparation of the compound. Assay treatment levels of $MeSO_2$-PCBs were in the μM range, and therefore a pM level amount of a dioxin-like impurity would represent less than 0.1% of the amount of $MeSO_2$-PCB present in the standard used.

The antiestrogenicity of persistent $MeSO_2$-PCB towards E2-stimulated gene expression *in vitro* appears to be more significant than for environmentally relevant OH-PCB metabolites. Whereas the 4′-$MeSO_2$-CB49 and –CB101 were effective inhibitors of E2-responsive gene expression in the present assay systems, environmentally relevant OH-PCBs have shown, i) little affinity for either the α or β subtype ER in humans, fish or amphibians, and ii) are weakly inhibitive of E2-induced gene expression *in vitro* (Kuiper *et al*., 1998; Matthews *et al*., 2000).

The physiological significance of the inhibitive potency and efficacy observed for the $MeSO_2$-PCBs is best compared to the competitive ER antagonist, tamoxifen. Tamoxifen (ICI 46,474; Nolvadex) is used clinically as a protective agent against estrogen-dependent breast cancer. The inhibitive LOEC potency for tamoxifen relative to 4′-$MeSO_2$-CB49 or -CB101 was only 100-fold and about 10-fold lower in the ER-CALUX and CARP-HEP/vtg assays, respectively. Human plasma concentrations of tamoxifen after a typical clinical dose is about 190 nM (Tukker *et al*., 1986), whereas concentrations of 4′-$MeSO_2$-CB49 and -CB101 have been reported to be about 0.02 nM in Swedish subjects (Weistrand *et al*., 1997). Comparison of these data may suggest that the potential antiestrogenic impact to humans from $MeSO_2$-PCB exposure is possibly many orders of magnitude lower then that of tamoxifen used in therapeutic doses. However, differences in metabolic susceptibility, and thus bioavailability, and bioaccumulative potential may suggest otherwise. Tamoxifen is given chronically to maintain steady-state levels in preventative treatment for breast cancers. After oral administration, the half-life of tamoxifen in human plasma was about 10 h, and is thus rapidly metabolized *in vivo* (Tukker *et al*., 1986). Tamoxifen is also metabolized *in vivo* to a hydroxylated form, which is about 10 times more potent. The 4′-$MeSO_2$-CB49 and -CB101 concentrations of 0.02 nM reported in the plasma of Swedish subjects (Weistrand *et al*., 1997) is about 5 orders of magnitude lower than the 1.0 μM LOEC found for antagonistic activity in the ER-CALUX assay (Table 1). However, whether formed metabolically or transferred within food webs, the $MeSO_2$-PCBs can accumulate in lipid-bearing target organs. For example, sum $MeSO_2$-PCB concentrations in the Baltic grey seal have been recorded as high as 110,000 ng/g (lipid wt) in adipose tissue (Letcher *et al*., 2000a), whereas levels ranging from 1.0 to 6.0 ng/g (lipid wt) have been reported in the plasma of Swedish human subjects (Weistrand *et al*., 1997).

In HEK293 cells, $MeSO_2$-PCBs antagonized E2-induced luciferase activity preferentially via an ERα- versus a ERβ-mediated mechanism of action. However, this is assuming that the two ER subtypes are expressed at equivalent levels in the two HEK293 cell lines, which was not verified in this study. Preference for ERα over ERβ may also be true in the T47D.luc cells since they express both the α and β forms of the ER subtypes, but were not differentiated in our experiments (Legler *et al*., 1999). Differences in ligand structure can have profound effects on ERα versus ERβ transactivational activity. Our observations of preferential interactions with human ERα for the tested $MeSO_2$-PCBs are similar to findings with other xenobiotics. A number of estrogenic chemicals, including OH-PCBs and polycyclic musk compounds, preferentially transactivate luciferase expression in HEK293-ERα-ERE.luc relative to HEK293-ERβ-ERE.luc cells (Kuiper *et al*., 1998; Seinen *et al*., 1999; Lemmen *et al*., submitted). Other examples include the efficacy and potency of a hydroxylated triaryl-pyrazole in comparison to E2, where the induction of CAT activity in ERα human endometrial cancer cells (ERα-HEC-1.CAT) were less than in ERβ-HEC-1.CAT cells (Sun *et al*., 1999). Sun *et al.* (1999) also showed that a structurally related tetrahydrocannabinol compound was also inductive in ERα-HEC-1.CAT cells, but was a full antagonist in ERβ -HEC-1.CAT cells. The physiological significance of the higher affinity toward ERα relative to ERβ is suggestive that $MeSO_2$-PCBs differentially affect the expression of the two ER sub-types in humans (Kuiper *et al*., 1997).

In conclusion, the antagonism of E2-induced gene expres-

sion *in vitro* by persistent 3′-, and especially 4′-$MeSO_2$-CB49 and -CB101, was concentration- and structure-dependent, regardless of the human or fish *in vitro* assay system used. The mechanism of 3′- and 4′-$MeSO_2$-CB101 antiestrogenicity appears to be competitive antagonism of E2 interaction with the ER. However, further research, using multiple antagonist concentrations, on the complete E2 dose-response curve is necessary to unambiguously show that the $MeSO_2$-PCBs are competitive antagonists. $MeSO_2$-PCB interactions apparently favored ERα over ERβ in the human embryonic cell lines. Comparisons to tamoxifen indicated that especially 4′-$MeSO_2$-PCBs are relatively potent antiestrogens in these cell assays. The results of this study suggest that persistent and bioaccumulative $MeSO_2$-PCB metabolites in exposed wildlife may be of concern as antiestrogens with the potential to modulate estrogen action at the level of the ER. It is also important to note that our findings with $MeSO_2$-PCBs indicate that, in terms of environment contaminant exposures in wildlife and humans, antiestrogenicity might be equally important as estrogenicity with respect to impacts and risks to development and reproduction.

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