In Vitro Estrogenicity and Anti-estrogenicity of Hydroxylated Chlorinated Biphenyls in Human Breast Tumor (MCF-7) Cells

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Introduction

The role of PCB's (polychlorinated biphenyls) in the development of estrogen-related pathogenesis is the subject of much research. A number of studies have related estrogenic responses of rats and mice to PCB exposure[1,2]. Impairment of reproduction in fish and wildlife have implicated chlorinated organic contaminants that include PCB's[3,4]. The potential for interfence of PCB's with estrogen action and metabolism has been established[5,6]. However, a complete understanding of the interactions of PCB's with estrogen receptor (ER), aryl hydrocarbon receptor (AhR), estrogen metabolism, and PCB metabolism has not been achieved.

The human breast tumor cell line, MCF-7, has been used as an *in vitro* model for evaluation of (anti)estrogenic compounds using endpoints such as cellular proliferation[7], formation of multicellular foci[8], native gene expression[9,10] (pS2 and Cathepsin D), and non-native gene expression[11] (firefly luciferase). Expression of a luciferase reporter gene was used to measure estrogenicity of 17 hydroxylated chlorinated biphenyls (hydroxy-CBs)[12] (including 9 confirmed CB metabolites) in stably transfected MCF-7 cells. The inhibition of binding of radio-labelled 17ß-estradiol (E2) to calf uterine estrogen receptor (ER) was measured and compared to the results of the expression assay.

The aim of the research was: 1) to measure the potency of (anti)estrogenicity of hydroxy-CB's in a rapid *in vitro* model; 2) to compare ER binding inhibition to (anti)estrogenic potency. The second aim was undertaken as a first step in determining whether the effects of hydroxy-CB's were mediated by the ER through direct binding or through other mechanisms.

Methods

Seventeen hydroxy-CB's and CB-75 were dissolved in ethanol and stored at -20°C in

glass vials. These solutions were used in both the luciferase expression assay and in the ER binding assay. Nine of the hydroxy-CB's (Compound IDs: A1-A9) were synthesized as authentic standards of mammalian-derived PCB metabolites[13]. All other hydroxy-CB's and CB-75 (Compound IDs: U1-U9), obtained from Ultra Scientific (North Kingstown, RI, USA), were tested to develop structure activity relationships and to compare results with previously published data[2].

The MCF-7 cell line (a gift of M. Pons, INSERM, Montpellier, France) was stably transfected with pVit-tk-Luc, a plasmid containing the *Xenopus laevis* vitellogenin A2 promoter region (containing estrogen responsive elements) upstream of the *Herpes simplex* virus thymidine kinase promoter and the firefly luciferase gene[11]. Cells, maintained in Full Medium (Dulbecco's Modified Eagle's Medium with Ham's F-12 Nutrient Mixture, 10% fetal bovine serum, 1 mM sodium pyruvate, 1 μg/ml bovine insulin, 10 U/ml penicillin, 10 μg/ml streptomycin, pH 7.4) were transferred into Assay Medium (Full Medium without 10% FBS and with 5% Dextran-coated charcoal stripped FBS, 3% CPSR-2 (Sigma Chemical Co.), 1.0 μg/ml human transferrin, 200 μg/ml BSA) and grown for three days reaching 80-95% confluence. Despite Dextran-coated charcoal stripping, the serum concentration of 17ß-estradiol was 5 pg/ml (18 pM), which accounts for the basal expression of luciferase in Assay Medium. Cells were transferred to 96 well tissue culture plates at a density of 1000 cells/well. Forty eight hours after plating, cells were exposed to the test compound dissolved in Assay Medium with a final ethanol concentration of 0.5%. Four concentrations were tested (except for 17ß-estradiol in which 8 concentrations were tested) such that the concentrations were 1/5, 1/10, and 1/50 of the highest concentration tested. The highest concentration tested for each compound was: A6, 37 μM; A8, A9, 30 μM; A1, A2, A3, A5, A7, 29 μM; A4, 27 μM; U1, U4, U7, U8, U9, 25 μM; E2, U2, U3, U5, U6, 5 μM. Assay Medium containing the test chemical was renewed after 24 hours. The assay was terminated 48 hours after initiation of exposure by chemical lysis of the cells (100 μl lysis buffer, Promega Corp. Madison, WI, USA). Luciferase activity was measured using a Dynatech ML3000 plate reading luminometer and calibrated to standard solutions of firefly luciferase dissolved in lysis buffer supplemented with 5 mg/ml BSA. Luciferase activity was normalized to total soluble protein measured by the coomassie blue dye binding assay. The EC50 for induction (or inhibition) of luciferase expression was calculated with a non-linear regression algorithm[14]. Note that the more potent inducer (inhibitor) of luciferase expression had a lower EC50. A compound was considered "not active" if there was no significant treatment effect (ANOVA, $\alpha = 0.2$).

The inhibition of specific binding of 2,4,6,7-^{3}H-17ß-estradiol to a cytosolic preparation of calf uterine estrogen receptor by the hydroxy-CB's was measured[15]. The IC50 for inhibition was calculated with a non-linear regression algorithm[14]. Note that a compound with a higher affinity for ER had a lower IC50. A compound was considered "not active" if there was less than a 15% reduction in specific binding at the highest concentration tested. Four concentrations were tested (except for 17ß-estradiol in which 8 concentrations were tested) such that the concentrations were 1/5, 1/10, and 1/50 of the highest concentration tested. The highest concentration tested for each compound was: A6, 37 μM; A8, A9, 30 μM; A1, A2, A3, A5, A7, 29 μM; A4, 27 μM; U1, U4, U7, U8, U9, 25 μM; E2, U2, U3, U5, U6, 5 μM.

RESULTS

All but two compounds tested caused a measurable reduction in specific binding of radio-labelled 17ß-estradiol to ER (Table 1). These two compounds were CB-75 and 4-hydroxy-3,5,3',4',5'-pentaCB. Five hydroxy-CB's caused a concentration dependent increase in estrogen-inducible luciferase expression. Eleven hydroxy-CB's caused a concentration dependent decrease (anti-estrogenic) in estrogen-inducible luciferase expression. Eight of nine confirmed PCB metabolites (A1-A8) were anti-estrogenic. The ninth CB metabolite, 4-hydroxy-3,5,2',3',4'-pentaCB (A9) had no effect on luciferase expression. There was no significant correlation, omitting 17ß-estradiol, between ER binding inhibition and induction of luciferase expression (Figure 1, Spearman rank correlation; $r_s = -0.257$, $p > 0.05$, $n = 6$). Similarly, there was no significant correlation between ER binding inhibition and reduction in luciferase expression (Figure 2, Spearman rank correlation; $r_s = 0.027$, $p > 0.2$, $n = 11$).

DISCUSSION

Sixteen of seventeen hydroxy-CB's caused a significant change in estrogen-inducible luciferase expression. However, the most potent estrogenic CB tested was CB-75. Broadly speaking, two factors determine the (anti)estrogenic potency of a compound: 1) the concentration of the compound in the cell at the receptor; 2) the binding affinity of the compound for the target receptor. Of the four hydroxy-CB's with the lowest ER binding inhibition IC50's, three were estrogenic. However, their luciferase induction EC50's were high suggesting low estrogenic potency. To explain the low estrogenic potency in light of the strong ER binding potential, it can be hypothesized that the concentration of these three hydroxy-CB's in the cell at the receptor was reduced by some combination of factors including low plasma membrane permeability or conjugation. Hydroxy-CB's are expected to be more polar and less lipophilic than their non-hydroxylated parent compounds. The higher potency of CB-75 may be explained, in part, by a greater ability to cross the cell membrane with subsequent metabolism to the suspected hydroxylated estrogenic form. The anti-estrogenicity of the hydroxy-CB's cannot be explained solely by their ER binding inhibition potential (Figure 2). Aryl hydrocarbon receptor (AhR) agonists can cause significant alterations in 17ß-estradiol metabolism in MCF-7 cells[16] as well as changes in ER[6]. Also, the anti-estrogenic activity of several AhR agonists has been correlated with increased metabolism and concomitant reduction in the concentration of 17ß-estradiol in the growth medium of MCF-7 cells[8]. Although the AhR agonist activity (EROD induction, UDPGT induction) of the hydroxy-CB's has not been determined, a hypothesis to be tested in future research based on these results states that a subset of hydroxy-CB's display anti-estrogenicity by altering the metabolism and reducing the concentration of 17ß-estradiol in MCF-7 cells.

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Table 1 Summary of calf uterine ER binding inhibition and MCF-7 cell estrogen-inducible luciferase expression.

Compound		ER Binding Inhibition		Estrogen-Inducible Luciferase Expression	
ID	Formula	IC50[a] (nM)	RBII[b]	Response[c]	EC50[d] (nM)
E2	17ß-estradiol	7.3	1.0	↑ $p<0.001$	1.6
U2	4-OH-2',3',4',5'-tetraCB	160	0.044	↑ $p<0.2$	7.0×10^{4} [g]
U1	4-OH-2',4',6'-triCB	550	0.013	↑ $p<0.01$	5.4×10^{5} [g]
U3	3-OH-2',3',4',5'-tetraCB	2800	2.6×10^{-3}	↓ $p<0.05$	3800
U7	4-OH-2,2',5'-triCB	4900	1.5×10^{-3}	↑ $p<0.2$	2.8×10^{9} [g]
A6	4-OH-3,3',4'-triCB	7400	9.8×10^{-4}	↓ $p<0.05$	NC[e]
A2	4-OH-2,3,5,3',4'-pentaCB	1.6×10^{4}	4.6×10^{-4}	↓ $p<0.001$	2300
A1	3-OH-4,5,2',4',5'-pentaCB	2.0×10^{4}	3.6×10^{-4}	↓ $p<0.001$	3700
U8	3-OH-6-monoCB	2.4×10^{4}	3.0×10^{-4}	↑ $p<0.2$	6.1×10^{6} [g]
A7	2-OH-3,4,2',3',4'-pentaCB	2.7×10^{4}	2.7×10^{-4}	↓ $p<0.001$	1.0×10^{4}
A3	3-OH-4,5,2',3',4'-pentaCB	2.9×10^{4}	2.5×10^{-4}	↓ $p<0.001$	1900
U5	2-OH-5,2',3',4',5'-pentaCB	3.8×10^{4}	1.9×10^{-4}	↓ $p<0.01$	2500
A5	4-OH-3,5,2',4',5'-pentaCB	4.2×10^{4}	1.7×10^{-4}	↓ $p<0.01$	7.0×10^{4} [g]
U4	4,4'-diOH-3,5,3',5'-tetraCB	7.4×10^{4} [g]	9.8×10^{-5}	↑ $p<0.001$	3.4×10^{6} [g]
U6	2-OH-3,2',3',4',5'-pentaCB	8.3×10^{4} [g]	8.7×10^{-5}	↓ $p<0.001$	1900
A9	4-OH-3,5,2',3',4'-pentaCB	4.5×10^{5} [g]	1.6×10^{-5}	$p>0.2$	NA[f]
A4	4-OH-3,5,2',3',4',5'-hexaCB	1.2×10^{11} [g]	6.0×10^{-11}	↓ $p<0.001$	1900
A8	4-OH-3,5,3',4',5'-pentaCB	NA[f]	0	↓ $p<0.1$	8500
U9	2,4,6,4'-tetraCB (CB-75)	NA[f]	0	↑ $p<0.05$	470

a IC50 = Concentration causing 50% inhibition of specific binding of ^{3}H-17ß-estradiol.
b RBII = Relative Binding Inhibition Index = $IC50_{E2}/IC50$. If IC50 = NA then RBII = 0.
c Response = ↑ = induction of basal expression; ↓ = reduction of basal expression.
d EC50 = Concentration causing 50% change in expression of luciferase.
e NC = Not calculated, non-linear regression algorithm failed to converge.
f NA = Not active, compound did not cause a significant reduction in specific binding or expression.
g Prediction extrapolated beyond highest concentration tested.

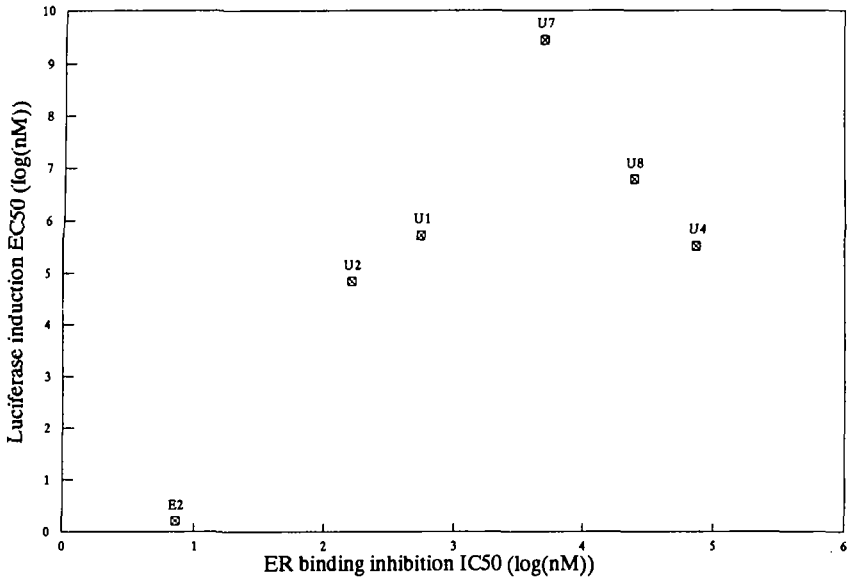

Figure 1. Relationship between calf uterine ER binding inhibition and induction of MCF-7 luciferase expression. Note log-log scale. Not shown: U9. See Table 1 for compound ID's.

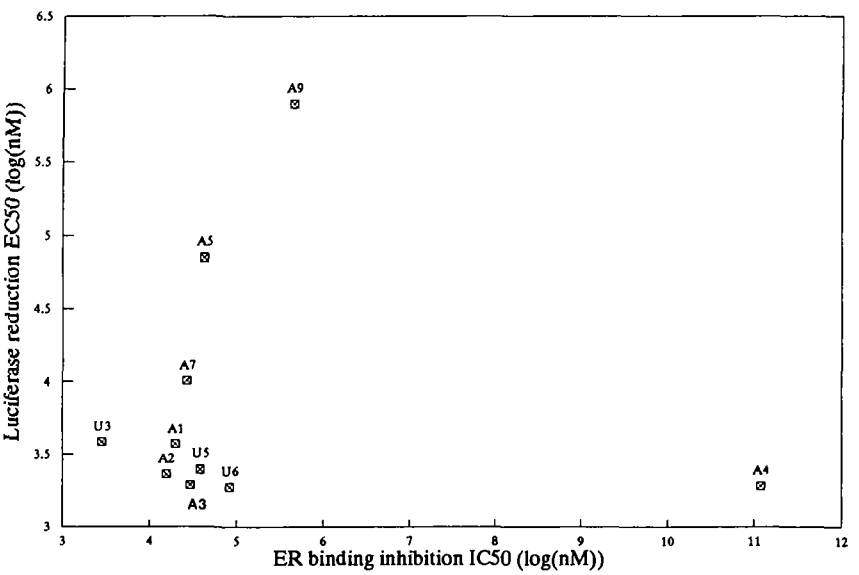

Figure 2. Relationship between calf uterine ER binding inhibition and reduction of MCF-7 luciferase expression. Note log-log scale. Not shown: A6, A8.