

Research article

Inhibition of E2-induced expression of *BRCA1* by persistent organochlorines

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Abstract

Background: Environmental persistent organochlorines (POCs) biomagnify in the food chain, and the chemicals are suspected of being involved in a broad range of human malignancies. It is speculated that some POCs that can interfere with estrogen receptor-mediated responses are involved in the initiation and progression of human breast cancer. The tumor suppressor gene *BRCA1* plays a role in cell-cycle control, in DNA repair, and in genomic stability, and it is often downregulated in sporadic mammary cancers. The aim of the present study was to elucidate whether POCs have the potential to alter the expression of *BRCA1*.

Methods: Using human breast cancer cell lines MCF-7 and MDA-MB-231, the effect on *BRCA1* expression of chemicals belonging to different classes of organochlorine chemicals (the pesticide toxaphene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and three polychlorinated biphenyls [PCB#138, PCB#153 and PCB#180]) was measured by a reporter gene construct carrying 267 bp of the *BRCA1* promoter. A twofold concentration range was analyzed in MCF-7, and the results were supported by northern blot analysis of *BRCA1* mRNA using the highest concentrations of the chemicals.

Results: All three polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin reduced 17 β -estradiol (E2)-induced expression as well as basal reporter gene expression in both cell lines, whereas northern blot analysis only revealed a downregulation of E2-induced *BRCA1* mRNA expression in MCF-7 cells. Toxaphene, like E2, induced *BRCA1* expression in MCF-7.

Conclusion: The present study shows that some POCs have the capability to alter the expression of the tumor suppressor gene *BRCA1* without affecting the cell-cycle control protein p21^{Waf/Cip1}. Some POCs therefore have the potential to affect breast cancer risk.

Keywords: *BRCA1*, estrogen receptor, polychlorinated biphenyls, tetrachlorodibenzo-*p*-dioxin, toxaphene

Introduction

The incidence of breast cancer has been increasing steadily over the past 60 years [1], today affecting one in eight women in the United States [2]. Despite tremendous efforts to understand the disease, less than 50% of all cases are of known etiology such as genetic inheritance, first-degree relatives with breast cancer, and age of men-
struation and menopause [3].

A large group of lipophilic organochlorines are found to persist in the environment and to biomagnify through the food chain. This group includes pesticides, polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-dioxins/polychlorinated dibenzo-furans [4–8]. Many of these compounds can interfere with a wide range of hormonal responses [9–13], including estrogen receptor (ER)-mediated responses. Due to their lipophilic nature,

bp = base pair; DMEM = Dulbecco's modified Eagle's medium; E2 = 17 β -estradiol; ER = estrogen receptor; PCB = polychlorinated biphenyl; PCR = polymerase chain reaction; POC = persistent organochlorine; TCDD = 2,3,7,8 tetrachlorodibenzo-*p*-dioxin.

organochlorines accumulate in the adipose tissue, can cross the placenta and are found in the breast milk [14]. The chemicals give rise to life-long exposure and are supposed to disturb early development. There is, however, a great epidemiological disparity in the literature regarding whether an association exists between POCs and breast cancer [15,16].

The breast cancer susceptibility gene, *BRCA1* [17], is linked to familial breast cancer [18], and it is found to be downregulated in sporadic breast and ovarian cancers [19,20]. Decreased expression of *BRCA1* promotes cell proliferation and can cause cell transformation [20,21]. The function of *BRCA1* remains unknown, but strong evidence suggests a role in cell-cycle control and DNA repair [22,23]. Furthermore, *BRCA1* expression is regulated by 17 β -estradiol (E2) [24,25].

To address whether POCs could affect *BRCA1* expression, we studied the effect on *BRCA1* promoter activity using a transient reporter gene assay and the effect on the *BRCA1* mRNA level by northern blot analysis in two human breast cancer cell lines. Furthermore, the effect on the estrogen-responsive gene (*pS2*) was studied, as well the effect on the cell-cycle regulator (*p21^{Waf/Cip1}*), in order to shed some light on the mechanistic pathways involved.

Materials and methods

Chemicals

Toxaphene (technical mixture; Dr Ehrenstorfer, Augsburg, Germany), 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) (Wellington Laboratories Inc., Guelph, Ontario, Canada), PCB#138, PCB#153 and PCB#180 (Dr Ehrenstorfer), E2 (Sigma-Aldrich Denmark A/S, Copenhagen, Denmark), and ICI 182780 (AstraZeneca, Luton, UK) were used. All compounds were dissolved in ethanol except TCDD, which was dissolved in dimethyl sulfoxide. All concentrations used were nontoxic as revealed by the Cytotoxicity Assay (Promega Corporation, Madison, Wisconsin, USA) and by measurement of total protein (BCA protein reagent; Perbio Science, Helsingborg, Sweden).

Plasmids

The plasmid pGL12 contains 267 bp, spanning -224 to +43 bp of the *BRCA1* promoter- α , subcloned upstream of the *luciferase* gene [26]. The plasmid pHEO1 contains full-length human *ER α* cDNA [27]. The plasmid pON249 encodes β -galactosidase under control of a *CMV* promoter [28], and was used as the internal standard for transfection efficiencies.

Reporter gene assay

MCF-7(BUS) cells (ER responsive) and MDA-MB-231 cells (ER nonresponsive) were cultured as described elsewhere [9,29]. For experiments, 7.3×10^5 MCF-

7(BUS) cells or 5×10^5 MDA-MB-231 cells were seeded in six-well trays in DMEM (without phenol red) media containing charcoal-depleted fetal bovine serum (Hyclone Europe, Perbio Science, Erembodegem-Aalst, Belgium). MCF-7(BUS) cells were exposed to the given chemicals and transfected with 1.4 μ g pGL12 and 0.4 μ g pON249 using 5 μ l fugene (Roche, Mannheim, Germany). Cells were harvested in lysis buffer (Roche) 24 hours after exposure and transfection. Luciferase activity was measured using lysis buffer containing 0.5 mM luciferin (Amersham Pharmacia Biotech, Uppsala, Sweden) and 0.5 mM ATP (Sigma). The β -galactosidase activity was measured by a chemiluminescence kit (Roche). MDA-MB-231 cells were exposed and harvested as MCF-7(BUS) but were transfected with 1.5 μ g pGL12 using 6 μ l fugene for 48 hours. Cotransfection with ER was carried out with 0.5 μ g pHEO1.

Total RNA isolation

On day 1, 2.2×10^6 cells were seeded in 75 cm² culture flasks and grown for 24 hours in fetal calf serum containing media. The cells were starved in serum-free media on day 2 to obtain G₁-synchronization, and they were exposed to chemicals in charcoal-depleted fetal bovine serum media on day 3. Total RNA was harvested after 22 hours of chemical exposure (PUREScript; Gentra Systems, Minneapolis, Minnesota, USA).

Northern blot and hybridization

A *BRCA1* probe was produced by PCR on genomic DNA against exon 11 (5'-GAGAGGCATCCAGAAAAGTATC-AGG-3' and 5'-CTCTGGGAAAAGTATCGCTGTTCATG-3') of the *BRCA1* gene (882–2216 bp of *BRCA1* cDNA [17]). Twenty micrograms of total RNA was used for northern blot analysis (Zeta-probe membrane; Bio-Rad, Hercules, California, USA). The ³²P-labeled *BRCA1* probe was made by random primed labeling (Megaprime; Amersham Pharmacia Biotech), and was hybridized overnight at 42°C in 50% formamide (as described by the manufacturer). The *pS2*-specific probe and the internal control (the *GADPH* probe) were used as described elsewhere [13,29]. The blots were visualized by a PhosphorImager and quantified with ImageQuant (Molecular Dynamics Amersham Biosciences, Sunnyvale, California, USA).

p21^{Waf/Cip1} protein level

The p21^{Waf/Cip1} protein was quantified on 24 hour exposure of MCF-7(BUS) to the given chemicals using the Waf1 Elisa kit (Oncogene Research Products, San Diego, California, USA).

Statistics

Statistical analysis was carried out in the Microsoft Excel standard diagram for independent samples test. *P* values were calculated using the two-tailed *t*-test two sample, assuming unequal variances.

Results

Reporter gene assay

The dose–response effects of the chemicals on the *BRCA1* promoter–*luciferase* construct (pGL12) in MCF-7(BUS) are presented in Table 1. In MCF-7(BUS), TCDD, ICI 182780, and the PCBs all significantly reduced the basal (ethanol) and the E2-induced promoter activity at the maximum concentrations tested (TCDD, 100 nM; ICI 182780 and PCBs, 10 μM). However, 10 μM toxaphene significantly induced basal promoter activity. TCDD and the pure ER antagonist ICI 182780 had the potential to decrease basal and E2-induced promoter activity at 100 and 10 pM, respectively, while PCB#138 had the potential to decrease E2-induced promoter activity at 100 pM. All chemicals (TCDD, 10 nM; ICI 182780 and PCBs, 10 μM) significantly reduced the *BRCA1*-promoter activity in MDA-MB-231. Cotransfection of the ER into MDA-MB-231 showed that the *BRCA1* promoter is indeed induced by E2 (Table 1); however, only twofold induction occurred compared with the fivefold induction in MCF-7(BUS).

Northern blot analysis

To assess whether the findings in the reporter gene assay are also valid for genomic *BRCA1* mRNA expression, northern blot analysis was carried out to measure the effect on the *BRCA1* mRNA level with each compound at 10 μM, except for TCDD at 10 nM and ICI 182780 at 100 nM. Figure 1 shows a PhosphorImager image of *BRCA1*, *pS2* and *GADPH* hybridizations to a northern blot. The analyzed data for *BRCA1* are presented in Fig. 2. E2 induced *BRCA1* mRNA expression twofold, and all compounds except toxaphene significantly decreased the E2-induced *BRCA1* expression in MCF-7. Toxaphene increased the basal *BRCA1* mRNA expression, while ICI 182780 downregulated basal and E2-induced expression. No effect of the chemicals on *BRCA1* mRNA expression was measured in the ER non-responsive MDA-MB-231 cells.

To further address whether ER mediates the observed deregulation of *BRCA1* mRNA, expression of the ER responsive gene *pS2* was measured in parallel (Fig. 3a). A 2.5-fold induction of *pS2* by E2 was observed, and a twofold induction by toxaphene confirmed its ER agonistic behavior. ICI 182780 significantly decreased basal and E2-induced *pS2* expression, whereas TCDD significantly reduced E2-induced *pS2* mRNA expression. The three PCBs did not significantly affect the *pS2* expression.

p21^{Waf/Cip1} study

To address whether the observed response could be a direct consequence of changes in cell-cycle regulation, we determined the effect of the POCs on the negative cell-cycle regulator p21^{Waf/Cip1}. As shown in Fig. 3b, the POCs generally did not affect the p21^{Waf/Cip1} protein level. Toxaphene, however, decreased the basal p21^{Waf/Cip1}

level like E2. Similar to ICI 182780, TCDD elicited an anti-estrogenic effect on cotreatment with E2.

Discussion

BRCA1 mRNA expression is frequently downregulated in sporadic breast and ovarian cancers [19,20]. This altered regulation is generally not correlated with mutations within the gene [30]. The present study demonstrated that POCs *in vitro* had the potential to deregulate *BRCA1* promoter activity and mRNA expression in human breast cancer cell lines. Using an E2-responsive construct carrying the key positive regulatory element of the *BRCA1* promoter upstream of the *luciferase* gene [26,31], we found that TCDD, PCB#138, PCB#153, and PCB#180 can downregulate the basal as well as the E2-induced *BRCA1* promoter activity in both the ER-positive cell line (MCF-7(BUS)) and in the ER-negative cell line (MDA-MB-231). However, toxaphene induced basal promoter activity (Table 1).

The inhibiting effect of TCDD and the three PCBs were confirmed by northern blot analysis on the E2-induced *BRCA1* mRNA level in MCF-7 (Fig. 2). E2 induced the *BRCA1* mRNA level twofold, and the observed estrogen-like activity of toxaphene supports earlier findings [32,33]. At concentrations of 10 μM, the three PCBs had the capacity to downregulate both basal and E2-induced *BRCA1* promoter activity, while they only decreased the E2-induced *BRCA1* mRNA level. Furthermore, the PCBs antagonized E2-induced *pS2* mRNA expression, but it was not statistically significant (Fig. 3a). The anti-estrogenic potential of the three PCBs mediated via the ER, causing an antiproliferative effect in MCF-7, has previously been published by our laboratory [9].

Jeffy *et al.* found *BRCA1* to be downregulated by benzo[a]pyrene in an ER-dependent fashion, probably through an aryl hydrocarbon receptor-mediated pathway [34]. Using RT-PCR, however, Jeffy *et al.* did not observe TCDD to downregulate *BRCA1* in MCF-7. In the present study, TCDD significantly reduced both *BRCA1* promoter activity as well as E2-induced *BRCA1* (Fig. 2) and *pS2* mRNA expression in MCF-7 (Fig. 3a; see [13]).

All the POCs except toxaphene significantly decreased E2-induced *BRCA1* mRNA expression in MCF-7. No effect was measured on the mRNA level in MDA-MB-231 (Fig. 2), which indicates the mechanism of action to be via the ER. In MDA-MB-231, however, the POCs also decreased the *BRCA1* promoter activity, suggesting that other pathways than the ER-mediated pathway should be considered. We have shown that the three PCBs in MCF-7(BUS) are antiproliferative and anti-estrogenic at 10 μM [9], as has also been reported for TCDD [34], whereas toxaphene has been shown to increase MCF-7 proliferation [33,35]. In the present study, the negative cell-cycle

Table 1

Effect of persistent organochlorines on transient *BRCA1-luciferase* (pGL12) reporter gene expression in MCF-7(BUS) and MDA-MB-231

MCF-7(BUS)	Tox.Tech.	Tox.Tech. + 100 pM E2	ICI 182780	ICI 182780 + 100 pM E2	TCDD	TCDD + 100 pM E2
Control	18 ± 2.9	100 ± 11.8	18 ± 0.3	100 ± 6.3	18 ± 0.8	100 ± 8.4
10 µM	36 ± 8.3*	59 ± 8.6*	5 ± 1.1*	8 ± 3.4*	n.d.	n.d.
1 µM	20 ± 2.5	96 ± 25.2	7 ± 0.5*	7 ± 0.6*	n.d.	n.d.
100 nM	20 ± 6.8	102 ± 16.8	5 ± 0.4*	5 ± 1.7*	7 ± 0.9*	46 ± 4.6*
10 nM	24 ± 8.1	95 ± 7.4	5 ± 0.7*	8 ± 2.4*	7 ± 0.8*	33 ± 2.6*
1 nM	23 ± 8.1	88 ± 21.8	5 ± 0.7*	46 ± 10.8*	8 ± 1.3*	39 ± 3.5*
100 pM	24 ± 9.3	108 ± 11.4	9 ± 0.3*	81 ± 5.0*	14 ± 2.5*	66 ± 12.6*
10 pM	17 ± 1.7	112 ± 4.2	12 ± 0.8*	109 ± 25.1	16 ± 4.0	110 ± 24.1
1 pM	21 ± 3.4	92 ± 18.9	15 ± 2.3	101 ± 55.8	25 ± 6.7	107 ± 13.8

MCF-7(BUS)	PCB#138	PCB#138 + 100 pM E2	PCB#153	PCB#153 + 100 pM E2	PCB#180	PCB#180 + 100 pM E2
Control	18 ± 3.0	100 ± 7.8	18 ± 2.1	100 ± 6.9	18 ± 1.7	100 ± 12.7
10 µM	7 ± 1.2*	59 ± 14.9*	8 ± 0.7*	75 ± 9.0*	9 ± 3.5*	70 ± 11.5*
1 µM	17 ± 1.5	81 ± 19.5	16 ± 3.5	92 ± 17.2	13 ± 4.0	105 ± 11.7
100 nM	16 ± 1.7	90 ± 4.9*	21 ± 0.9	97 ± 12.9	16 ± 1.4	110 ± 24.7
10 nM	17 ± 3.9	82 ± 10.5*	22 ± 4.4	113 ± 10.6	19 ± 5.4	107 ± 28.9
1 nM	19 ± 3.0	87 ± 4.0*	20 ± 5.1	131 ± 28.9	23 ± 7.9	94 ± 10.2
100 pM	18 ± 4.1	88 ± 6.5*	21 ± 6.4	95 ± 16.5	17 ± 6.4	108 ± 31.1
10 pM	19 ± 2.2	85 ± 21.2	22 ± 0.9	112 ± 26.7	21 ± 7.4	112 ± 11.3
1 pM	24 ± 5.3	86 ± 20.3	19 ± 6.2	111 ± 9.5	16 ± 5.0	109 ± 21.2

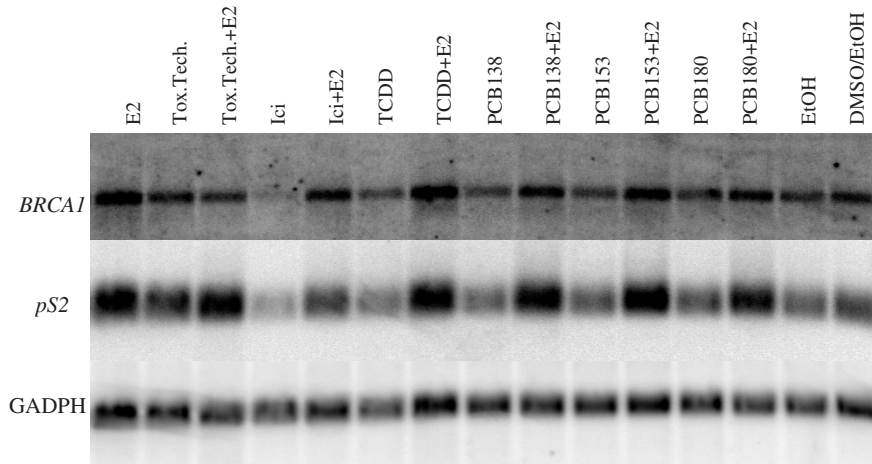
MDA-MB-231	Control	Tox.Tech. (10 µM)	TCDD (10 nM)	PCB#138 (10 µM)	PCB#153 (10 µM)	PCB#180 (10 µM)
pGL12	93 ± 19.3	21 ± 8.1*	65 ± 11.5*	35 ± 1.3*	50 ± 10.6*	49 ± 7.0*
pGL12 + 10 nM E2	100 ± 15.0	21 ± 6.6*	69 ± 8.6*	32 ± 6.9*	53 ± 6.0*	44 ± 5.6*
pGL12/ER	57 ± 10.4	35 ± 11.6*	55 ± 15.5	45 ± 10.6	46 ± 12.8	48 ± 13.5
pGL12/ER + 10 nM E2	100 ± 13.8	37 ± 21.3*	74 ± 35.8	65 ± 23.9*	60 ± 24.5*	58 ± 36.5

All values are corrected with β -galactosidase values (MCF-7(BUS)) or protein (MDA-MB-231) and then normalized to 17 β -estradiol (E2)-induced cells (set to 100). The data are mean \pm standard deviations of at least five or six measurements from two independent experiments. pGL12/ER, cotransfection with the pHEO1-expressing estrogen receptor alpha; Tox.Tech., toxaphene (technical mixture); TCDD, 2,3,7,8 tetrachlorodibenzo-p-dioxin; PCB, polychlorinated biphenyl. * $P < 0.05$ relative to the given control (ethanol/DMSO or E2).

regulator p21^{Waf/Cip1} was not affected by the PCBs, while the effect of E2 was as expected [36] and toxaphene decreased the p21^{Waf/Cip1} protein level. ICI 182780 and TCDD increased the E2 reduced level to its normal level (Fig. 3b). This suggests that the deregulation of *BRCA1* observed in this study is not a simple consequence of altered cell-cycle regulation. Since the reporter gene assay also showed an effect of the POCs in MDA-MB-231,

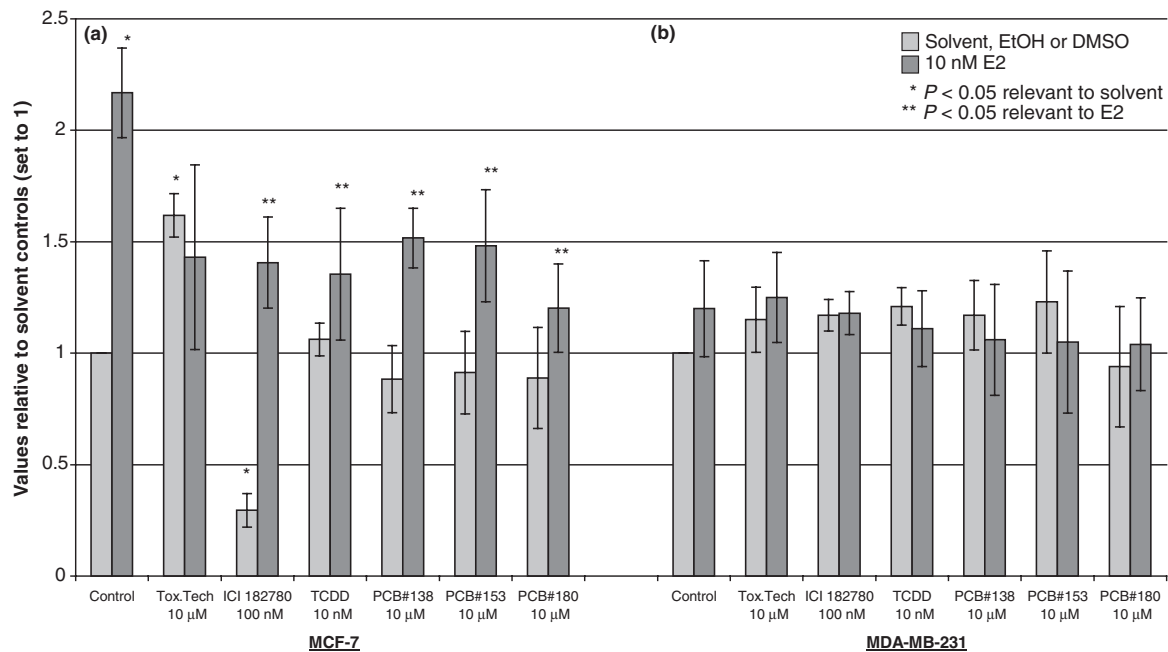
further research is needed to elucidate the pathways of the *BRCA1* deregulation as well as the involvement of other regulatory sites outside of the 267 bp key regulatory element of the *BRCA1* promoter cloned in the pGL-12 vector. In addition, a possible effect on *BRCA1* expression of the chemicals in combination and in a mimic of the concentrations found in the adipose tissue should be addressed.

Figure 1



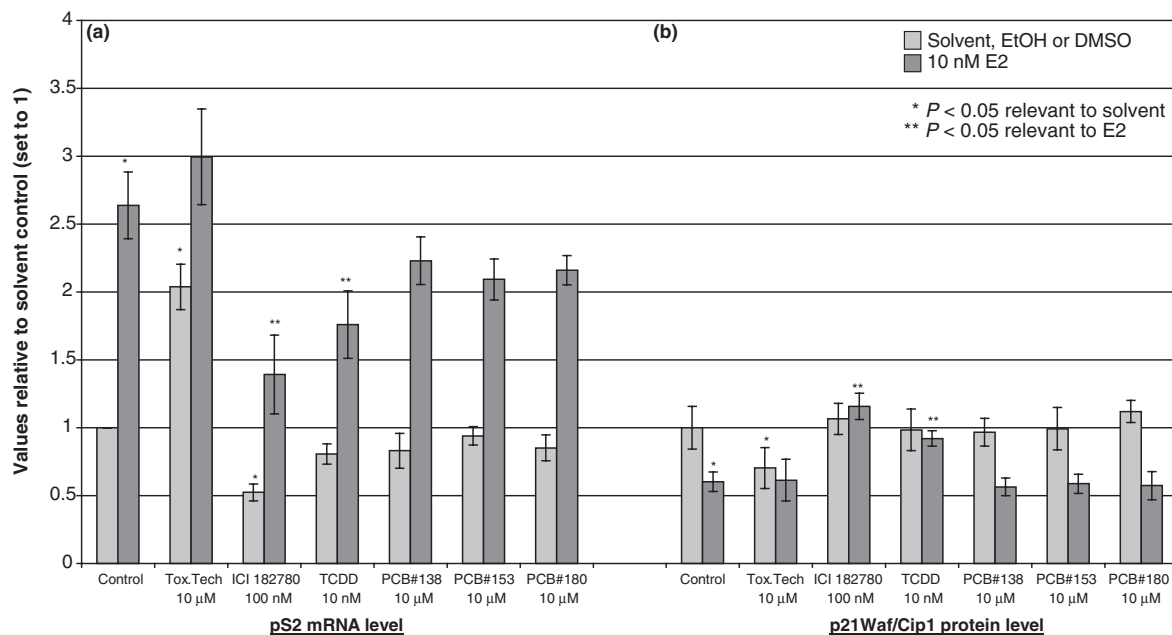
PhosphorImager images of hybridizations with *BRCA1* (7.8 kb), *pS2* (500 bp), and *GADPH* (1.3 kb) probes against the same northern blot membrane with 20 µg/lane total RNA from MCF-7. Concentrations of the chemicals are as shown in Fig. 2. The filter represents only one experiment. The collective data are presented in Figs 2 and 3. After hybridization with *BRCA1*, the filter was stripped and rehybridized with *pS2* and then with *GADPH*. MDA-MB-231 data are not shown. E2, 17β-estradiol; Tox.Tech., toxaphene (technical mixture); Ici, ICI 182780; TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin; PCB, polychlorinated biphenyl; EtOH, ethanol; DMSO, dimethyl sulfoxide.

Figure 2



Effect of persistent organochlorines on *BRCA1* mRNA expression in (a) MCF-7 and (b) MDA-MB-231. All values are normalized to the internal control (*GADPH*) and then related to the solvent control (set to 1). Dark bars indicate cotreatment of 17β-estradiol and the given compound. * $P < 0.05$ compared with solvent-treated cells, ** $P < 0.05$ compared with 17β-estradiol (10 nM)-exposed cells. Data are presented as mean ± standard deviation of at least three independent experiments. E2, 17β-estradiol; Tox.Tech., toxaphene (technical mixture); TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin; PCB, polychlorinated biphenyl; EtOH, ethanol; DMSO, dimethyl sulfoxide.

Figure 3



Effect of persistent organochlorines on (a) *pS2* mRNA expression in MCF-7 and (b) *p21^{Waf1/Cip1}* in MCF-7(BUS). The *pS2* data are normalized to *GADPH* and the related to solvent control (set to 1). Dark bars indicate cotreatment of 17β-estradiol and the given compound. * $P < 0.05$ compared with solvent-treated cells, ** $P < 0.05$ compared with 17β-estradiol (10 nM)-exposed cells. Data are presented as mean \pm standard deviation of at least three independent experiments (*pS2*) or two independent experiments assayed in duplicate (*p21^{Waf1/Cip1}*). E2, 17β-estradiol; Tox.Tech., toxaphene (technical mixture); TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin; PCB, polychlorinated biphenyl; EtOH, ethanol; DMSO, dimethyl sulfoxide.

Conclusion

It was found that TCDD, PCB#138, PCB#153, and PCB#180 decreased E2-induced *BRCA1* promoter activity and mRNA expression in human breast cancer cell lines. Toxaphene induced the basal *BRCA1* expression, probably via inducing cell proliferation since it was found to downregulate the cell-cycle protein *p21^{Waf1/Cip1}*. The presented data show that the ER is likely to play an essential role in the deregulation of *BRCA1*, but the data indicate a possible involvement of other pathways as well. The effect of these POCs on *BRCA1* expression may, however, for a large part be a consequence of their anti-estrogenic, and hence antiproliferative, effect. By deregulating *BRCA1*, therefore, as has been found in sporadic breast cancers, the POCs could impair the DNA repair machinery, the cell-cycle control and the stress-induced apoptosis [37]. POCs consequently could affect the overall risk of breast cancer.

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