



Estrogenic Activity of Persistent Organic Pollutants and Parabens Based on the Stably Transfected Human Estrogen Receptor- α Transcriptional Activation Assay (OECD TG 455)

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Screening of estrogenic activity on dichloro diphenyl trichloroethane (DDT), dichloro diphenyl dichloro ethylene (DDE), dieldrin, heptachlor, aldrin, chlordane, lindane, polybrominated diphenyl ethers (PBDE) and parabens was compared using Organization for Economic Cooperation and Development (OECD) test guideline 455 (TG455). The estrogenic activity of DDT was 58,000-fold (PC_{50} , 1.67×10^{-6} M) less than 17β -estradiol(E_2) (PC_{50} , 2.88×10^{-11} M) but DDE, dieldrin, heptachlor, aldrin, chlordane, lindane and PBDE did not show any estrogenic activity in this assay system. In the case of paraben compounds, the rank of relative transcriptional activation (logRTA) was butyl paraben -1.63752 (PC_{50} , 1.25×10^{-7} M) > isobutyl paraben -2.34008 (PC_{50} , 6.3×10^{-7} M) > ethyl paraben -2.64016 (PC_{50} , 1.26×10^{-6} M) > isopropyl paraben -2.73993 (PC_{50} , 1.58×10^{-6} M) > propyl paraben -2.84164 (PC_{50} , 2.0×10^{-6} M). Our data suggest that OECD test guideline TG455 may be useful as a screening tool for potential endocrine disruptors.

Key words: Estrogenic activity, Endocrine disruptors, OECD test guideline 455, DDT, Parabens

INTRODUCTION

Endocrine disruptors (EDCs) may be interfered the endocrine system in wildlife and humans reproduction (Colborn *et al.*, 1993; Vos *et al.*, 2000; Bonefeld-Jorgensen *et al.*, 2004). They mimic, compete, block or alter the activities of endogenous hormones. Previous studies have been reported that endocrine disruptors interrupt interactions of ligand-receptor binding that act as receptor agonist or antagonist (Hunter *et al.*, 1999; Dandimopoulos *et al.*, 2008). For rapid alarming health and environmental consequences involved fast and reliable approaches are needed. Based on these situation, the Organization for Economic Cooperation and Development (OECD) conceptual framework for testing and assessment of man-made compounds comprised five levels and *in vitro* screening methods for alternative animal study ranked as level 2 (OECD, 2003). Recently, the stably transfected transcriptional activation assay (STTA)

developed by Chemicals Evaluation and Research Institute (CERI) have been accepted as the OECD test guideline 455 (TG455) in 2009 (OECD, 2009). The STTA assay is to evaluate the ability of chemicals to function as an estrogen receptor alpha ($ER\alpha$) ligand and activate an $ER\alpha$ agonistic responses.

Here, we validated the estrogenic activity of persistent organic pollutants DDT, DDE, dieldrin, heptachlor, aldrin, chlordane, lindane, PBDE) and parabens using STTA assay. Previous studies have been indicated that these compounds showed weak or potent estrogenic activity using *in vitro* and *in vivo* screening assay systems (Okubo *et al.*, 2001; Vo and Jeung *et al.*, 2009).

MATERIALS AND METHODS

Cell line and cell culture conditions. The h $ER\alpha$ -HeLa-9903 cell line (HeLa9903) which is stably transfected a human $ER\alpha$ gene containing a firefly luciferase gene as a reporter gene was provided from the CERI. The experiments were performed with 5~28 passages of the cells, which were seeded at 1×10^4 cells per well on 96 well plates and incubated cells for 3 hr at 37°C with 5% CO_2 . The

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reagents of cell culture used commercial products except Dextran-coated charcoal treated fetal bovine serum (DCC-FBS, provided from CERI). The cells were maintained and tested in 10% DCC-FBS-EMEM including 7.5% NaHCO₃, 4 mM L-glutamine and 10% DCC-FBS.

Test chemicals. Reference chemicals: 10 mM 17 α -estradiol (Sigma, St. Louis, MO, USA), 10 mM 17 β -estradiol (E₂, Sigma, St. Louis, USA), and 100 mM corticosterone (Wako, Japan) were prepared in dimethyl sulfoxide (DMSO, Sigma) as the stock solution. Test chemicals: dichloro diphenyl trichloroethane (DDT, Supelco, St. Louis), dichloro diphenyl dichloro ethylene (DDE, Sigma, St. Louis, USA), dieldrin (Chem Service, USA), heptachlor (Supelco, St. Louis, USA), aldrin (Chem Service, USA), chlordane (Chem Service, USA), lindane (Supelco, St. Louis, USA), polybrominated diphenyl ethers (PBDE, Chem Service, USA), butyl paraben (Sigma, St. Louis, USA), ethyl paraben (Aldrich, St. Louis, USA), propyl paraben (Aldrich, St. Louis, USA), isobutyl paraben (Wako, Japan), isopropyl paraben (Wako, Japan) were obtained in commercially available. All test chemicals were prepared at log-serial dilutions (10⁻⁵~10⁻¹⁰ M) with DMSO.

Chemical treatment and determine the luciferase activity. The luciferase activities were determined in accordance with OECD TG 455. Cells were exposed with reference chemicals and serially diluted test chemicals, and then were incubated for 20~24 hrs (at 37°C 5% CO₂). The media on the cell plates were removed then added 50l of luciferase solution (Steady-Glo Luciferase Assay kit, Promega) and the activities were measured by a Luminometer (US Microplate Scintillation and Luminescence Counter, Packard).

Determine of relative transcriptional activation (RTA). The fold induction of E₂ (1 nM), at least 4-fold greater than the mean vehicle control (VC), and the values of the PC₅₀ (17 α -estradiol, corticosterone and test compounds) were obtained from provided spreadsheet using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). The RTA value of the estrogen activities were obtained from ratios of PC₅₀ of test chemicals with PC₅₀ of E₂.

RESULTS AND DISCUSSION

Estrogenic activities of persistent organic pollutants by STTA. The estrogenic activities of persistent organic pollutants (DDT, DDE, dieldrin, heptachlor, aldrin, chlordane, lindane, PBDE) were determined by STTA assay. In the validation study for this assay system, the quality control and standard curve were coincident with criteria TG455 (Table 1 and Fig. 1). In this study, DDT showed a weak estrogenic activity which was 58,000-fold lower than that of

Table 1. Performance and quality control criteria for the STTA assay

Reference chemicals	Acceptable criteria Log[PC ₅₀ M]	Result
17 β -estradiol (E ₂)	-11.4~-10.1	-10.54~
17 α -estradiol (α E ₂)	-9.6~-8.1	-8.64~
Corticosterone*	-	-

*Corticosterone is used for negative control.

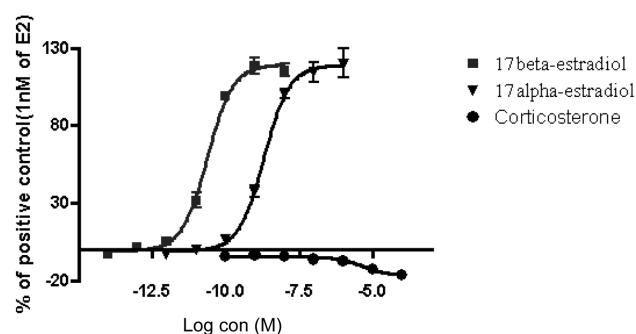


Fig. 1. The final concentration of each well for reference chemicals. E₂ and α E₂, positive control; cor, negative control.

Table 2. PC₅₀ and logRTA values for STTA assay (persistent organic pollutants)

Test chemicals	STTA assay	
	PC ₅₀ (M)	logRTA*
17 β -estradiol	2.88 × 10 ⁻¹¹	2
17 α -estradiol	3.0 × 10 ⁻⁹	-0.01773
Dichloro diphenyl trichloroethane (DDT)	1.67 × 10 ⁻⁶	-2.76447
Dichloro diphenyl dichloro ethylene (DDE)	Negative	Negative
Dieldrin	Negative	Negative
Heptachlor	Negative	Negative
Aldrin	Negative	Negative
Chlordane	Negative	Negative
Lindane	Negative	Negative
Polybrominated diphenyl ethers (PBDE)	Negative	Negative

*Relative transcriptional activation is calculated as 100 × (PC₅₀) of E₂ / (PC₅₀) of test compound; a value of 100 indicates that the compound tested is a full agonist.

Abbreviations: RTA, Relative transcriptional activation; PC₅₀, the concentration of chemical estimated to cause 50% of activity of the positive control response on a plate by plate basis.

E₂. However, DDE, dieldrin, heptachlor, aldrin, chlordane, lindane and PBDE did not show any estrogenic activity (Table 2 and Fig. 2). As showed in Table 4, previous studies indicated that the relative estrogenic activity of DDT against E₂ detected by 3 different assay systems (Coldham *et al.*, 1997; Soto *et al.*, 1995). DDT showed higher relative estrogenic activity in STTA assay as compare with that of Yeast and E-screen assays.

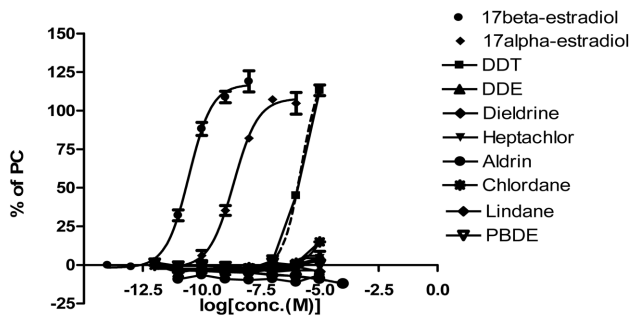


Fig. 2. The final concentration of each well for persistent organic pollutants and reference chemicals.

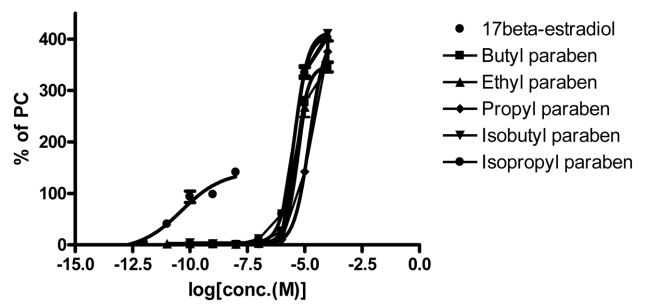


Fig. 3. The final concentration of each well for parabens and reference chemicals.

Table 3. PC₅₀ and logRTA values for STTA assay (parabens)

Test chemicals	STTA assay	
	PC ₅₀ (M)	logRTA ^a
17β-estradiol	2.88 × 10 ⁻¹¹	2
17α-estradiol	3.0 × 10 ⁻⁹	-0.01773
Butyl paraben	1.25 × 10 ⁻⁷	-1.63752
Ethyl paraben	1.26 × 10 ⁻⁶	-2.64016
Propyl paraben	2.0 × 10 ⁻⁶	-2.84164
Isobutyl paraben	6.3 × 10 ⁻⁷	-2.34008
Isopropyl paraben	1.58 × 10 ⁻⁶	-2.73993

^aRelative transcriptional activation is calculated as 100 × (PC₅₀) of E₂/(PC₅₀) of test compound; a value of 100 indicates that the compound tested is a full agonist.

Abbreviations: RTA, Relative transcriptional activation; PC₅₀, the concentration of chemical estimated to cause 50% of activity of the positive control response on a plate by plate basis.

Estrogenic activities of parabens by STTA. The estrogenic activities of parabens (butyl-, ethyl-, propyl-, isobutyl-, and isopropyl- paraben) were determined by STTA assay. Butyl-, ethyl-, propyl-, isobutyl- and isopropyl paraben showed lower estrogenic activity than E₂ (Table 3 and Fig. 3). The rank of logRTA on the parabens was butyl paraben -1.63752 (PC₅₀, 1.25 × 10⁻⁷ M) > isobutyl paraben -2.34008 (PC₅₀, 6.3 × 10⁻⁷ M) > ethyl paraben -2.64016 (PC₅₀, 1.26 × 10⁻⁶ M) > isopropyl paraben -2.73993 (PC₅₀, 1.58 × 10⁻⁶ M) > propyl paraben -2.84164 (PC₅₀, 2.0 × 10⁻⁶ M). Butyl-, isobutyl-, ethyl-, isopropyl-, and propyl paraben

Table 5. Comparison of *in vitro* assay for parabens

Test chemicals	MCF-7 cell proliferation assay	STTA assay
	Okubo ^a logRPP	This study logRTA
17β-estradiol	2	2
Butyl paraben	-3.82	-1.64
Ethyl paraben	-3.82	-2.64
Propyl paraben	-3.82	-2.84
Isobutyl paraben	-2.22	-2.34
Isopropyl paraben	-2.22	-2.74

^a: data from Okubo *et al.* (2001).

Abbreviations: E₂, 17β-estradiol; RPP, relative proliferative potency is the ratio between 17β-estradiol and xenoestrogen doses needed to produce maximal cell yields ×100; RTA, Relative transcriptional activation.

were shown weak estrogenic activities which were approximately 4,300-fold, 22,000-fold, 44,000-fold, 55,000-fold, 69,000-fold lower than E₂. Butyl-, ethyl- and propyl paraben were all estrogenic in a yeast-based estrogen assay, with the most potent butyl paraben being 10,000 times less potent than E₂ (Routledge *et al.*, 1998; Terasaki *et al.*, 2009). The relative estrogenic activity of parabens against E₂ using 2 different assays were indicated in Table 5. Okubo *et al.* (2001) had shown that the relative estrogenic activity of parabens using MCF-7 cell proliferation assays. Butyl paraben, ethyl paraben and propyl paraben showed higher

Table 4. Comparison of *in vitro* assay for dichloro diphenyl trichloroethane (DDT)

Test chemicals	Yeast assay	E-screen assay	STTA assay
	Coldham ^a logRP	Soto ^b logRPP	This study logRTA
17β- estradiol	2	2	2
Dichloro diphenyl trichloroethane (DDT)	-4.52	-4	-2.76

^a: data from Coldham *et al.* (1997), ^b: data from Soto *et al.* (1995).

Abbreviations: E₂, 17β-estradiol; RP, relative potency compared to E₂ (100) by molar mass determined with the recombinant yeast bioassay; RPP, relative proliferative potency is the ratio between 17β-estradiol and xenoestrogen doses needed to produce maximal cell yields ×100; RTA, Relative transcriptional activation.

relative estrogenic activity in STTA assay as compare with that of MCF-7 cell proliferation assay.

In conclusion, STTA assay is useful and corresponds with other *in vitro* studies based on relative estrogenic activity. Our present results indicate that DDT and parabens (butyl-, ethyl-, propyl-, isobutyl- and isopropyl- paraben) act as weak estrogen agonist by human ER α mediated transcriptional activation.

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