Supplementary Information

New insights in the endocrine disruption effects of three primary metabolites of organophosphate flame retardants

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Materials and Methods

S.1 Cells culture and plasmids

The rat estrogen receptor (*r*ER) expression plasmid *rERα/pCI* and the response element *pERE-AUG-Luc+* were kindly provided by Dr. M. Takeyoshi (Chemicals Assessment Center, Chemicals Evaluation and Research Institute, Oita, Japan). The human mineralocorticoid receptor (*h*MR) expression plasmid *EGFP-C1-hMR* was kindly provided by Dr. Claudia Großmann (Julius Bernstein Institute for Physiology, Martin Luther University, Halle, Germany) and Dr. Jing Liu (Zhejiang University, Hangzhou, China). The plasmid *pRL-TK* (Promega, Madison, WI) was used as an internal control in the dual-luciferase reporter gene assay.

S.2. Dual-luciferase reporter gene assay for ER and MR

The plasmids proportions in each well to detect ER, MR activity are as follows:

ER activity: *rERα/pCI* 25 ng + *pERE-AUG-Luc+* 145 ng + *pRL-TK* 10 ng;

MR activity: *EGFP-C1-hMR* 20 ng + *pMMTV-luc* 160 ng + *pRL-TK* 10 ng;

Table S1 Information on three metabolites of organophosphate flame retardants

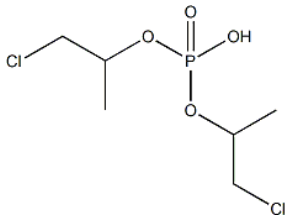
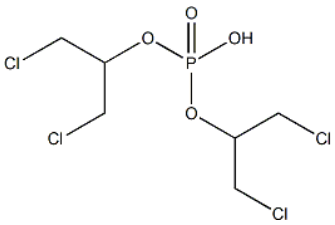
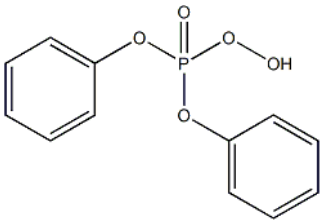
chemicals and abbreviation	CAS No.	Chemical structure
Bis (1-chloro-2-propyl) phosphate (BCIPP)	789440-10-4	
Bis (1,3-dichloro-2-propyl) phosphate (BDCIPP)	72236-72-7	
Diphenyl phosphate (DPHP)	838-85-7	

Table S2. The primer sequences used for qRT-PCR of zebrafish

Genes	Primers sequence (5'-3')	Accession number
β-actin	F: CTGCTATGTGGCTCTTGACT R: CAGGTCCTTACGGATGTCG	EF597101.1
ERα	F: TGGGCTAAGAAAGTACCA R: CTTGAGTTTGAGACTGCGGAAT	HM045496.1
VTG	F: AGGGAGTATGCAGGACC R: CTCAGTGTATGCCAAACCAAT	JN004056.1
CYP17	F: AGGGACCCGAGTCATTAT R: GGGCAGCACAAACCATCAC	JN858107.1
Cyp19a	F: GGCTACAAAGTGAAG R: GAACGGCTGAAAGAAACGAC	GU220394.1
Cyp19b	F: CCTGGTGACCCTGTTGT R: GTGGCTGTCCGTCGTTTCT	GU220393.1
StAR	F: CTGAGAATGGACCCACCTGT R: GCAATAAACGTCAGCAAGCA	NM_131663
HMGRA	F: GAGCCATCGACTCTCTCCTG R: GAACACGACTGCTAGCACCA	BC155135
MR	F: ATTGGGCCTAGTGCAAAATG R: TCTCTGTTTGGCTCGGTCTT	EF567113
POMC	F: AGGTCGACTATCCGCAAGAA R: CAACCTCTCCCCCTTAAAGC	AY158003
CRH	F: TTCGGGAAGTAACCACAAGC R: CTGCACTCTATTCGCCTTCC	NM_001007379
3βHSD	F: AGAGACCCGGAGAAAAGAGC R: GGGTGGAGTGAATCTCAGGA	AY279108
CRHR2	F: AAAGATGCTGGTTTGGGAAG R: CCCAGTAAAGGCAGAAGCAC	XM_681362

Table S3. The prime sequence of detected genes of H295R cells.

Genes	Primers sequence (5'-3')	Accession number
CYP11A1	F: GAGATGGCACGCAACCTGAAG R: CTTAGTGTCTCCTTGATGCTGGC	NM_001099773
CYP11B1	F: GGTTTGCCAGGCTAAGC R: CAAACTGCCCAGAGGACAG	NM_000497
CYP11B2	F: TCCAGGTGTGTTCAGTAGTTCC R: GAAGCCATCTCTGAGGTCTGTG	NM_000498
CYP17	F: GGCACCAAGACTACAGTGATTGG R: AGAGTCAGCGAAGGCGATAC	M14564
CYP19	F: GCCAGTGAGGAGCAGGAC R: TTGGAAATGCTGAACCCGATAC	M74714.1
CYP21	F: ACCTCAGTTTCTCCTTTATTGC R: AGAGCCAGGGTCCTTCAC	NM_000500
GAPDH	F: GAAGGTGAAGGTCGGAGT R: GAAGATGGTGATGGGATTTC	NC_000012
HMGR	F: TTCAGGTTCCAATGGCAACAAC R: GCCACGAGTCATCCCATCTG	NM_000859
3βHSD	F: AGCATCTTCTGTTTCCTGGCA R: TCTCCTTCAGTTCCTTCTCTTC	NM_000198
17βHSD	F: AAGACTTGCTTGCTGTGG R: TTCATGGAGAAGGTGTTGG	KF742611
STAR	F: ATGAGTAAAGTGGTCCCAGATG R: ACCTTGATCTCCTTGACATTGG	NM_000349

Table S4 The fold changes of 10 steroidogenic genes in H295R cells exposed to various concentrations of BCIPP, BDCIPP and DPHP for 24 h (*$p < 0.05$ compared with the value of DMSO control).

Chemicals	Genes	*3βHSD*	*CYP11A1*	*CYP11B1*	*CYP11B2*	*CYP17*
	Control	1.00±0.14	1.00±0.16	1.00±0.17	1.00±0.38	1.00±0.09
	10^{-5} M	1.15±0.55	0.82±0.12*	3.13±0.70*	1.91±0.61*	1.22±0.55
BCIPP	**10^{-6} M**	0.96±0.25	0.94±0.20	2.07±1.13*	1.69±0.64*	0.98±0.25
	10^{-7} M	0.98±0.13	0.92±0.12	1.48±0.51*	1.24±0.21	0.98±0.17
	10^{-5} M	0.73±0.33*	0.83±0.07*	1.85±0.20*	1.28±0.36	1.01±0.08
BDCIPP	**10^{-6} M**	0.88±0.29	0.95±0.06	1.26±0.19*	1.14±0.29	0.93±0.09
	10^{-7} M	1.04±0.03	0.90±0.12	1.15±0.25	1.08±0.19	0.92±0.07
	10^{-5} M	1.22±0.27*	0.92±0.19	1.35±0.11*	1.71±0.30*	0.99±0.19
DPHP	**10^{-6} M**	1.16±0.12*	0.89±0.22	1.28±0.22*	1.29±0.14*	1.04±0.12
	10^{-7} M	1.12±0.33	0.93±0.32	1.08±0.29	1.09±0.18	0.97±0.33
Chemicals	**Genes**	***CYP21***	***HMGR***	***STAR***	***17βHSD***	***CYP19***
	Control	1.00±0.09	1.00±0.11	1.00±0.11	1.00±0.07	1.00±0.06
	10^{-5} M	0.48±0.08*	1.03±0.15	0.73±0.23*	1.65±0.25*	1.53±0.79*
BCIPP	**10^{-6} M**	0.66±0.18*	1.12±0.32	0.77±0.14*	1.34±0.42*	1.23±0.20*
	10^{-7} M	1.13±0.19	0.97±0.11	0.82±0.14*	1.19±0.27	1.06±0.18
	10^{-5} M	1.01±0.19	0.93±0.08	0.81±0.11*	1.40±0.38*	1.17±0.10*
BDCIPP	**10^{-6} M**	1.04±0.13	0.96±0.09	0.84±0.14*	1.33±0.15*	0.98±0.08
	10^{-7} M	1.03±0.12	0.95±0.17	0.87±0.06*	1.28±0.04*	0.95±0.10
	10^{-5} M	1.09±0.16	0.99±0.30	0.92±0.15	1.55±0.37*	0.97±0.21
DPHP	**10^{-6} M**	0.98±0.34	1.16±0.18	1.11±0.15	1.21±0.18*	1.11±0.08
	10^{-7} M	1.01±0.34	1.05±0.27	1.01±0.25	1.09±0.23	1.12±0.38

Table S5 Docking scores of BCIPP, BDCIPP, DPHP for ER and MR.

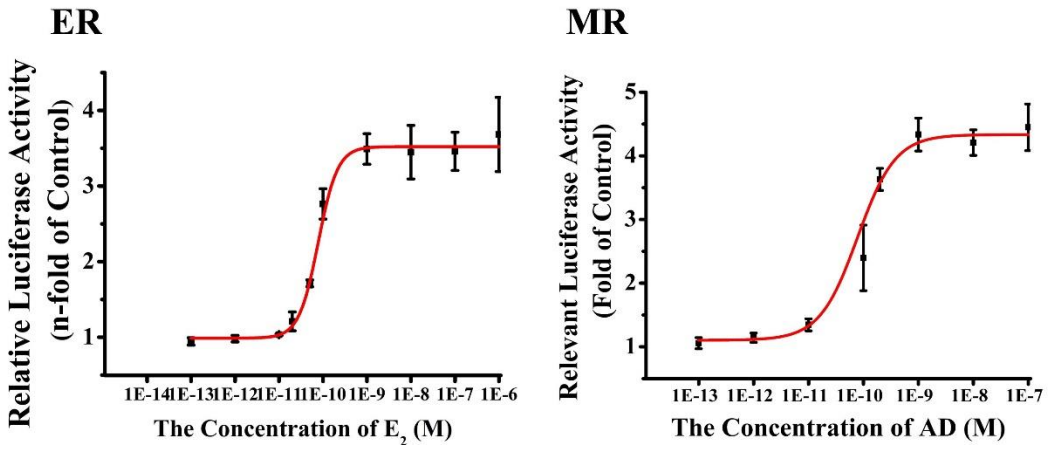
Compounds	**BCIPP**	**BDCIPP**	**DPHP**
Score for ER	-8.7	-7.3	-6.9
Score for MR	-7.6	-8.8	-6.7

Table S6 The fold changes of 5 genes of hypothalamic-pituitary-gonadal (HPG) axis in zebrafish exposed to various concentrations of BCIPP, BDCIPP and DPHP for 96 h (*$p < 0.05$ compared with the value of DMSO control).

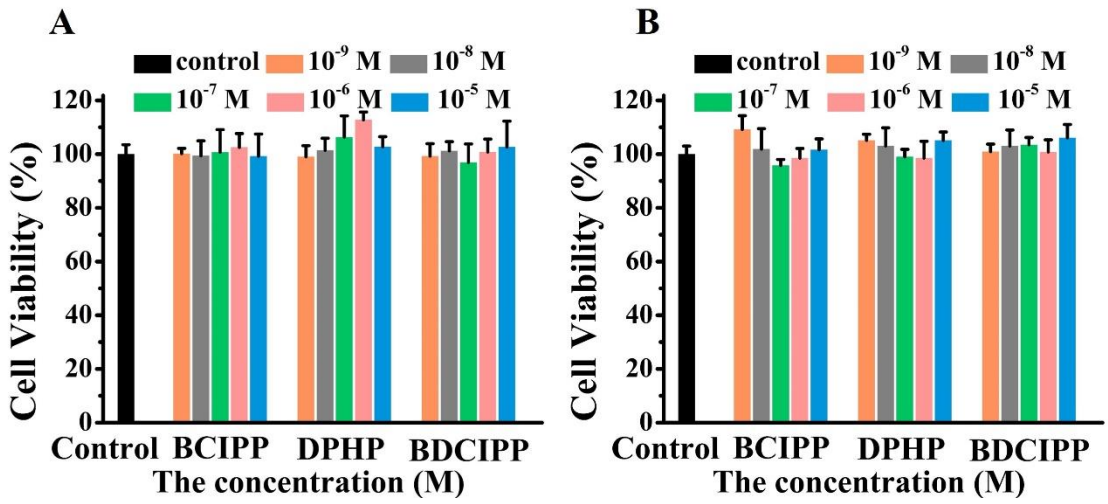
Chemicals	Genes	*ERα*	*VTG*	*CYP17*	*Cyp19a*	*Cyp19b*
	Control	1.00±0.06	1.00±0.06	1.00±0.06	1.00±0.41	1.00±0.05
	10^{-5} M	3.61±0.77*	3.66±0.44*	7.14±0.71*	11.14±0.98*	15.54±3.37*
BCIPP	**10^{-6} M**	1.29±0.07*	2.12±0.57	5.80±2.03*	1.54±0.004	1.70±0.09*
	10^{-7} M	0.79±0.41	1.73±0.48	0.71±0.23	0.95±0.19	0.98±0.14
	10^{-5} M	0.20±0.17*	10.79±5.03*	9.02±0.28*	0.97±0.16	5.27±0.55*
BDCIPP	**10^{-6} M**	0.58±0.23*	2.26±0.48*	2.90±1.33*	0.94±0.09	2.67±0.31*
	10^{-7} M	0.65±0.29	1.78±0.65	0.22±0.16*	0.82±0.40	2.69±0.22*
	10^{-5} M	0.04±0.006*	2.07±0.14*	0.25±0.03*	0.83±0.29	0.88±0.16
DPHP	**10^{-6} M**	0.83±0.77	1.71±0.57	0.33±0.07*	0.92±0.14	1.20±0.25
	10^{-7} M	0.75±0.44	1.78±0.30	0.39±0.05*	1.44±0.29	1.77±0.16

Table S7 The fold changes of 8 genes of hypothalamic-pituitary-adrenal (HPA) axis in zebrafish exposed to various concentrations of BCIPP, BDCIPP and DPHP for 96 h (*$p < 0.05$ compared with the value of DMSO control).

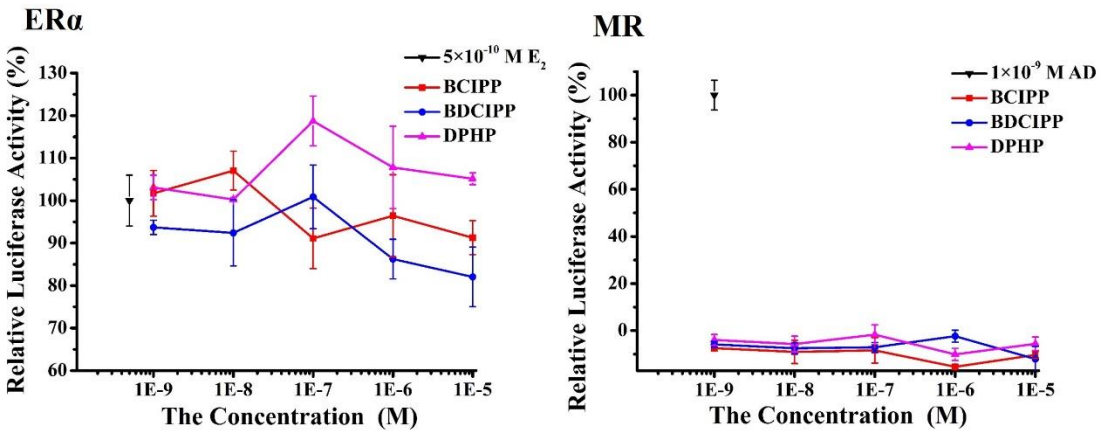
Chemicals \ Genes		*GR*	*MR*	*STAR*	*3βHSD*
	Control	1.00±0.10	1.00±0.02	1.00±0.24	1.00±0.15
BCIPP	**10^{-5} M**	3.07±0.36*	3.67±0.89*	1.50±0.63	3.89±0.32*
	10^{-6} M	0.79±0.04	1.64±0.32	0.76±0.01	1.51±0.35
	10^{-7} M	0.69±0.04	0.86±0.23	1.55±0.19	0.98±0.04
BDCIPP	**10^{-5} M**	0.56±0.02*	0.47±0.05*	0.72±0.06	2.10±0.48
	10^{-6} M	0.87±0.24	0.47±0.25*	0.73±0.30	2.16±0.21
	10^{-7} M	0.97±0.20	0.48±0.11*	0.78±0.11	2.06±0.49
DPHP	**10^{-5} M**	3.68±0.08*	2.17±0.13*	0.22±0.02*	2.82±0.17*
	10^{-6} M	0.89±0.08	1.64±0.30	1.93±0.74	2.13±0.34
	10^{-7} M	1.32±0.18	2.00±0.50	1.65±0.52	1.18±0.39
Chemicals \ Genes		***CRH***	***CRHR2***	***HMGRA***	***POMC***
	Control	1.00±0.06	1.00±0.30	1.00±0.18	1.00±0.37
BCIPP	**10^{-5} M**	4.02±1.08*	1.45±0.05	1.14±0.66	0.64±0.14
	10^{-6} M	1.38±0.50	0.81±0.15	1.13±0.17	0.67±0.20
	10^{-7} M	1.15±0.37	0.84±0.08	0.90±0.02	1.42±0.34
BDCIPP	**10^{-5} M**	2.57±0.36*	0.80±0.10	0.70±0.10	0.72±0.13
	10^{-6} M	0.96±0.03	1.48±0.35	1.30±0.11	0.83±0.13
	10^{-7} M	1.15±0.20	0.74±0.20	0.89±0.15	0.99±0.22
DPHP	**10^{-5} M**	2.24±0.12*	0.93±0.26	0.83±0.20	0.84±0.23
	10^{-6} M	1.48±0.03*	0.82±0.09	1.56±0.20	0.92±0.13
	10^{-7} M	1.03±0.61	1.55±0.21	1.00±0.18	1.00±0.37



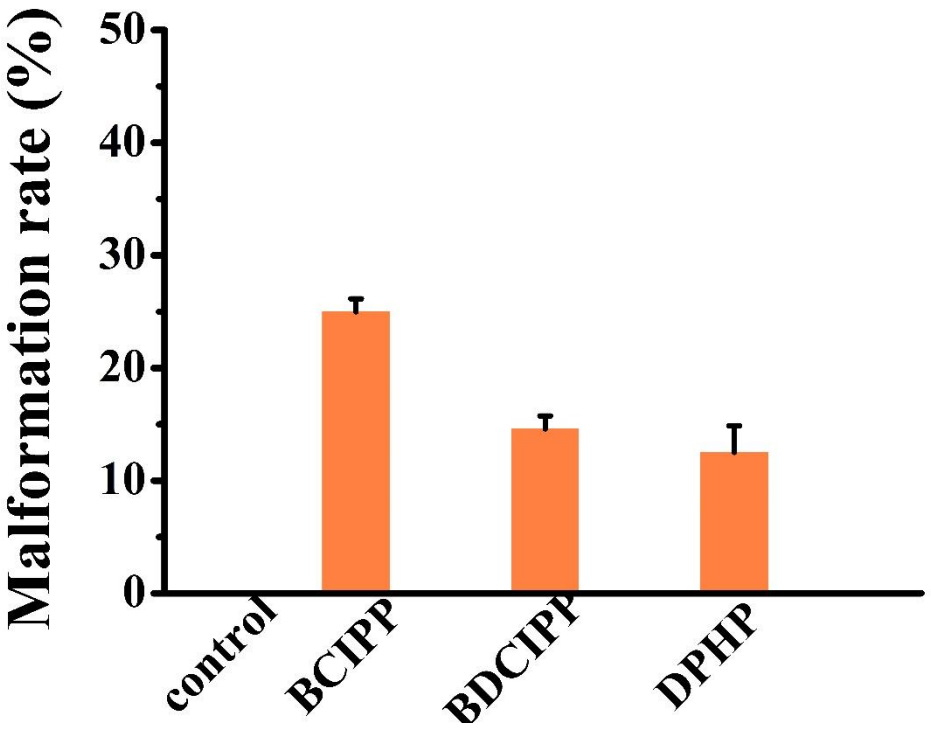
Figures S1 Dose response curves of E_2 and AD obtained from the ER and MR transactivation assays. *Firefly* luciferase activity was normalized based on the *Renilla* luciferase activity. Results were presented as the fold of control. Each value represents the mean ± SD of at least three independent experiments (n=3 samples, $*p<0.05$). The dose response curves were fitted by logistic model.



Figures S2 The cytotoxicity of three metabolites of organophosphate flame retardants examined in MTS assay. A: the cytotoxicity of CHO cells, B: the cytotoxicity of H295R cells.



Figures S3 The antagonist activities of the three OPFRs metabolites to ERα and agonist activities to MR by dual-luciferase reporter gene assay. *Firefly* luciferase activity was normalized based on the *Renilla* luciferase activity. The positive control was defined as 100%. Results were presented as the percentage quotient of the response of metabolites to positive control. Each value represents the mean ± SD of at least three independent experiments (n=3 samples, **p*<0.05).



Figures S4 Proportion of short tail (st) of zebrafish larvae exposed to three metabolites of organophosphate flame retardants at 10^{-5} M (96hpf). The results are expressed as mean ± SD of three replicates.