Supplemental Material

Hydroxylated Polybrominated Biphenyl Ethers Exert Estrogenic Effects via Non-Genomic G Protein-Coupled Estrogen Receptor Mediated Pathways

Lin-Ying Cao, Xiao-Min Ren, Yu Yang, Bin Wan, Liang-Hong Guo, De Chen, and Yong Fan

Table of Contents

The full name of the 12 PBDEs and 18 OH-PBDEs

Figure S1. Scheme of synthesis of E2-F. The abbreviations: Pd(OAc)$_2$, palladium acetate; PPh$_3$, triphenylphosphine; Cul, copper iodide; (C$_2$H$_5$)$_2$NH, diethylamine; HCl, hydrochloric acid; C$_2$H$_5$OH, ethanol; NHS-Fluorescein, 5-carboxyfluorescein succinimidyl ester; DMSO, dimethylsulfoxide.

Figure S2. Analysis of E2-F by high performance liquid chromatography (HPLC).

Figure S3. Analysis of E2-F by mass spectra (MS).

Figure S4. Analysis of E2-F by proton nuclear magnetic resonance spectra ($^1$H-NMR).

Figure S5. Expression of G protein-coupled estrogen receptor (GPER) protein in SKBR3 and HEK293 cells as detecting using a Western blotting assay.
Figure S6. Establishment of the SKBR3 cell-based fluorescence competitive binding method using E2-F probe. (A) The binding curves of E2-F or fluorescein to SKBR3 or control HEK293 cells, the inset means the binding curve at low concentrations from 5 nM to 100 nM. (B) The binding of E2-F to SKBR3 or HEK293 cells relative to fluorescein. The relative fluorescence means the fluorescence of cells binding with E2-F substrate the fluorescence of cells binding with fluorescein. (C) Determination of the FITC fluorescence signals of E2-F or fluorescein incubated SKBR3 and HEK293 cells by FCM. Red: SK+E2-F, SKBR3 cells incubated with 50 nM E2-F; purple: SK+fluorescein, SKBR3 cells incubated with 50 nM fluorescein; blue: HEK+E2-F, HEK293 cells incubated with 50 nM E2-F; yellow: HEK+ fluorescein, SKBR3 cells incubated with 50 nM fluorescein; green: SK+E2-F+E2 means SKBR3 cells incubated with 50 nM E2-F and 25 μM E2. (D) The corresponding fluorescence intensity of SKBR3 and HEK293 cells with treatment as indicated in Figure S6 C. (E) Determination the binding affinities of α-E2, G1 and G15. The maximum specific binding of 50 nM E2-F was determined in the presence of 25 μM E2. (F) Binding of 50 nM E2-F to control HEK293 cells in the presence of various concentrations of E2. (G) Binding of 50 nM E2-F to control HEK293 cells in the presence of the highest concentration of OH-PBDEs (10 μM for 3'-OH-BDE-007, 3'-OH-BDE-028, 5'-OH-BDE-099, 3'-OH-BDE-154, 6-OH-BDE-180, 4-OH-BDE-187, 4'-OH-BDE-201, and 20 μM for 2'-OH-BDE-003, 3-OH-BDE-047, 4'-OH-BDE-049, 3-OH-BDE-100) based on the results of the SKBR3 cells-based competition experiments. (H) Binding of 50 nM fluorescein to SKBR3 cells in the presence of the highest concentration of OH-PBDEs based on the results of the SKBR3 cells-based competition experiments.

Figure S7. The competition curves of PBDEs and OH-PBDEs with GPER determined by the SKBR3 cell-based fluorescence competitive binding assay.

Figure S8. Docking simulation for the interactions of G1, G15, α-E2, PBDEs, and OH-PBDEs with GPER. The hydrogen bond is represented by green dotted line.

Figure S9. Effects of G1 on calcium mobilization, cAMP production and cell migration of SKBR3 cells. (A) Calcium mobilization induced by G1 (0.1–1000 nM) in the absence or presence of G15 (1000 nM). (B) The cAMP production induced by G1 (0.1–1000 nM) in the absence or presence of G15 (1000 nM). (C) Effects of G1 (0.1–1000 nM) on SKBR3 cell migration determined by wound-healing assay in the absence or presence of G15 (1000 nM). The relative cell migration was quantified by setting the wound area change of control group (without treatment of G1 or G15) as 100%. (D) Effects of G1 (0.1–1000 nM) on SKBR3 cell migration determined by Boyden chamber assay in the absence or presence of G15 (1000 nM). The relative migrated cells was calculated by setting the migrated cells induced by control group (without treatment of G1 or G15) as 100%.

Figure S10. Effects of different concentrations of OH-PBDEs and E2 on the calcium mobilization in SKBR3 cells.

Figure S11. Effects of different concentrations of OH-PBDEs and E2 on the cAMP production in SKBR3 cells.
The full name of the 12 PBDEs and 18 OH-PBDEs

The twelve PBDEs include 4-bromodiphenyl ether (BDE-003), 2,4-dibromodiphenyl ether (BDE-007), 2,4,4'-tri-bromodiphenyl ether (BDE-028), 2,2',4,4'-tetrabromodiphenyl ether (BDE-047), 2,2',4,5'-tetrabromo diphenyl ether (BDE-049), 2,2',3,4,4'-pentabromodiphenyl ether (BDE-085), 2,2',4,4',5-pentabromodiphenyl ether (BDE-099), 2,2',4,4',6-pentabromodiphenyl ether (BDE-100), 2,4,4',5,6'-hexabromodiphenyl ether (BDE-154), 2,2',3,4',5,5',6-heptabromodiphenyl ether (BDE-187), 2,2',3,4',5,5',6-heptabromodiphenyl ether (BDE-180), and 2,2',3,3',4,5,6,6'-octabromodiphenyl ether (BDE-201).

The eighteen OH-PBDEs include 2'-hydroxy-4-monobromodiphenyl ether (2'-OH-BDE-003), 3'-hydroxy-2,4-dibromodiphenyl ether (3'-OH-BDE-007), 2'-hydroxy-2,4-dibromodiphenyl ether (2'-OH-BDE-007), 3'-hydroxy-2,4,4'-tribromodiphenyl ether (3'-OH-BDE-028), 2'-hydroxy-2,4,4'-tribromodiphenyl ether (2'-OH-BDE-028), 3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether (3-OH-BDE-047), 5-hydroxy-2,2',4,4'-tetrabromodiphenyl ether (5-OH-BDE-047), 6-hydroxy-2,2',4,4'-tetrabromodiphenyl ether (6-OH-BDE-047), 4'-hydroxy-2,2',4,5'-tetrabromodiphenyl ether (4'-OH-BDE-049), 6-hydroxy-2,2',3,4,4'-pentabromodiphenyl ether (6-OH-BDE-085), 6'-hydroxy-2,2',4,4',5-pentabromodiphenyl ether (6'-OH-BDE-099), 5'-hydroxy-2,2',4,4',5-pentabromodiphenyl ether (5'-OH-BDE-099), 5'-hydroxy-2,2',4,4',6-pentabromodiphenyl ether (5'-OH-BDE-100), 3-hydroxy-2,2',4,4',6-pentabromodiphenyl ether (3-OH-BDE-100), 3'-hydroxy-2,2',4,4',5,6'-hexabromodiphenyl ether (3'-OH-BDE-154), 4-hydroxy-2,2',3,4,5,5'-heptabromodiphenyl ether (4-OH-BDE-187), 6-hydroxy-2,2',3,4,4',5,5'-heptabromodiphenyl ether (6-OH-BDE-180), and 4'-hydroxy-2,2',3,3',4,5',6,6'-octabromodiphenyl ether (4'-OH-BDE-201).
Figure S1. Scheme of synthesis of E₂-F. The abbreviations: Pd(OAc)$_2$, palladium acetate; PPh$_3$, triphenylphosphine; CuI, copper iodide; (C$_2$H$_5$)$_2$NH, diethylamine; HCl, hydrochloric acid; C$_2$H$_5$OH, ethanol; NHS-Fluorescein, 5-carboxyfluorescein succinimidyl ester; DMSO, dimethylsulfoxide.
**Figure S2.** Analysis of $E_2$-F by high performance liquid chromatography (HPLC).
Figure S3. Analysis of E₂-F by mass spectra (MS).
Figure S4. Analysis of E2-F by proton nuclear magnetic resonance spectra ($^1$H-NMR).
Figure S5. Expression of G protein-coupled estrogen receptor (GPER) protein in SKBR3 and HEK293 cells as detecting using a Western blotting assay.
Figure S6. Establishment of the SKBR3 cell-based fluorescence competitive binding method using E2-F probe. (A) The binding curves of E2-F or fluorescein to SKBR3 or control HEK293 cells, the inset means the binding curve at low concentrations from 5 nM to 100 nM. (B) The binding of E2-F to SKBR3 or HEK293 cells relative to fluorescein. The relative fluorescence means the fluorescence of cells binding with E2-F substrate the fluorescence of cells binding with fluorescein. (C) Determination of the FITC fluorescence signals of E2-F or fluorescein incubated SKBR3 and HEK293 cells by FCM. Red: SK+E2-F, SKBR3 cells incubated with 50 nM E2-F; purple: SK+fluorescein, SKBR3 cells incubated with 50 nM fluorescein; blue: HEK+E2-F, HEK293 cells incubated with 50 nM E2-F; yellow: HEK+fluorescein, SKBR3 cells incubated with 50 nM fluorescein; green: SK+E2-F+E2 means SKBR3 cells incubated with 50 nM E2-F and 25 μM E2. (D) The corresponding fluorescence intensity of SKBR3 and HEK293 cells with treatment as indicated in Figure S6 C. (E) Determination the binding affinities of α-E2, G1 and G15. The maximum specific
binding of 50 nM E$_2$-F was determined in the presence of 25 μM E$_2$. (F) Binding of 50 nM E$_2$-F to control HEK293 cells in the presence of various concentrations of E$_2$. (G) Binding of 50 nM E$_2$-F to control HEK293 cells in the presence of the highest concentration of OH-PBDEs (10 μM for 3’-OH-BDE-007, 3’-OH-BDE-028, 5’-OH-BDE-099, 3’-OH-BDE-154, 6-OH-BDE-180, 4-OH-BDE-187, 4’-OH-BDE-201, and 20 μM for 2’-OH-BDE-003, 3-OH-BDE-047, 4’-OH-BDE-049, 3-OH-BDE-100) based on the results of the SKBR3 cells-based competition experiments. (H) Binding of 50 nM fluorescein to SKBR3 cells in the presence of the highest concentration of OH-PBDEs based on the results of the SKBR3 cells-based competition experiments.

The error bars represent the mean of 3 replicates ± SD.
Figure S7. The competition curves of PBDEs and OH-PBDEs with GPER determined by the SKBR3 cell-based fluorescence competitive binding assay.
Continued above figure

- 2'-OH-BDE-028
- BDE-047
- 3-OH-BDE-047
- 5-OH-BDE-047
- 6-OH-BDE-047
- BDE-049
- 4'-OH-BDE-049
- BDE-085
- 6-OH-BDE-085
- BDE-099
- 6'-OH-BDE-099
- BDE-100
Figure S8. Docking simulation for the interactions of G1, G15, α-E2, PBDEs, and OH-PBDEs with GPER. The hydrogen bond is represented by green dotted line.
Figure S9. Effects of G1 on calcium mobilization, cAMP production and cell migration of SKBR3 cells. (A) Calcium mobilization induced by G1 (0.1–1000 nM) in the absence or presence of G15 (1000 nM). (B) The cAMP production induced by G1 (0.1–1000 nM) in the absence or presence of G15 (1000 nM). (C) Effects of G1 (0.1–1000 nM) on SKBR3 cell migration determined by wound-healing assay in the absence or presence of G15 (1000 nM). The relative cell migration was quantified by setting the wound area change of control group (without treatment of G1 or G15) as 100%. (D) Effects of G1 (0.1–1000 nM) on SKBR3 cell migration determined by Boyden chamber assay in the absence or presence of G15 (1000 nM). The relative migrated cells was calculated by setting the migrated cells induced by control group (without treatment of G1 or G15) as 100%.

*p < 0.05, compared with the control group (Ctrl, 0.1% DMSO). #p < 0.05, compared with the groups treated with compounds in absence of G15.
Figure S10. Effects of different concentrations of OH-PBDEs and E$_2$ on the calcium mobilization in SKBR3 cells.

*p < 0.05, compared with the control group (concentration at 0 nM, 0.1% DMSO).
Figure S11. Effects of different concentrations of OH-PBDEs and E\textsubscript{2} on the cAMP production in SKBR3 cells.

\*\(p < 0.05\), compared with the control group (concentration at 0 nM, 0.1% DMSO).