Supporting Information

Chlorinated Polyfluoroalkylether Sulfonic Acids Exhibit Stronger Estrogenic Effects than Perfluorooctane Sulfonate by Activating Nuclear Estrogen Receptor Pathways

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The supporting information (17 pages) contains 6 figures and 3 tables.

Contents

Zebrafish Husbandry and Breeding. All male and female adult zebrafish were maintained on a 14 h:10 h light/dark cycle in an automatic circulation system at 28 °C, and fed twice daily with fresh Artemia nauplii. For breeding, two male zebrafish and two female zebrafish were independently housed in a breeding tank overnight. When the light was turned on the next morning, spawning occurred after removing the clapboard in the breeding tank. Zebrafish embryos of 2 h post fertilization (hpf) were collected and cleaned carefully, and examined according to the standard characteristics under an optical microscope (Olympus CKX31, Japan). The healthy fertilized embryos were manually selected and used in subsequent experiments.

Zebrafish Exposure and Sex Hormones Determination. In order to evaluate the effect of chemicals on the survival of zebrafish larvae and select suitable exposure concentrations, embryos were first exposed to Cl-PFESAs and PFOS to conduct the toxicity tests. In brief, healthy fertilized embryos of 2 hpf were randomly distributed into 12-well plates (Corning, USA) with 20 embryos per well, and exposed to a series concentrations (0, 0.4, 0.8 1.6, 3.1, 6.3, 12.5, and 25.0 μM) of 6:2 Cl-PFESA, 8:2 Cl-PFESA, and PFOS for 7 d, with the total volume of exposure solution in each well of 4 mL. All exposure experiments were performed in a light incubator with the conditions being the same as the culture conditions. The solution in each well was replaced daily with fresh test solution. The treatment with 0.1% (*v/v*) DMSO was employed as solvent control. Each exposure was carried out in triplicates (3 plates). Any dead embryos or larvae were removed daily and the number of survivors was

recorded every 24 h. The concentration-response curves for survival were created by OriginPro 8 soft-ware. The 168 h LD_{50} (50% lethal dosage) value for each compound was calculated from survival curves with a nonlinear curve fit.

On the basis of the results of toxicity tests, two non-lethal concentrations (0.1 μM and 0.5 μM) were selected for the following exposure experiments. Embryos were placed in 300 mL glass beakers (200 embryos/beaker), and then exposed to 6:2 Cl-PFESA, 8:2 Cl-PFESA, and PFOS for 7 d. All groups were replaced daily with fresh test solution. The treatments with 0.1% (*v/v*) DMSO and 10 nM E2 were employed as solvent control and positive control, respectively. Every exposure (168 hpf) was carried out in triplicates (3 plates). Exposed zebrafish larvae were collected for the determination of sex hormones and VTG. The procedures were same as described in our previous study.[1]

Histopathological Analysis. Four-month-old male and female zebrafish were acclimated for 2 week and separately placed in 6 L glass tanks (15 males/females per tank). Then, zebrafish were exposed to 0.5 μM 6:2 Cl-PFESA, 8:2 Cl-PFESA, and PFOS for 28 d. The groups treated with 0.1% (*v/v*) DMSO and 10 nM E2 were employed as solvent control and positive control, respectively. During exposure period, exposure solution of each group was renewed daily, and all zebrafish were fed with fresh Artemia nauplii twice every day.

The gonads collected in the exposed zebrafish were fixed in paraformaldehyde solution (4%, *w/v*) for 24 h at 4 °C. Then, all samples were dehydrated in ethanol, embedded in paraffin wax, sectioned (3 μm) and stained with hematoxylin and eosin

(HE). Each section was examined carefully under a light microscope.

Transcriptional Analysis of Estrogen-related Genes. Zebrafish embryos of 2 hpf were manually selected and placed in 6-well plates (40 embryos/well), and then exposed to 0.1 μM and 0.5 μM of 6:2 Cl-PFESA, 8:2 Cl-PFESA PFOS for 7 d in three replicates. Embryos exposed to 0.1% (*v/v*) DMSO and 10 nM E2 were set as solvent control and positive control, respectively. After exposure, forty zebrafish larvae of each group were collected and washed three times with PBS to eliminate residual test solution. Then, total RNA was manually extracted using TRIzol Reagent (Life Technologies, USA) following the manufacturer's instructions. The quantity and quality of extracted RNA were evaluated by using a NanoDrop spectrophotometer (Thermo Scientific, USA). The first-strand complementary DNA (cDNA) was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) was used for quantification of abundance of gene expression. Real-time PCR reactions were performed on a Roche 480 Real-Time PCR system in 96-well PCR plates (Roche, USA).

Transcriptional levels of five genes (including ERα, ERβ1, ERβ2, CYP19a, and CYP19b) in exposed zebrafish larvae were quantified as markers of estrogenic effect. β-actin was selected as the housekeeping gene in the quantitative PCR analysis because the expression of this gene was not affected after Cl-PFESAs exposure. All primers used in our study were referenced to the previous studies.[2] The specific primer sequences are listed in SI, Table S1. The fold change of target gene was

calculated by the $2^{-\Delta\Delta Ct}$ method.[3]

E-screen Assay. Estrogenic effects of Cl-PFESAs and PFOS were further detected using E-screen assay. Briefly, MCF-7 cells were seeded in 96-well plates (Corning, New York, USA) at a density of 4×10^3 cells per well and allowed to attach overnight. The growth medium was replaced with steroid-free DMEM medium containing 5% CS-FBS and 2 mM L-glutamine. Cells were then starved for 48 h under culture condition to make them estrogen responsive. Then, cells were treated with different concentrations (0, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25.0, and 50.0 μM) of 6:2 Cl-PFESA, 8:2 Cl-PFESA or PFOS with or without 1 μM fulvestrant (ICI 182780). All plates were further incubated for 5 d in the cell incubator. Cells treated with different concentrations of E2 were employed as positive control. Each exposure group was carried out in five replicates. Thereafter, 10 μL of MTT solution (5 mg/mL) was added to each well. After incubation for 4 h at 37 °C, the exposure medium in each well was removed and 100 μL DMSO was added. The absorbance at 550 nm of each well was measured using a SpectraMax i3x multi-mode microplate reader (Molecular Devices, CA).

Quantification of Internalized Cl-PFESAs and PFOS. For the determination of intracellular concentration of Cl-PFESAs and PFOS, MVLN cells and MCF-7 cells were plated at a density of 2×10^5 cells/mL in 60-mm cell culture dishes (Corning, New York, USA) containing 3 mL of culture medium and allowed to attach overnight. Then, MVLN cells and MCF-7 cells were exposed to 6:2 Cl-PFESA, 8:2 Cl-PFESA or PFOS at concentration of 50 μM for 48 h (MVLN cells) and 5 d (MCF-7 cells),

respectively. After treatment, all cells were washed with ice-cold PBS, lysed with 400 μL 1 × cell culture lysis reagent (Promega, Madison, WI, USA), and then centrifuged at 12,000 rpm for 10 min at 4 °C. The concentration of total protein in each sample was quantified with BCA protein assay kit (ComWin Biotech, Beijing, China). Next, 1 ng of C_8-^{13}PFOS (Wellington Laboratories Inc., Guelph, ON, Canada) was added as an internal standard to each sample before quantification. Each group was carried out in triplicates (3 plates). Cells without exposure were employed as negative control.

For comparison of the internal exposure dose of Cl-PFESAs and PFOS in zebrafish larvae, forty zebrafish larvae were randomly selected from each group after 7 d exposure, and then washed with deionized water, and homogenized in 400 μL of deionized water. An aliquot of the homogenate was centrifuged at 12,000 rpm, 4 ° C for the determination of protein concentration. 200 μL of homogenized larvae was mixed with equal volume of HPLC-grade methanol and 1 ng of internal standard (C_8-^{13}PFOS). The mixtures were then vortexed for 10 s and centrifuged at 4000 rpm for 30 min. After dilution with HPLC-grade methanol, the concentrations of 6:2 Cl-PFESA, 8:2 Cl-PFESA and PFOS in all samples were measured according to the published method in Ruan et al.'s study.[4]

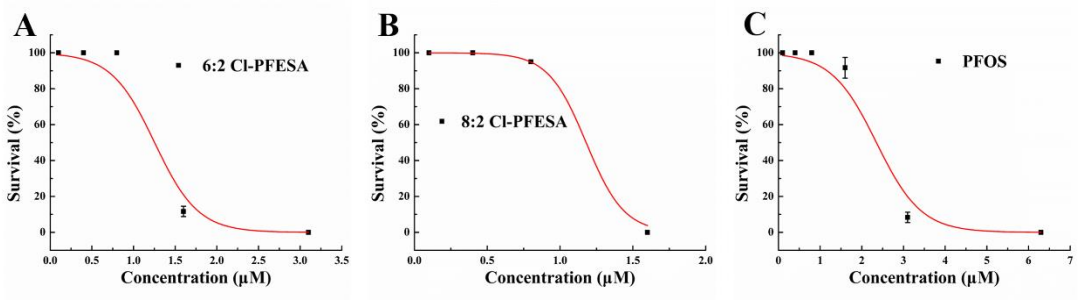

Figure S1. The results of zebrafish toxicity test. (**A**) The dose-response curve of 6:2 Cl-PFESA. (**B**) The dose-response curve of 8:2 Cl-PFESA. (**C**) The dose-response curve of PFOS. The *error bar* represents the standard deviation of three independent measurements.

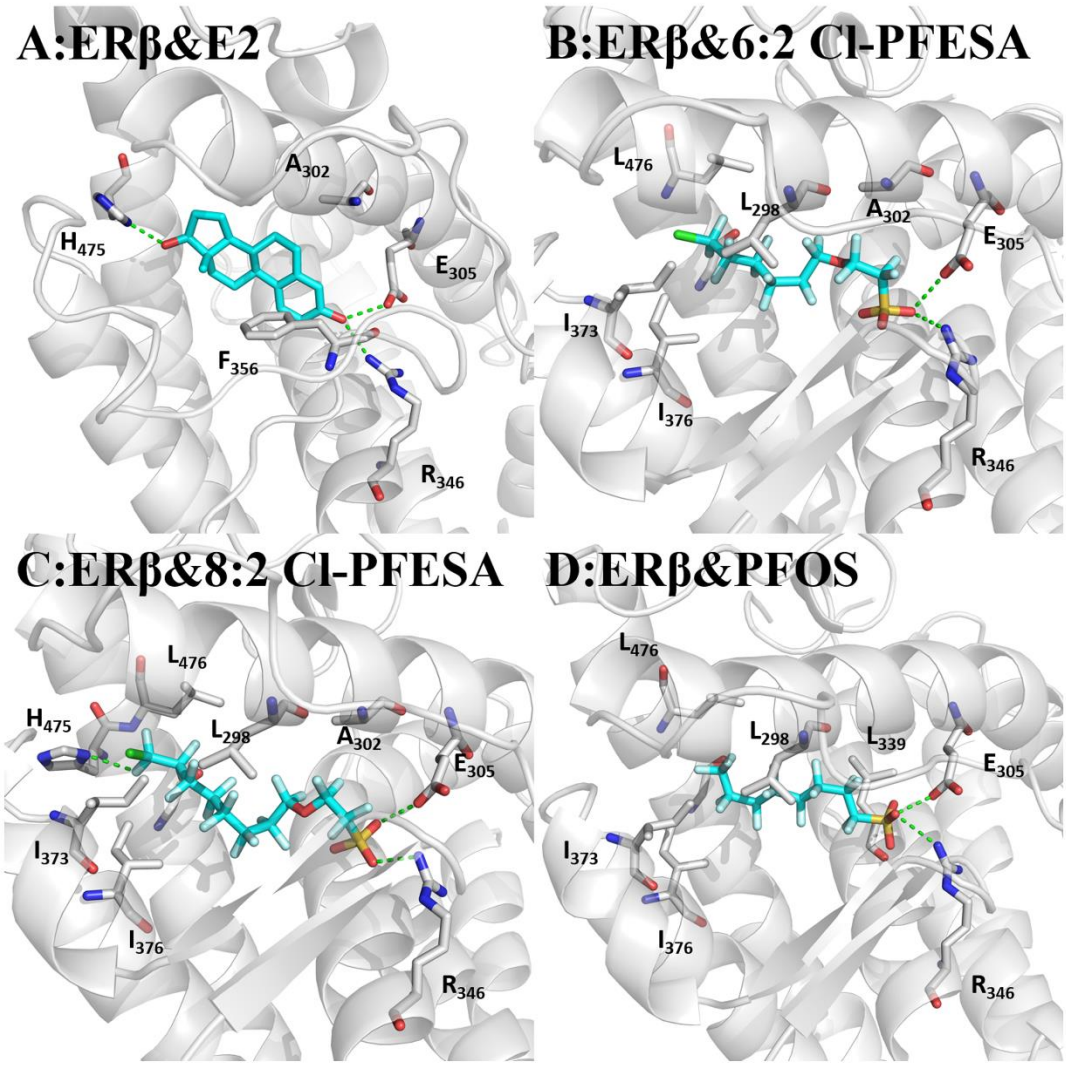

Figure S2. Molecular docking results of (**A**) E2, (**B**) 6:2 Cl-PFESA, (**C**) 8:2 Cl-PFESA, and (**D**) PFOS with human ERβ.

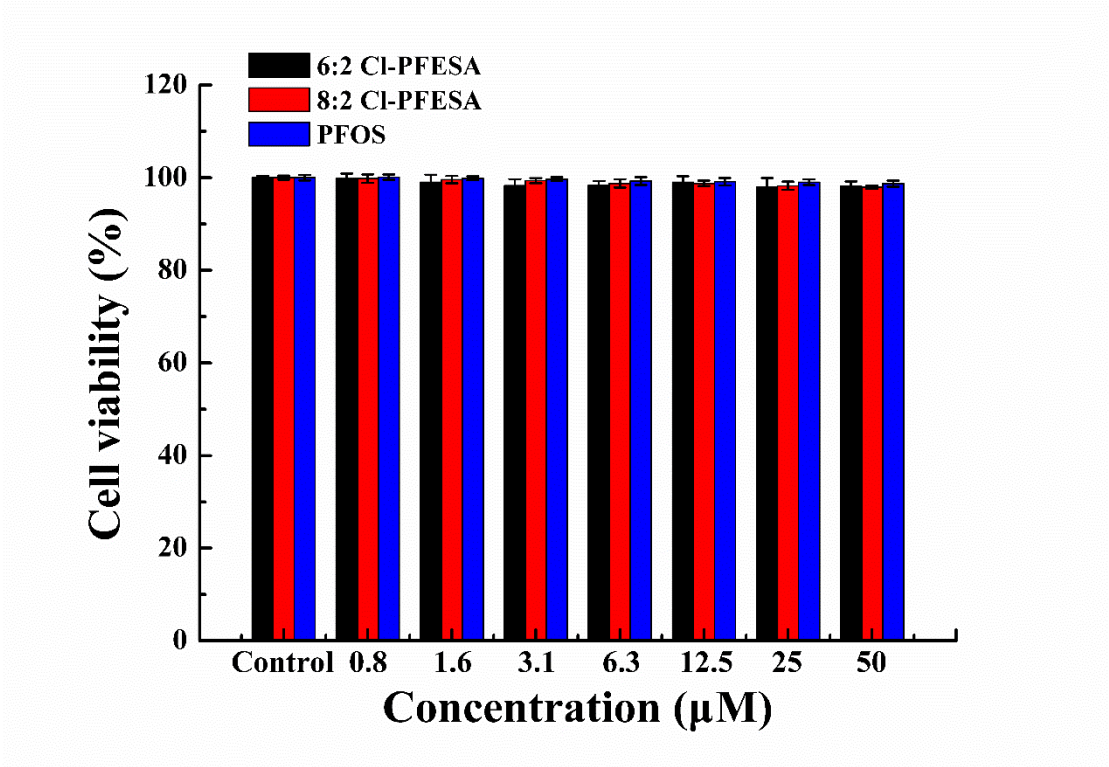

Figure S3. Effects of 6:2 Cl-PFESA, 8:2 Cl-PFESA, and PFOS on viability of MVLN cells. The *error bar* represents the standard deviation of three independent measurements.

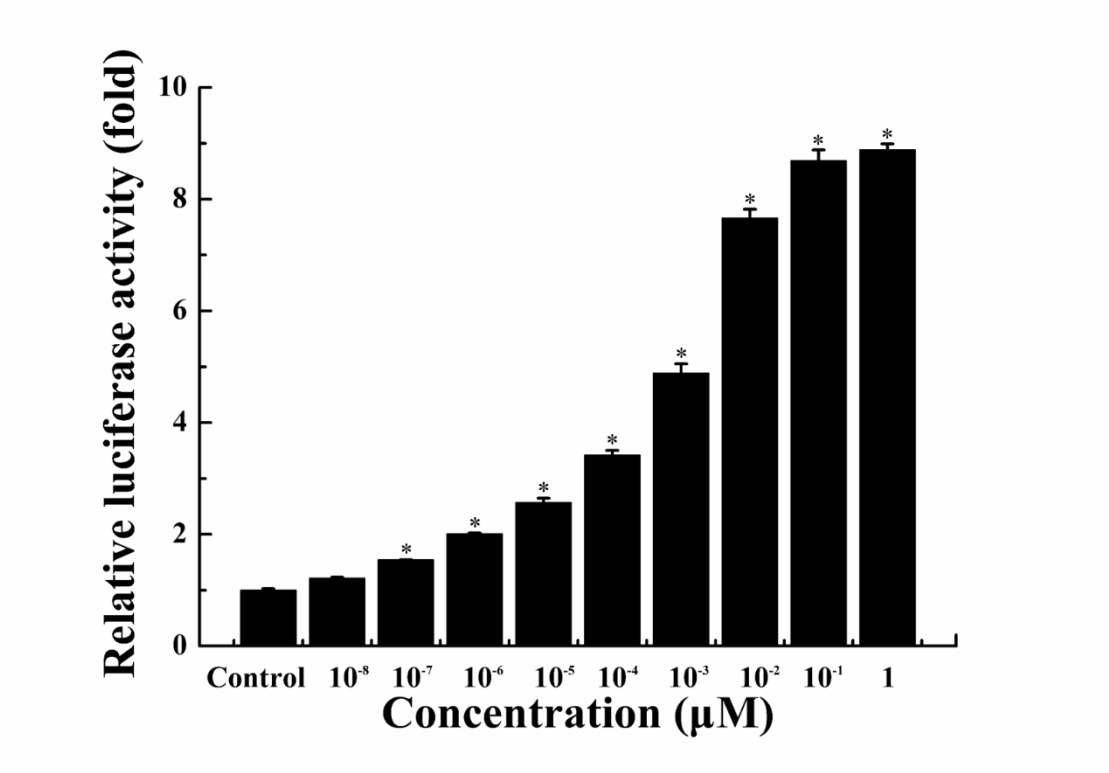

Figure S4. Activity of E2 towards estrogen receptor pathways. MVLN cells were exposed to different concentrations of E2 for 48 h. Afterward, treated cells were lysed and measured using luciferase assay system. The *error bar* represents the standard deviation of three independent measurements. * $p < 0.01$, compared with the control group.

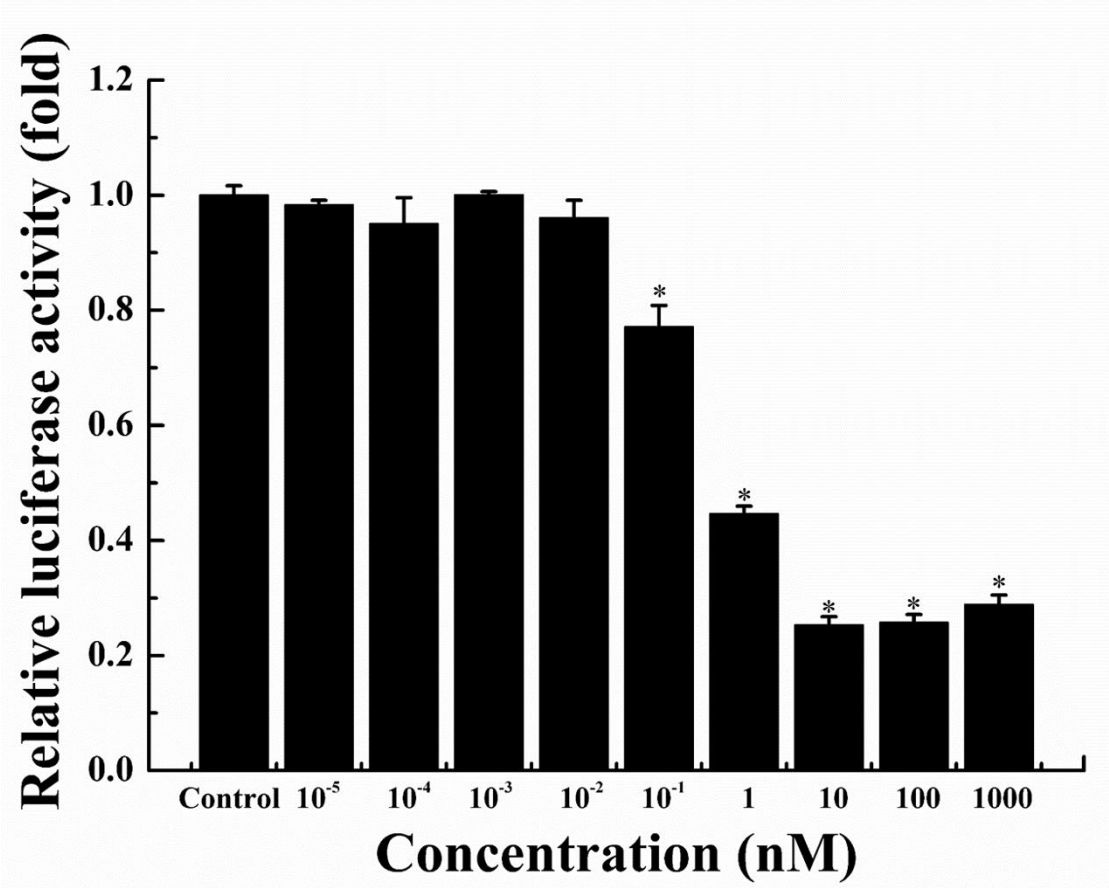

Figure S5. Activity of ICI 182780 towards estrogen receptor pathways. MVLN cells were exposed to different concentrations of E2 for 48 h. Afterward, treated cells were lysed and measured using luciferase assay system. The *error bar* represents the standard deviation of three independent measurements. * $p < 0.01$, compared with the control group.

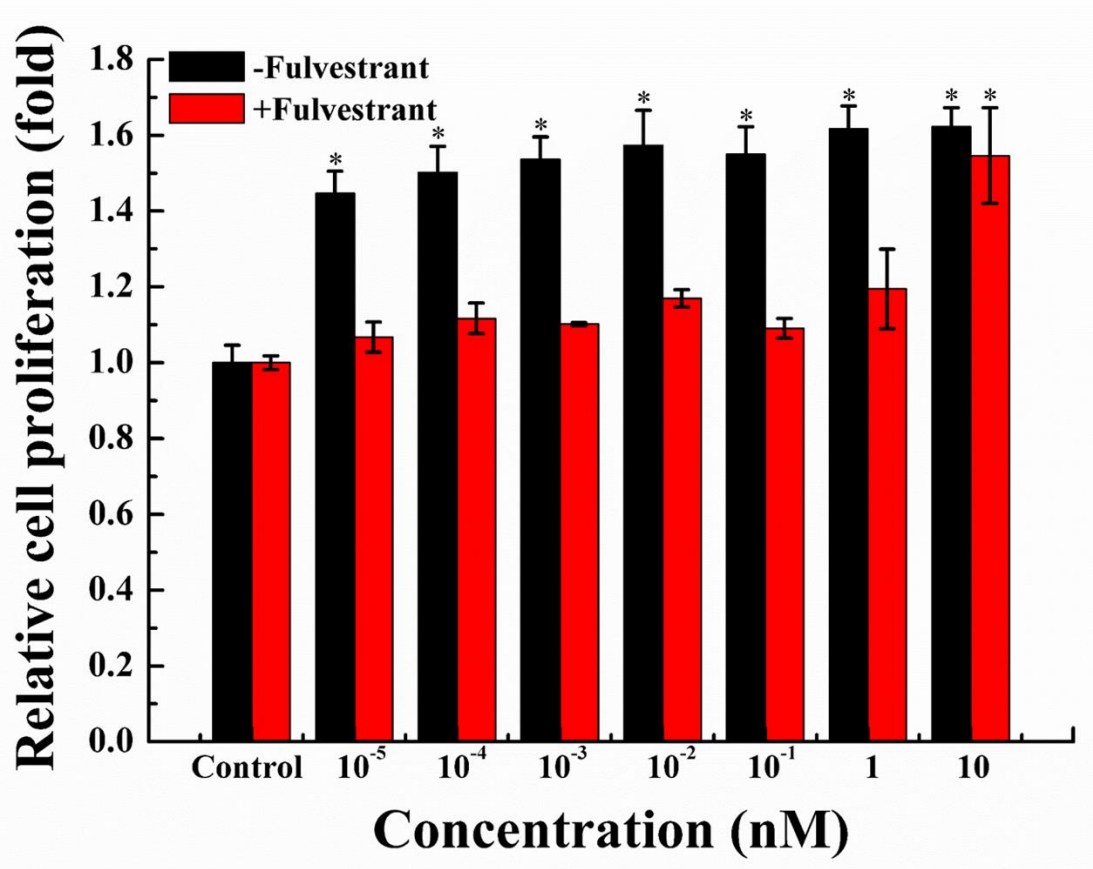

Figure S6. Effects of E2 on MCF-7 cell proliferation. MCF-7 cells were exposed to different concentrations of E2 with or without 1 μM ICI 182780 for 5 d. Afterward, the proliferation effects were assessed using MTT assay. The error bar represents the standard deviation of five independent measurements. * $p < 0.01$, compared with the control group.

Table S1. Primer sequences for the estrogen-related genes in zebrafish experiment

Gene name	Accession No.	Description	Sequence of primers (5'-3')
ERα	NM_152959.1	Forward	GGTCCAGTGTGGTGTCCTCT
		Reverse	AGAAAGCTTTGCATCCCTCA
ERβ1	NM_174862.3	Forward	GGGCGAAGAAGATACCAGGT
		Reverse	TCCTCCCTGTTGAGCTTGAG
ERβ2	NM_180966.2	Forward	TAGTGGGACTTGGACCGAAC
		Reverse	TTCACACGACCACACTCCAT
VTG	NM_001044897.3	Forward	AACGAACAGCGAGAAAGAGATTG
		Reverse	GATGGGAACAGCGACAGGA
CYP19a	NM_131154.3	Forward	AGATGTCGAGTTAAAGATCCTGCA
		Reverse	CGACCGGGTGAAAACGTAGA
CYP19b	NM_131642.2	Forward	GACACTCTCTCCATCAGTCTGTTCTT
		Reverse	CATTCAGTTTCTGCAAGTCAGCA
β-actin	NM_131031.2	Forward	ACCCACACCGTGCCCATCTA
		Reverse	CGGACAATTTCTCTTTCGGCTG

Table S2. *In vivo* concentrations and uptake ratios of Cl-PFESAs and PFOS in zebrafish larvae

		Exposure concentration	
		0.1 (μM)	**0.5 (μM)**
***In vivo* concentration (nM/mg protein)**	**6:2 Cl-PFESA**	0.8 (6.2%)	4.2 (10.9%)
	8:2 Cl-PFESA	5.8 (36.0%)	28.4 (36.8%)
	PFOS	0.8 (6.4%)	3.1 (7.0%)

Table S3. Cellular concentrations of Cl-PFESAs and PFOS in MVLN cells and MCF-7 cells

		MVLN cells	MCF-7 cells
Intracellular concentration (nM/mg protein)	**6:2 Cl-PFESA**	1.0	0.8
	8:2 Cl-PFESA	5.9	3.9
	PFOS	1.9	1.3

REFERENCES

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(2) Wang, J.; Cao, X.; Sun, J.; Huang, Y.; Tang, X. Disruption of endocrine function in H295R cell in vitro and in zebrafish in vivo by naphthenic acids. *J. Hazard. Mater.* **2015,** *299*, 1-9.

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