SUPPORTING INFORMATION

Organophosphate Esters Bind to and Inhibit Estrogen Related Receptor γ in Cells

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Synthesis and characterization of fluorescence probe GSK-FITC. A site-specific fluorescence probe (GSK-FITC) was designed and synthesized to study the binding potency of chemicals with ERRγ by conjugating FITC to an ERRγ inverse agonist GSK5182 (Figure S1). The crystal structure of ERRγ/GSK5182 showed the amino terminal of GSK5182 extended towards the entrance of binding pocket of ERRγ[1]. Thus, we designed a GSK5182 analogue by replacing the quaternary ammonium moiety of GSK5182 with an amino group for further derivatization (Figure S2). FITC was conjugated to the amino group through a four carbon alkyl chain linker. The synthesis of probe GSK-FITC involved 7 steps (Figure S2) and the characterization of GSK-FITC are detailed in the Figure S3–S5. The synthetic product (with purity > 99%, Figure S3) had the correct molecular weight of 878.6 $(M+H)^+$ (Figure S4). Proton nuclear magnetic resonance (^{1}H NMR) results also displayed the correct structure of GSK-FITC (Figure S5). Stock solutions of the fluorescence probe (1 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at -80^{o}C until use. The concentration of GSK-FITC was determined by the absorbance at 490 nm, using a molar extinction coefficient of $7.8\times10^4\ M^{-1}cm^{-1}$.

Reagents and solvents were used without further purification as obtained from commercial sources. The reaction progress was determine by thin layer chromatography (TLC) analysis using Merck Kieselgel 60 F254 plates (Merck, Darmstadt, Germany). Product purification was performed on Kieselgel 60 silica gel columns with 300−400 mesh (Merck). ^{1}H NMR spectra were detected by Bruker Ultra Shield-300 (Bruker, Karlsruhe, Germany). Abbreviations are used as follows: s

= singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublets of doublet. Mass spectra (MS) were determined on a Agilent liquid chromatography-mass spectrometer system (LC-MS), using a CAPCELL PAK UG-120 ODS column (Shiseido Co., Ltd., Tokyo, Japan) and an HP-1100 apparatus (Agilent Technologies, Palo Alto, California, USA). All MS were detected using electrospray ionization (ESI) in positive ion mode. Purity analysis was performed on a Agilent high performance liquid chromatography (HPLC) instrument (Agilent Technologies) equipped with a XBridge C18 S-3.5 μm column (Waters, Milford, MA, USA) and detected at 254 nm using UV detection (Waters). Preparative purifications were performed by Pre-HPLC using a Gilson pumping system (Gilson, Villiers le Bel, France), a Gemini C18 column (Phenomenex, Torrance, CA, USA), a Gilson 215 auto sampler (Gilson) and a photodiode array detector (Hewlett-Packard, Palo Alto, CA, USA).The following were the detailed synthetic process of GSK-FITC.

Methyl 5,5-bis(4-hydroxyphenyl)-4-phenylpent-4-enoate (GSK-001). Zinc powder (3.9 g) was suspended in dry THF (50 mL) at room temperature (rt) in a Schlenk tube under argon, and $TiCl_4$ (60 mL, 1 M in DCM) was added slowly via a syringe while stirring. After heating at reflux for 2 h, a THF solution containing 2.14 g SM1 and 1.92 g SM2 was added, and the mixture was heated at reflux for another 2 h. The reaction mixture was cooled, poured into H_2O, acidified with HCl until the dark color disappeared and extracted with dichloromethane. The organic layer was washed with H_2O, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude mixture was purified by silica gel column with DCM/CH_3OH=20: 1 to give GSK-001

(2.8 g, 75%) as a light yellow oil. MS shows the correct molecular weight of 375 $(M+H)^+$. ^1H NMR (400 MHz, DMSO): δ 9.44 (s, 1H), 9.19 (s, 1H), 7.19 (t, 2H), 7.11 (dd, 3H), 6.99 (d, 2H), 6.75 (d, 2H), 6.59 (d, 2H), 6.40 (d, 2H), 3.48 (s, 3H), 2.70–2.61 (m, 2H), 2.25–2.16 (m, 2H).

(Z)-Methyl5-(4-(2-(tert-butoxycarbonylamino)ethoxy)phenyl)-5-(4-hydroxy-phenyl)-4-phenylpent-4-enoate (GSK-002). The tert-butyl 2-bromoethylcarbamate (223 mg) was added to a mixture of GSK-001 (374 mg) and K_2CO_3 (276 mg) in DMF (10 mL). The resulting mixture was stirred at 65 ^oC for 3 h. The reaction mixture was diluted with H_2O (15 mL) and extracted with EtOAc (30 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by silica gel chromatography using DCM/CH_3OH= 30:1 to give GSK-002 (260 mg, 50%) as a light yellow solid. MS shows the correct molecular weight of 518 $(M+H)^+$.

(Z)-Methyl5-(4-(2-aminoethoxy)phenyl)-5-(4-hydroxyphenyl)-4-phenylpent-4-enoate (GSK-003). The GSK-002 (250 mg) was added to a solution of trifluoroacetic acid (3 mL) in dichloromethane (10 mL) and the resulting mixture was stirred at rt for 3 h. The resulting mixture was concentrated and the residue GSK-003 was used in the next step without further purification (180 mg, 90%). MS shows the correct molecular weight of 418 $(M+H)^+$.

(Z)-Methyl-5-(4-(2-(5-(tert-butoxycarbonylamino)pentanamido)ethoxy)phenyl)-5-(4-hydroxyphenyl)-4-phenylpent-4-enoate (GSK-004). GSK-003 (100 mg), 5-(tert-butoxycarbonylamino) pentanoic acid (62 mg), DIPEA (93 mg) and HATU

(137 mg) were dissolved in DMF (5 mL) and stirred at rt for 3 h. The reaction mixture was diluted with EtOAc (25 mL) and washed with H_2O (15 mL). The organic phase was concentracted and the residue was purified by silica gel with DCM/CH_3OH= 20:1 to give GSK-004 (95 mg, 65%) as a yellow solid. MS shows the correct molecular weight of 617 $(M+H)^+$.

(Z)-Methyl5-(4-(2-(5-aminopentanamido)ethoxy)phenyl)-5-(4-hydroxyphenyl)-4-phenylpent-4-enoate (GSK-005). The GSK-004 (50 mg) was added to a solution of trifluoroacetic acid (1 mL) in dichloromethane (5 mL) and the resulting mixture was stirred at rt for 3 h. The resulting mixture was concentrated and the residue GSK-005 (40 mg) was used in the next step without further purification. MS shows the correct molecular weight of 517 $(M+H)^+$.

(Z)-2-(3-hydroxy-6-oxo-6H-xanthen-9-yl)-5-(3-(5-(2-(4-(1-(4-hyd-roxyphenyl)-5-methoxy-5-oxo-2-phenylpent-1-enyl)phenoxy)ethylamino)-5-oxopentyl) thioureido) benzoic acid (GSK-006). GSK-005 (200 mg), FITC (150 mg) and DIPEA (150 mg) were dissolved in THF (10 mL) and stirred at rt for 2 h. The resulting mixture was concentrated and the residue GSK-006 (400 mg) was used in the next step without further purification. MS shows the correct molecular weight of 906 $(M+H)^+$.

(Z)-5-(3-(5-(2-(4-(5-hydroxy-1-(4-hydroxyphenyl)-2-phenylpent-1-enyl)phenoxy) ethylamino)-5-oxopentyl)thioureido)-2-(3-hydroxy-6-oxo-6H-xanthen-9-yl) benzoic acid (GSK-FITC). LiAlH4 (20 mg) was batchwise added to a solution of GSK-006 (400 mg) in THF (10 mL) at 0 ^oC. The mixture was stirred at 0 ^oC for 30

min and then quenched by 1 mL H_2O. The mixture were filtered and the filtrate was concentrated. Then the residue was purified by Pre-HPLC to give the final product GSK-FITC as a yellow solid. MS shows the correct molecular weight of 878 $(M+H)^+$. ^1H NMR (400 MHz, MeOD): δ8.339-8.321(m, 1H), 7.934-7.915(m, 1H) , 7.278-7.258(m, 1H) , 7.172-7.073(m, 7H) , 7.031-7.010(m, 2H) , 6.954-6.894(m, 3H) , 6.777-6.751(m, 2H) , 6.681-6.632(m, 1H) , 6.588-6.566(m, 1H) , 6.422-6.382(m, 1H) , 4.107-4.081(m, 1H) , 3.941-3.915(m, 1H) , 3.624-3.597(m, 3H) , 3.522-3.495(m, 1H) , 3.428-3.395(m, 2H) , 2.513-2.473(m, 2H) , 2.333-2.265(m, 2H) , 1.737-1.689(m, 4H) , 1.563-1.524(m, 2H).

Binding of GSK-FITC probe to ERRγ-LBD. Change of the fluorescence polarization (FP), which depends on the rotational relaxation time of a fluorescent molecule, can be used to detect the binding of a fluorescent probe with proteins[2, 3]. A small fluorescent molecule such as GSK-FITC rotates quickly in a solution and results in a low polarization value. Once bound to a large molecule such as a receptor, rotation of GSK-FITC is slowed down significantly and results in a high polarization value. Therefore, the free and protein-bound fluorescent probe can be differentiated by the polarization value.

Firstly, we determined the binding affinity of GSK-FITC with ERRγ-LBD by FP based direct binding assay. Briefly, various concentrations of ERR-LBDs (25−420 nM) were mixed with 50 nM GSK-FITC in Tris-HCl buffer (50 mM Tris-HCl/100 mM NaCl, pH 7.4) in a total volume of 100 μL. After incubation for 2 min at room temperature, the fluorescence polarization (FP) value of each sample was detected on

a Horiba Fluoromax-4 spectrofluorimeter (Edison, NJ, USA). The excitation and emission wavelengths were set at 490 nm and 520 nm, respectively. The FP value was plotted as a function of ERR-LBDs concentration. The apparent K_d values of the probe ($K_{d,GSK-FITC}$) with ERR-LBDs were then calculated by nonlinear regression curve-fitting of the binding data using Sigmaplot software (Systat Software Inc., San Jose, CA, USA).

As shown in Figure S6A, with the increase of ERRγ-LBD concentration, the FP value increased from 70 mP to 140 mP, reflecting the binding of GSK-FITC to ERRγ-LBD. By fitting the binding curve with sigmoidal mode, a K_d value of 54.5 ± 5.0 nM (R^2=0.98) was obtained. In previous studies, several radiolabeled probes such as [^{3}H]4-OHT and [^{3}H]BPA were synthesized and used to determine the binding potency of chemicals to ERRγ-LBD[4]. To the best of our knowledge, the GSK-FITC used in the present study is the first fluorescent probe targeting ERRγ-LBD.

GSK5182 was designed and proved to bind to ERRγ with higher affinity than ERRβ, but not bind with ERRα.[1,5] Therefore, we investigated the binding selectivity of GSK-FITC towards ERRγ by measuring the binding of GSK-FITC to the other two ERR subtypes, ERRα-LBD and ERRβ-LBD. As shown in Figure S6B, GSK-FITC bound to ERRβ-LBD with a K_d value of 200.6 ± 18.7 nM (R^2=0.97), which is about 4 folds larger than the value with ERRγ-LBD. For ERRα-LBD, no binding was found with GSK-FITC (Figure S6C). Our results are in accordance with the selectivity of GSK5182 towards ERRs reported before. Furthermore, in order to exclude the possible interference of FITC, we also detected the binding of FITC with ERRγ. The

results showed FITC could not bind to ERRγ-LBD (Figure S7). All the above results demonstrated that GSK-FITC bound to ERRγ-LBD through the GSK moeity of the probe.

Competitive Binding Assay. Binding potency of a chemical with ERRγ-LBD was determined by competitive binding assay using GSK-FITC as the fluorescence probe. In each assay, 50 nM GSK-FITC, 200 nM ERRγ-LBD and different concentrations of ligand were mixed in Tris-HCl buffer in a total volume of 100 μL. The content of DMSO in the final solution was kept below 1% to avoid solvent effect. After incubation for 2 min at room temperature, the fluorescence polarization at 520 nm was measured and plotted as a function of the ligand concentration. The competitive curve was fitted with a sigmoidal model using SigmaPlot software to obtain the IC_{50} value ($IC_{50,\ ligand}$). The dissociation constant of a ligand ($K_{d,\ ligand}$) was then calculated according to Eq.1:

$$IC_{50,ligand}/[GSK-FITC]_{total} = K_{d,ligand}/K_{d,GSK-FITC} \quad (1)$$

where $[GSK\text{-}FITC]_{total}$ is the total concentration of GSK-FITC (50 nM) used in the experiment, $K_{d,\ GSK\text{-}FITC}$ is the K_d value of the probe with ERRγ-LBD obtained in the direct binding assay described above.

To verify the new method, we first detected the binding potency of two known ERRγ ligands (an agonist GSK4716 and an inverse agonist 4-OHT) as positive controls and an inactive compound (E2) as negative control, respectively. As shown in Figure S6D, as the concentration of GSK4716 increased, the FP value decreased, suggesting the probe was displaced from ERRγ-LBD by GSK4716. Similar result was

obtained for 4-OHT (Figure S6E). Based on the competitive binding curves, K_d values of 3.25 ± 0.57 μM and 0.04 ± 0.01 μM were obtained for GSK4716 and 4-OHT, respectively (Table 1). For E2, it did not decrease the FP value even at the highest concentration tested (Figure S6F). In a previous study, a K_d value of 35 nM was obtained for 4-OHT by radio ligand competitive binding assay using [^{3}H]4-OHT as a tracer[6]. Based on ERRγ-fluorescence resonance energy transfer assay, IC_{50} values of 2 μM and 50 nM were also obtained for GSK4716 and 4-OHT respectively[6, 7]. The K_d values obtained by our fluorescence competitive method are in good agreement with the reported values[5], thus validating the new method.

Cell Culture and Cytotoxicity Assay. HeLa cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (HyClone Inc., Waltham, MA, USA), penicillin (100 units/mL) and streptomycin (100 μg/mL) (Invitrogen) at 37 °C in a humidified 5% CO_2 atmosphere. Cytotoxicity of OPEs was determined using water-soluble tetrazolium salt WST-1 assay (Roche Diagnostics, Mannheim, Germany). Briefly, HeLa cells were seeded in a 96-well plate at a density of 2×10^4 cells/well. After 24 h, the cells were treated with different concentrations of OPEs for another 24 h. Then, the cells were washed with PBS and incubated with 100 μl WST-1 in phenol red-free DMEM (Invitrogen). After 2 h, the cell viability was assessed by the absorbance at 450 nm (with a reference wavelength of 690 nm) using a Varioskan Flash microplate reader (ThermoFisher Scientific, Waltham, MA, USA).

References

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Identification and structure - activity relationship of phenolic acyl hydrazones as selective agonists for the estrogen-related orphan nuclear receptors ERR beta and ERR gamma. *J. Med. Chem.* **2005**, *48*, 3107-3109.

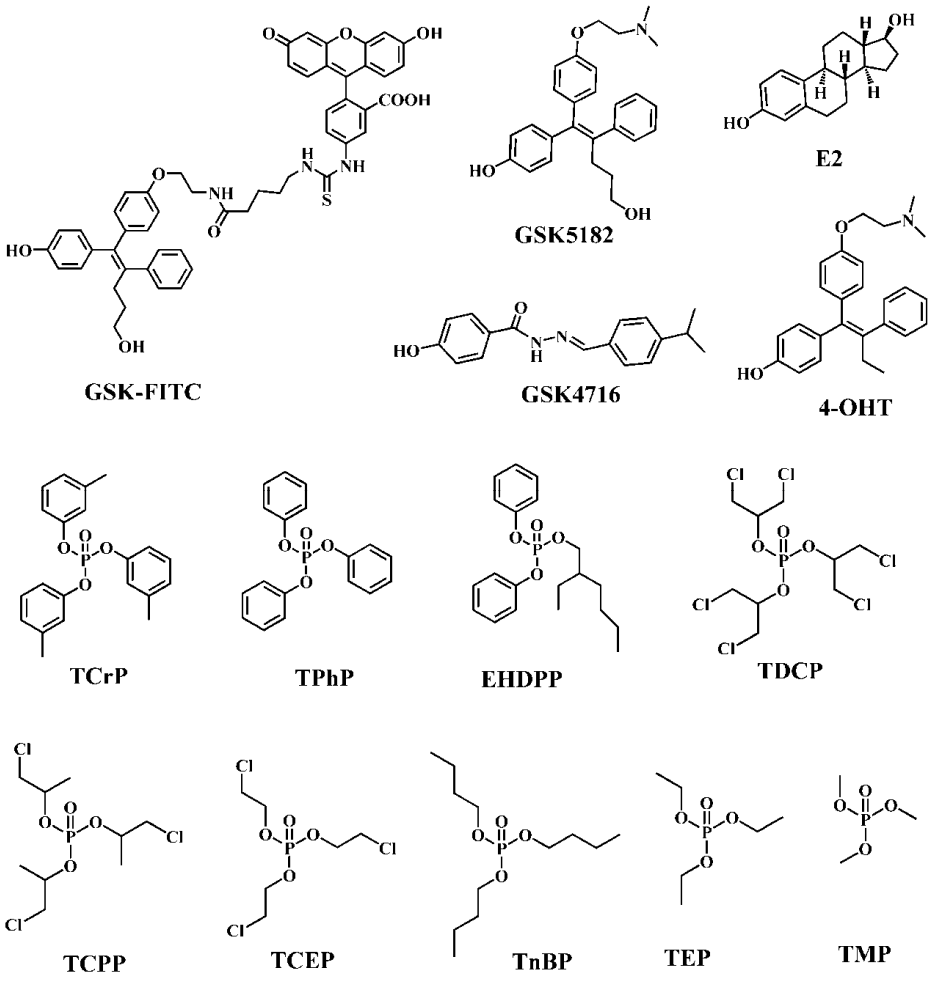


Figure S1. The chemical structures of the fluorescence probe GSK-FITC and the compounds used in the present study.

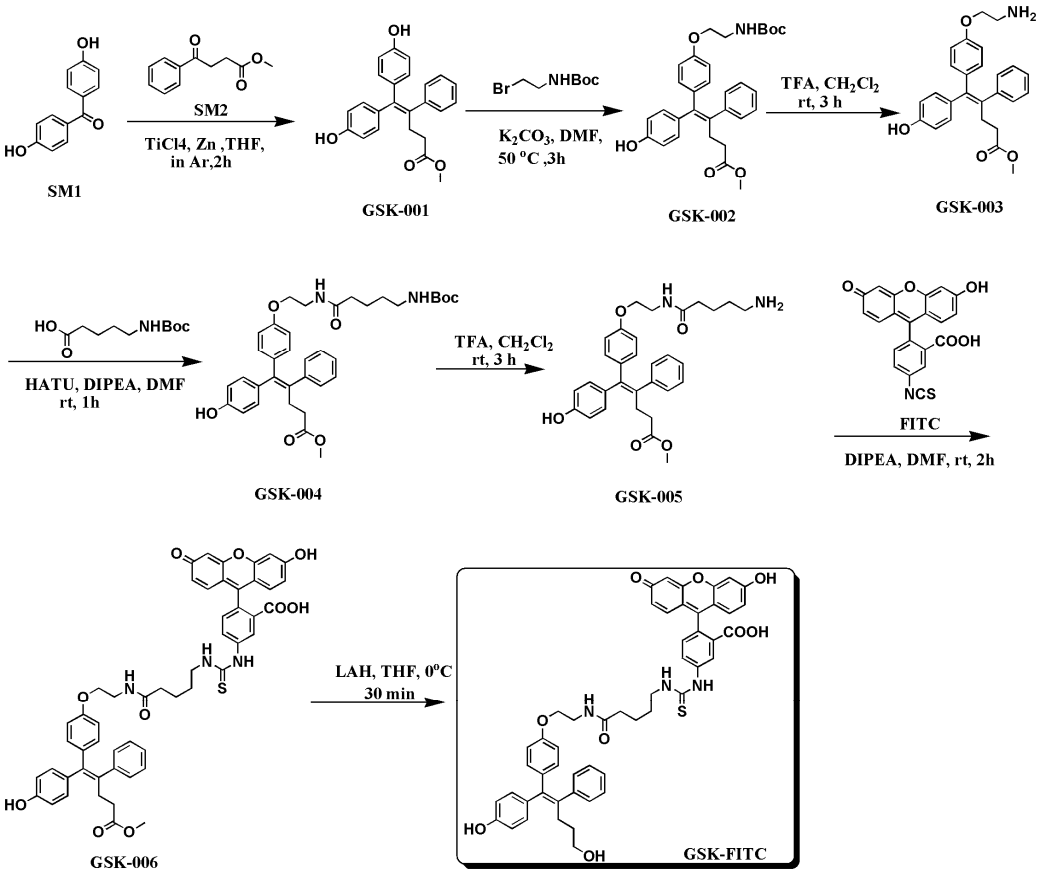
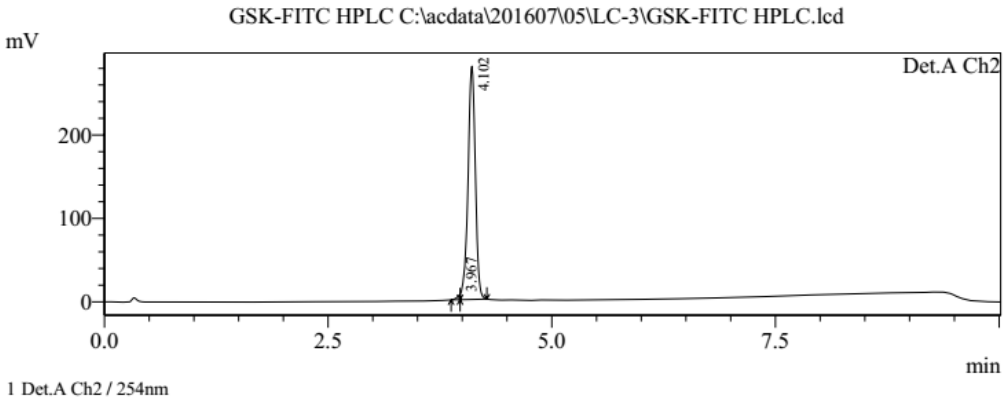


Figure S2. Scheme of synthesis of GSK-FITC.



<Result>

PeakTable

Detector A Ch2 254nm

Peak#	Ret. Time	Area	Height	Area %
1	3.967	15033	5719	0.935
2	4.102	1593219	279682	99.065
Total		1608252	285401	100.000

Figure S3. High performance liquid chromatography (HPLC) analysis of GSK-FITC.

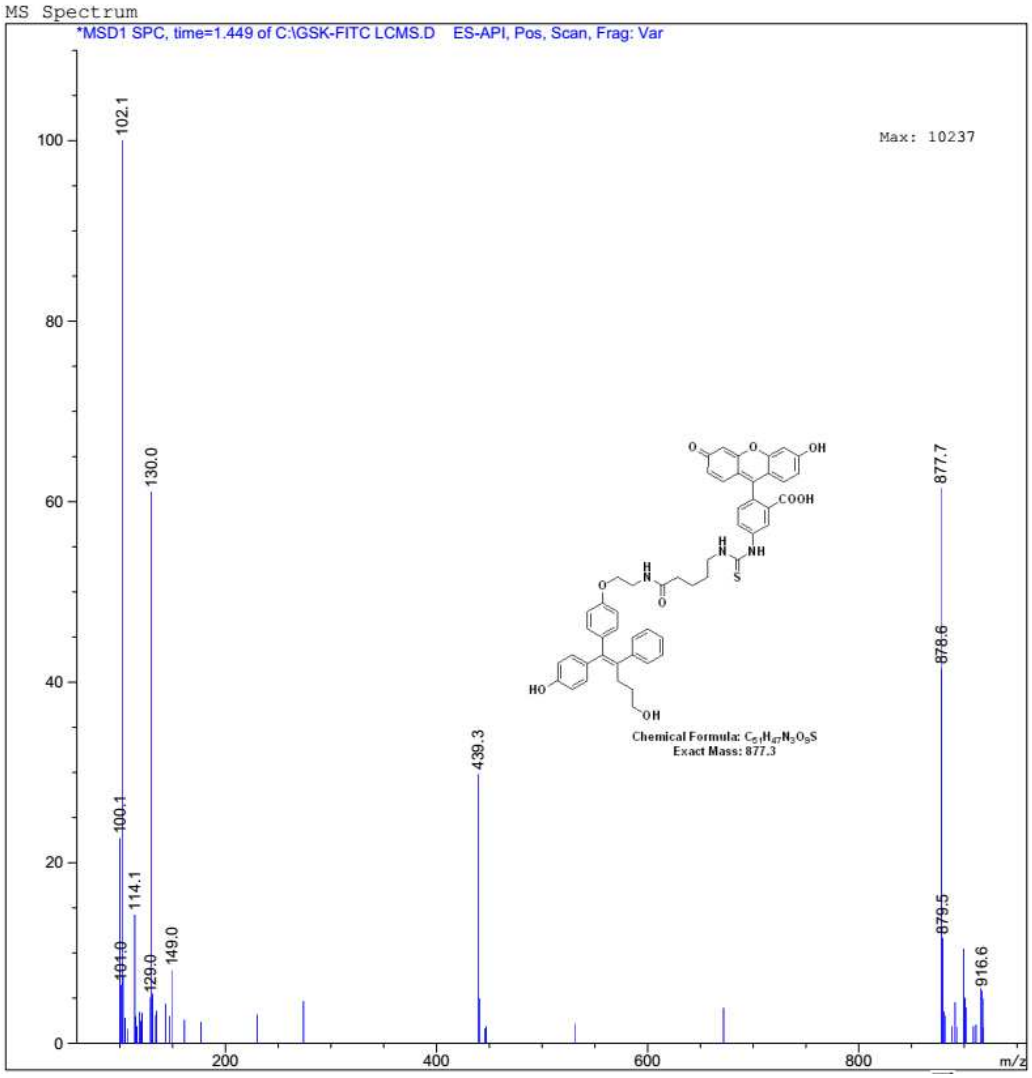


Figure S4. Mass spectra (MS) analysis of GSK-FITC.

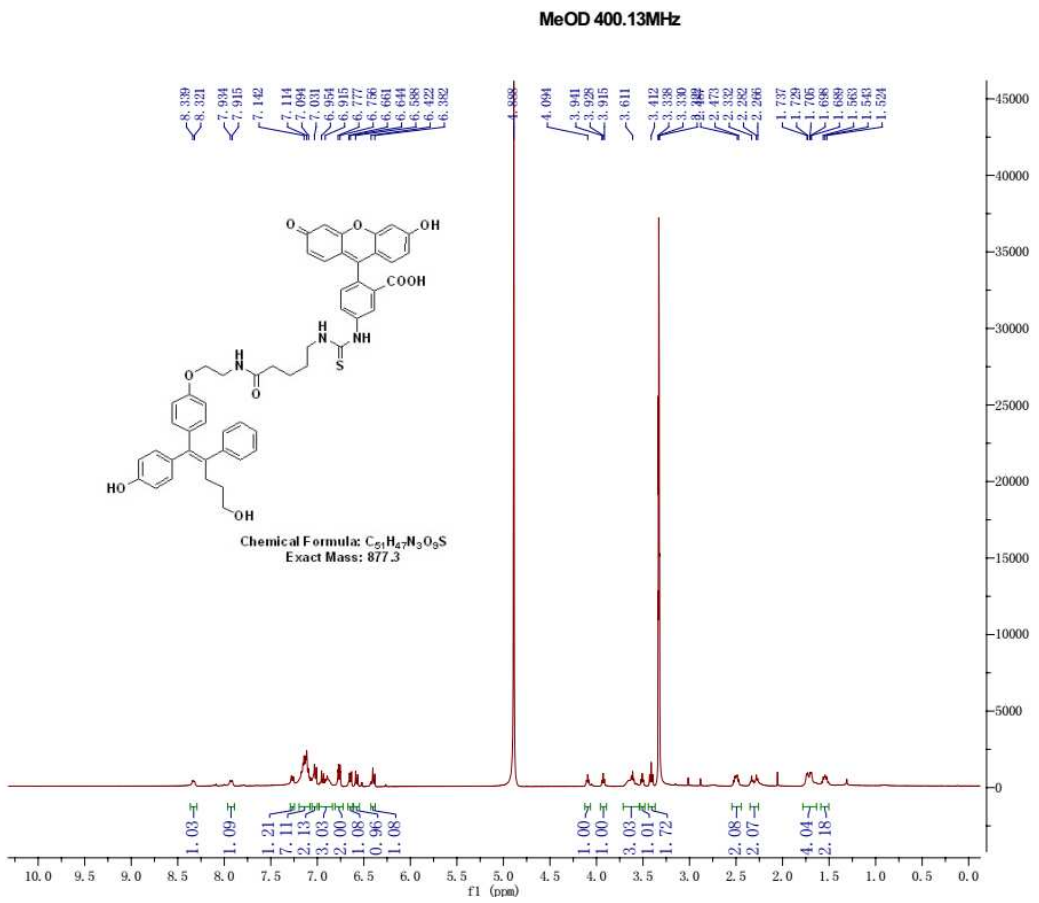


Figure S5. Proton nuclear magnetic resonance spectra (^{1}H-NMR) analysis of GSK-FITC.

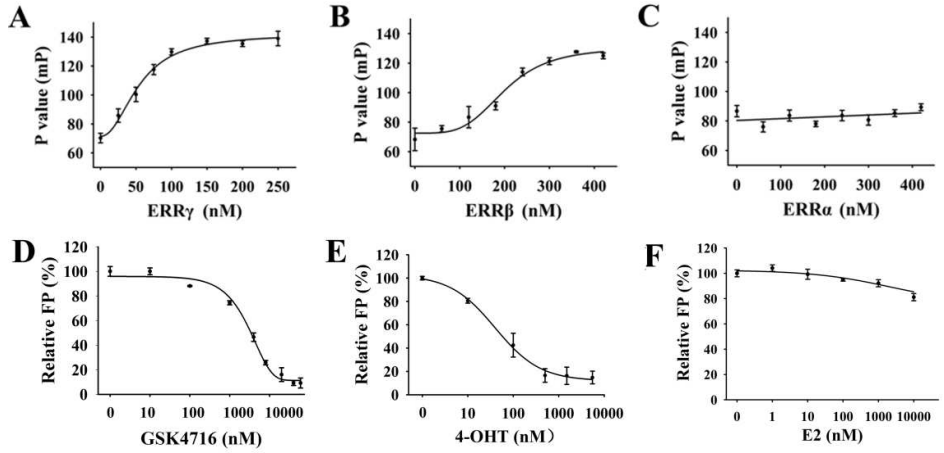


Figure S6. Binding of fluorescence probe GSK-FITC to ERRγ-LBD (A), ERRβ-LBD (B) and ERRα-LBD (C) and the competitive binding curves of GSK4716 (D), 4-OHT (E) and E2 (F) to ERRγ-LBD. The binding curves were plotted the fluorescence polarization of 50 nM GSK-FITC at 520 nm as a function of added protein concentrations. The fluorescence competitive binding curves were plotted the relative fluorescence polarization value of 50 nM GSK-FITC at 520 nm in 200 nM ERRγ-LBD as a function of added compounds concentrations.

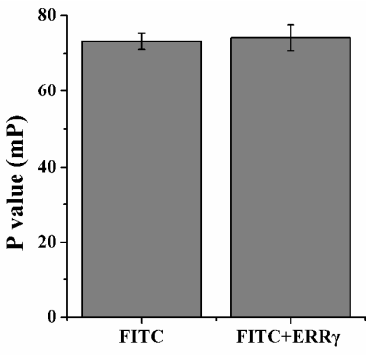


Figure S7. The binding of FITC to ERRγ. The fluorescence polarization value of 50 nM FITC at 520 nm in absence and presence of 200 nM ERRγ.

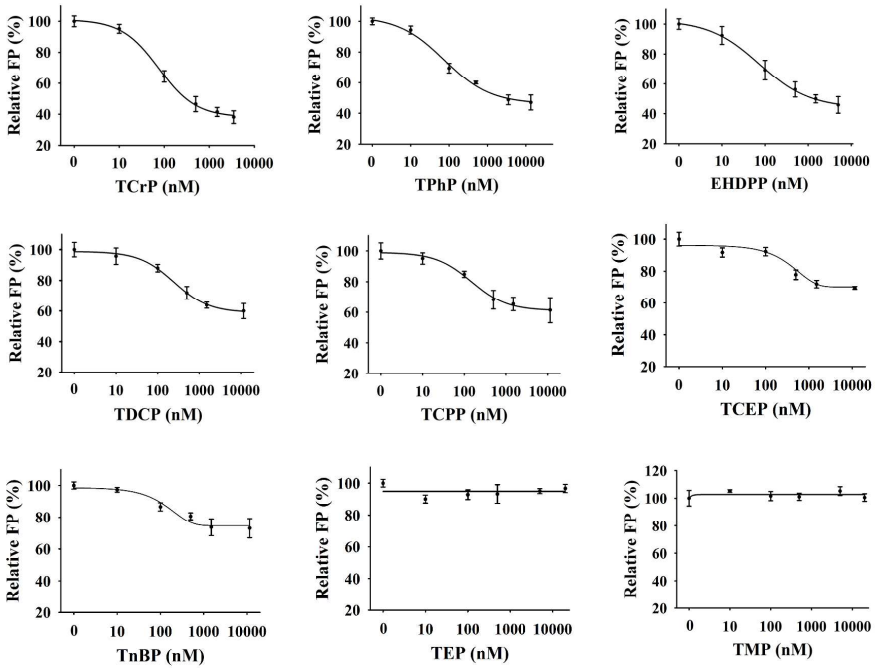


Figure S8. The competitive binding curves of 9 OPEs to ERRγ-LBD. The competitive binding curves were plotted the relative FP values of 50 nM GSK-FITC at 520 nm in presence of 200 nM ERRγ-LBD as a function of concentrations of tested compounds.

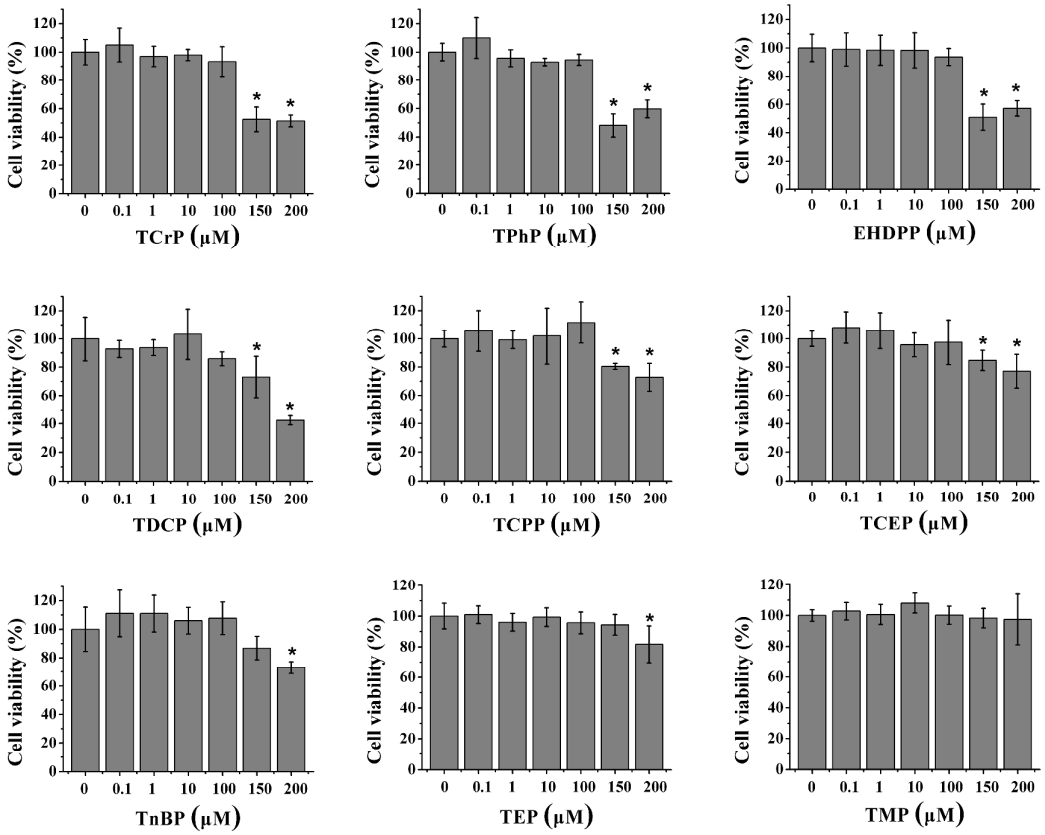


Figure S9. The cytotoxicity of OPEs on HeLa cells determined by WST-1 assay. *$p<$ 0.05, compared with the control group (concentration of 0 nM, 0.1% DMSO).

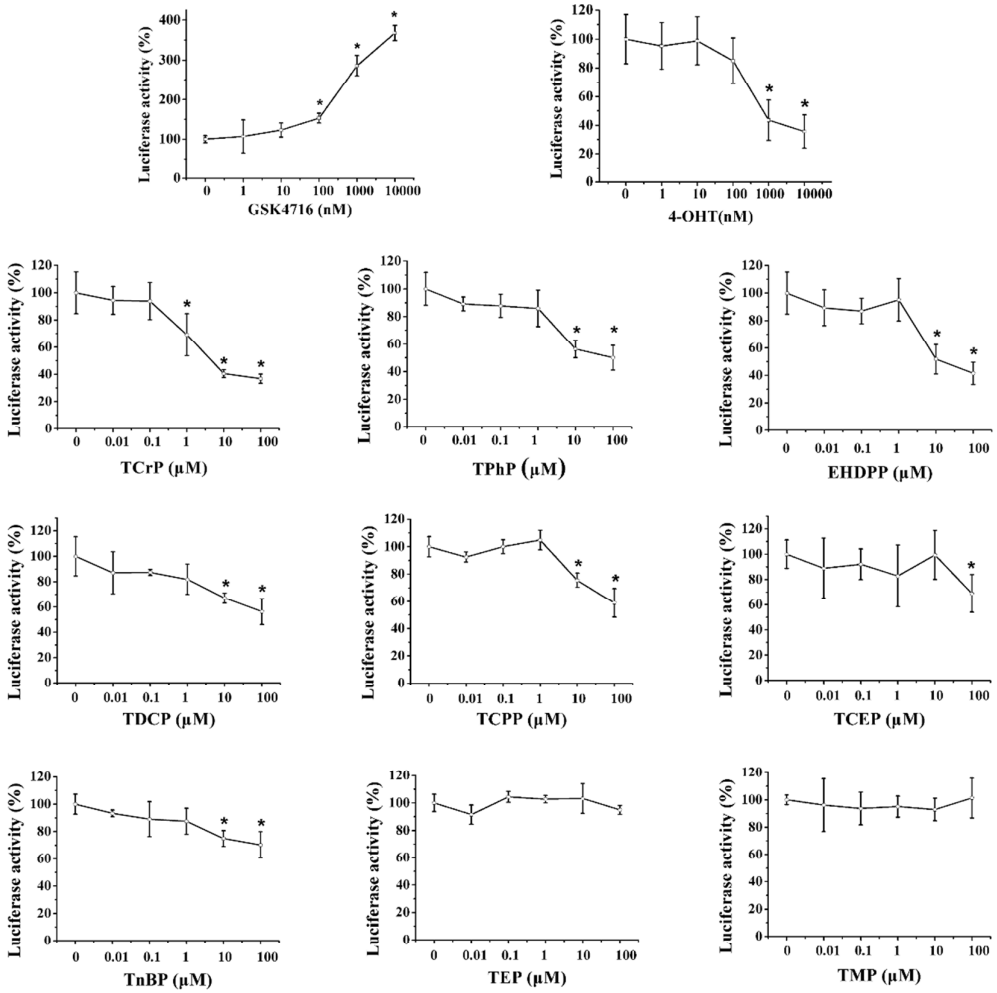


Figure S10. The effects of different concentrations of GSK4716, 4-OHT and 9 OPEs on ERRγ transcriptional activity. *$p < 0.05$, compared with the untreated group (concentration at 0 μM).

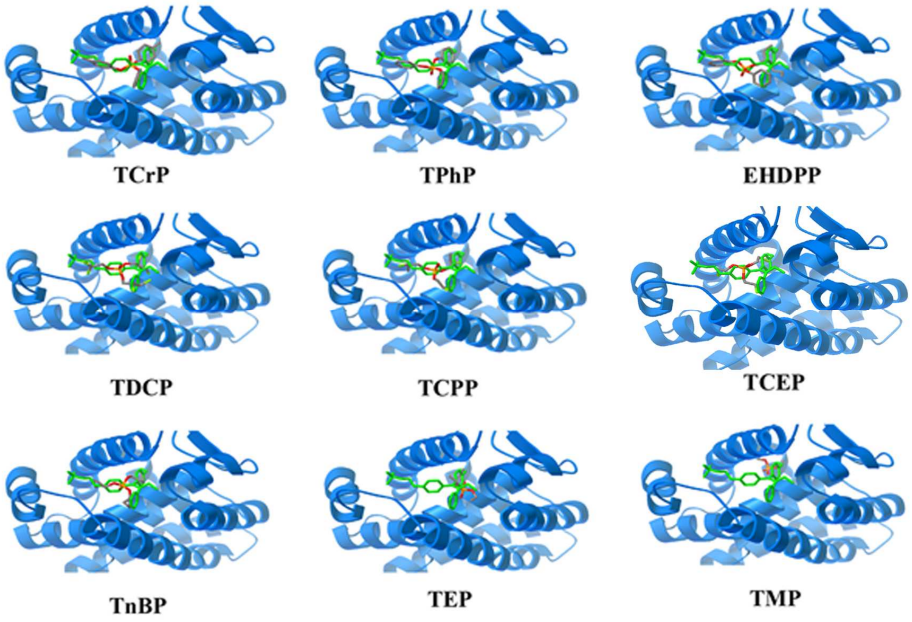


Figure S11. Molecular docking of 4-OHT and OPEs with ERRγ. For comparison, the binding pose of OPEs were overlaid with 4-OHT; ERRγ was shown as blue ribbons; 4-OHT was shown in green sticks; OPEs were shown in gray sticks [carbon (C), gray; oxygen (O), red; chlorine (Cl), green; phosphorus (P), yellow].