Supporting Information

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SI Methods

Crystallization and Data Collection. The protein was concentrated to ≈5 mg/ml in solution containing 20 mM Tris·HCl (pH 8.0), 100 mM NaCl, and 100 μM ligand. Crystallization was carried out by the hanging-drop vapor-diffusion method at room temperature. The reservoir solution is 100 mM Mes (pH 6.8), 0.5 M $MgCl_2$, 15% wt/vol PEG 4000, and 10% Ethylene glycol. Crystals of ER/LY156681 belong to space group C2 2 21 with unit cell parameters a = 56.47 Å, b = 102.761Å, c = 172.952Å. Crystals of ER/LY117018 belong to space group C2 2 21 with unit cell parameters a = 63.132 Å, b = 98.737 Å, c = 174.981 Å. There is one molecule of the complex per asymmetric unit. The crystal was cooled at 100 K, by using 15–20% ethylene glycol plus the mother liquor as cryoprotectant, before data collection. X-ray diffraction data sets were collected on IMCA (Industrial Macromolecular Crystallography Association) beam lines and processed by using HKL2000. The resolution of the datasets used for structure refinement is 2.0 Å.

HDX. Briefly, the protein/ligand complex was diluted into a D_2O-exchange buffer and subjected to HDX for 1, 30, 60, 900, and 4,200 sec. The exchange reaction was then quenched with an ice-cold, acidic solution, and the whole solution was loaded onto a house-packed pepsin column. Digested peptides were then eluted across a C_{18} analytical column and electrosprayed directly into a Thermo Finnigan LTQ mass spectrometer.

Protein and Reagents for HDX Analysis. The ER ligand-binding domain (amino acids 298–554) was cloned into a modified PET vector pMCSG7 with a ligation-independent cloning site, 6× His tag, and TEV protease site. The protein was induced in BL21 (DE3) Rosetta cells, and purified with nickel chromatography. The eluted protein mixed with a 1:30 ratio (by mg weight) of His-tagged TEV protease and dialyzed overnight in 20 mM Tris (pH 8), 100 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM imidazole, and 10% glycerol. The solution was then passed through a nickel column to remove uncut ER, the cut tags, and the TEV protease. The flowthrough was diluted five times in H_2O and subjected to ion-exchange chromatography with a Q-FF column (GE Healthcare). The protein was eluted in 100–200 mM NaCl, 20 mM Tris (pH 8.0), 10 mM 2-mercaptoethanol, and 10% glycerol, and then concentrated to 5.5 mg/ml. All compounds are from Eli Lilly except that raloxifene was from Sigma. In the HDX analysis, ERαLBD was incubated for 1 h with estradiol, a natural agonist of the receptor at two ratios: 1:5 and 1:10, respectively. The data listed in Table 2 was at 1:10 ratio. ERαLBD was also incubated for 1 h with ligands at 1:5 ratio for DES, ICI 182780, 4-hydroxytamoxifen, raloxifene, and lasofoxifene and 1:10 ratio for bazedoxifene and compounds LY88074, LY156681, LY165176, and LY117018. The protein concentration was 12 μM in the deuterium incubation buffer.

Structure Determination and Refinement. The crystal structure was determined by the method of molecular replacement with AMORE by using internal ERαLBD structures as a search model. The program suite QUANTA 2000 (Accelrys, Inc.R factors are R_{work} = 0.208, R_{free} = 0.257 for crystal ER/LY156681, and R_{work} = 0.210, R_{free} = 0.273 for crystal ER/LY117018 (Table S1). The coordinates have been deposited in the Protein Data Bank (PDB ID codes = 2R6W and 2R6Y).

ER Binding Assay. The competition binding assay was performed in a buffer containing 50 mM Hepes (pH 7.5), 1.5 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mg/ml ovalbumin, and 5 mM DTT, using 0.025 μCi per well [^{3}H]estradiol (NET517 at 118 Ci/mmol, 1.5 nM E2; NEN/PerkinElmer), and 10 ng per well ERα or ERβ. Nonspecific binding was determined in the presence of 1 μM 17-β estradiol (E2). The binding reaction was incubated 4 h at room temperature, and then a cold DCC buffer was added to each reaction [The DCC buffer contains per 50 ml of assay buffer, 0.75 g of charcoal (Sigma), and 0.25 g of dextran (Amersham Pharmacia)]. Plates were mixed for 8 min on an orbital shaker at 4°C and then centrifuged at 1,000 × g at 4°C for 10 min. An aliquot of the mix was added to Wallac Optiphase "Hisafe 3" scintillation fluid, incubated for 5 h, and read in a Wallac Microbeta counter. The K_d for [^{3}H]estradiol was determined by saturation binding for ERα or ERβ receptors. The IC_{50} values for compounds were converted to K_i using Cheng–Prusoff equation.

MCF-7 Cell Assay. MCF-7 breast adenocarcinoma cells (ATCC HTB 22) were grown in assay medium MEM (phenol red-free, GIBCO–BRL) supplemented with 10% DCC-FBS. Cells were assayed by plating 8,000 cells in each well of 96-well Cytostar T scintillation plates (Amersham) and incubated at 37°C for 24 h before adding compounds. For antagonist mode, 10 pM E2 was added along with dilutions of compounds for 48 h, and then medium containing 0.01 μCi of [^{14}C]thymidine (52 mCi/ul; Amersham) was added to each well. The plates were incubated overnight and then quantitated on a Wallac counter. The data were averaged to calculate an IC_{50} and percentage of inhibition at 1 μM.

Ishikawa Assay. Estrogen stimulation and antagonism were measured in Ishikawa human endometrial tumor cells by alkaline phosphate quantitation. The cells were incubated in DMEM/F-12 (3:1) supplemented with 5% DCC-stripped FBS (HyClone), L-glutamine (2 nM), MEM sodium pyruvate (1 mM), Hepes (2 mM) (all from Life Technologies). For the agonist mode, plates received diluted compounds only, whereas antagonist mode plates additionally received 1 nM estradiol (E2; Sigma). Cells were incubated for 48 h, and then fresh compounds were added for an additional 72 h. The assay was quenched by removing medium and rinsing plates twice in PBS, and the plates were dried for 5 min and frozen at −70°C. After thawing, 100 μl of 1-Step p-nitrophenyl phosphate (PNPP) (Pierce) were added for 20 min, and plates were read on a spectrophotometer at 405 nm. For the agonist mode, a percentage increase over control was calculated. The data were fitted to a linear interpolation to derive IC_{50} values for the antagonist mode, and a percentage efficacy was calculated that blocks the E2 (1 nM) stimulus.

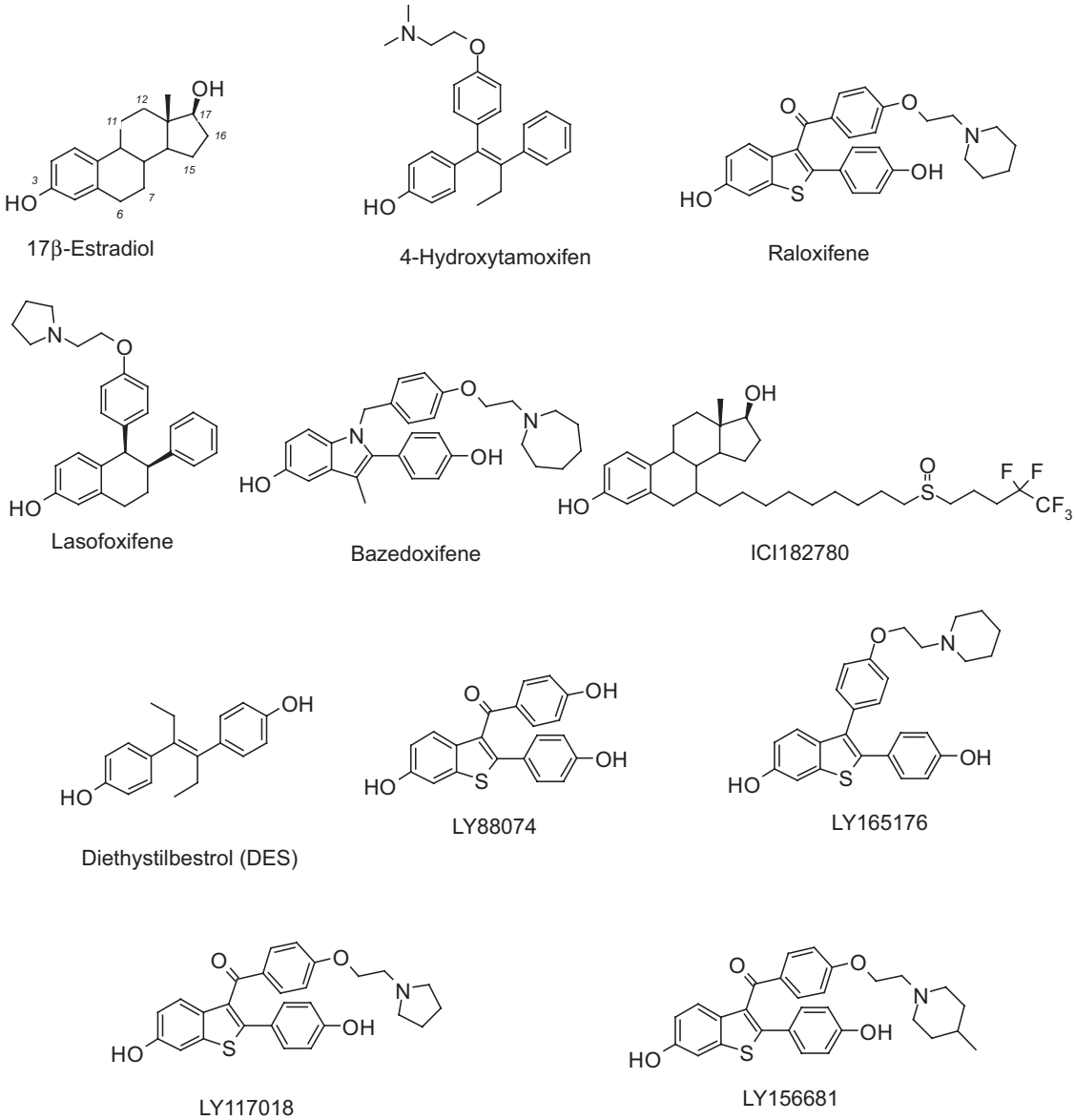

Fig. S1. Chemical structures of 11 ER ligands.

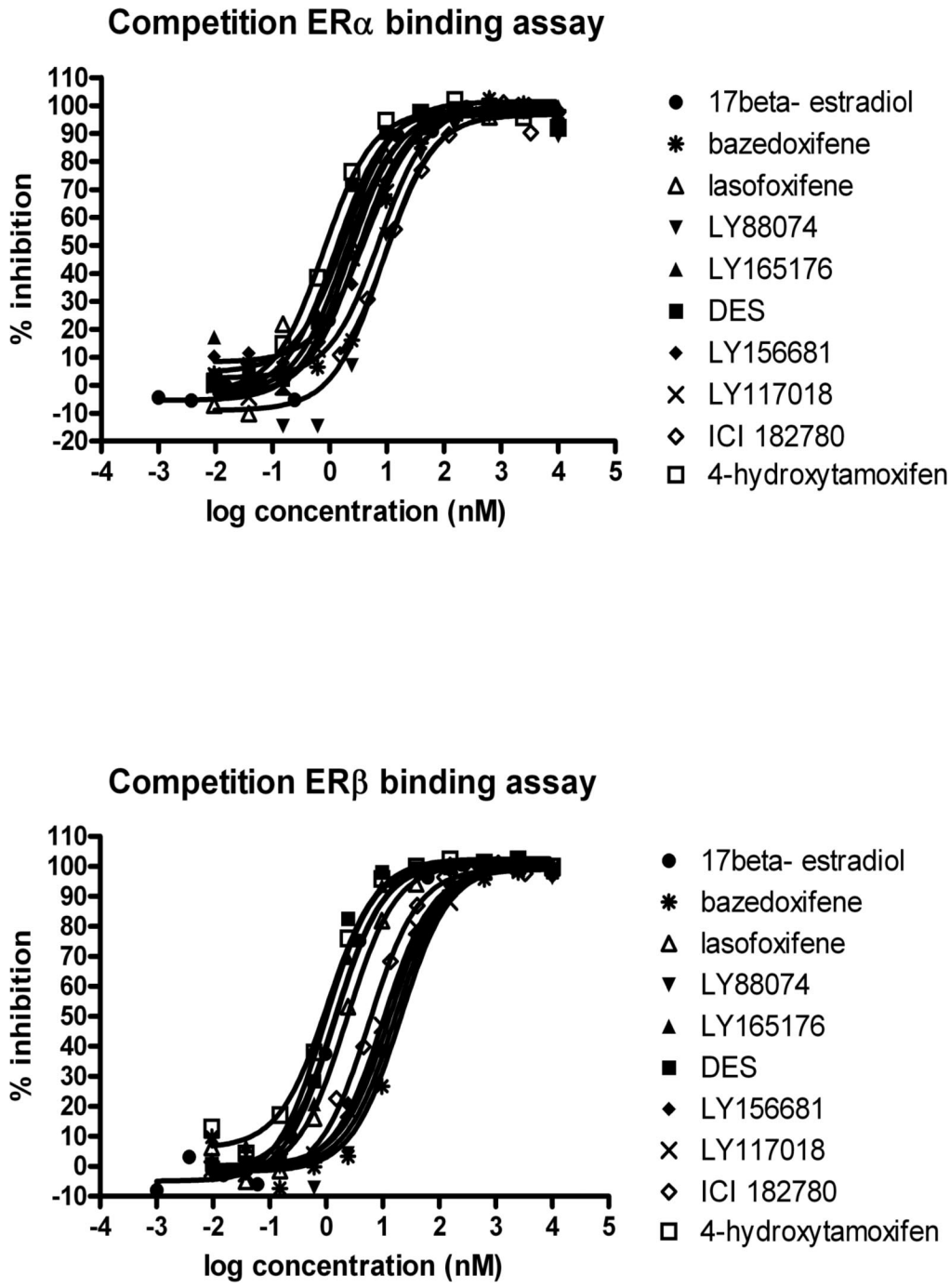

Fig. S2. ER ligand-binding curves. The percentage of inhibition of [^{3}H]estradiol was measured for ERα (*Upper*) and ERβ (*Lower*) in competition binding assays. This representative experiment demonstrates that all compounds tested bound to ERα and ERβ receptors with IC_{50} values <10 nM. The IC_{50} data were converted to K_i by using the Cheng–Prussof equation and represented in Table 1 as Mean $\pm$ SD of 3–6 experiments.

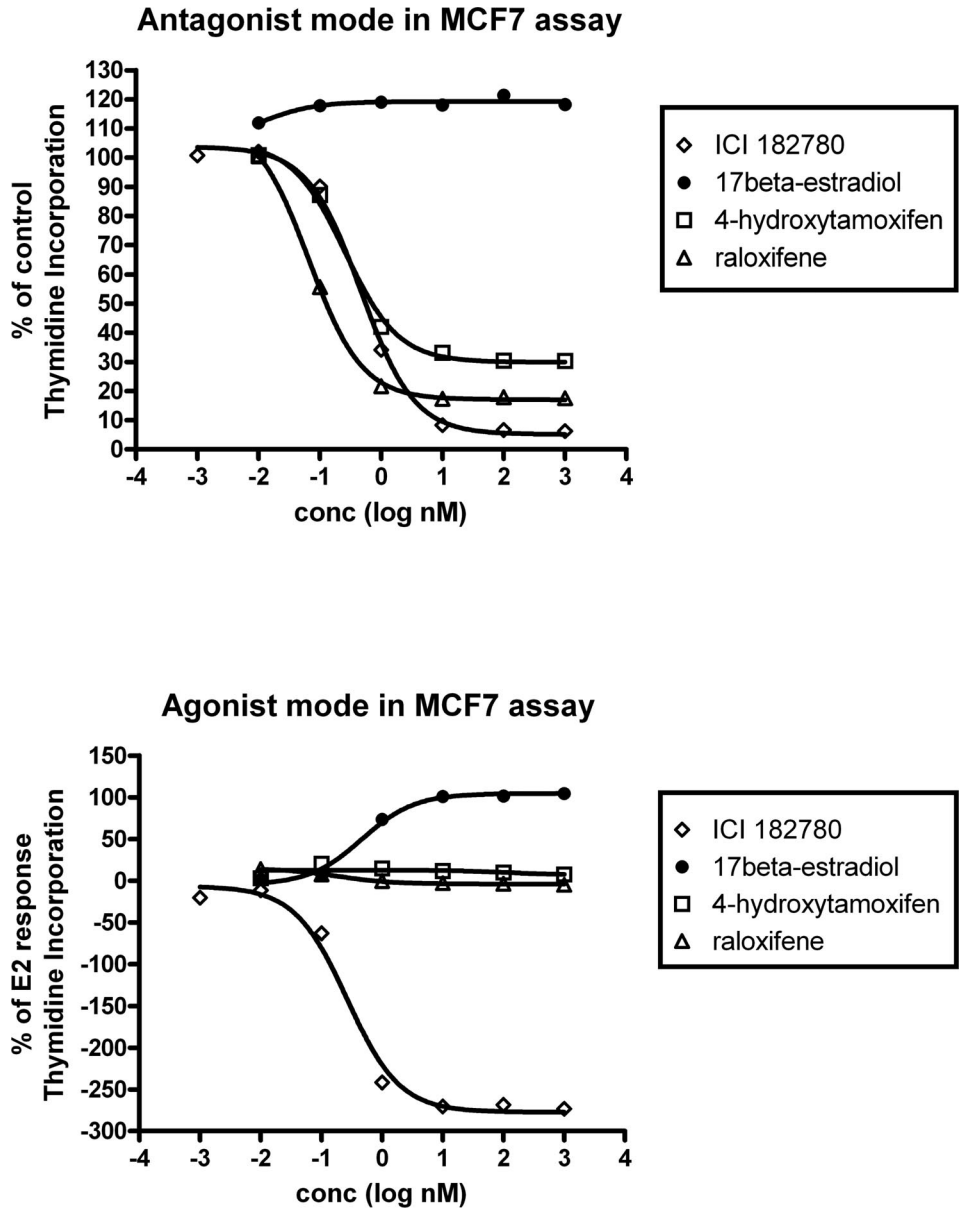

Fig. S3. Antagonist and agonist modes of ER ligands in MCF-7 cell assay. The representative curves were shown for E2, 4-hydroxytamoxifen, raloxifene, and ICI 182780. The percentage of thymidine incorporation is referred to the maximum response of E2 in agonist mode, or to the response of 10 pM E2 in the antagonist mode.

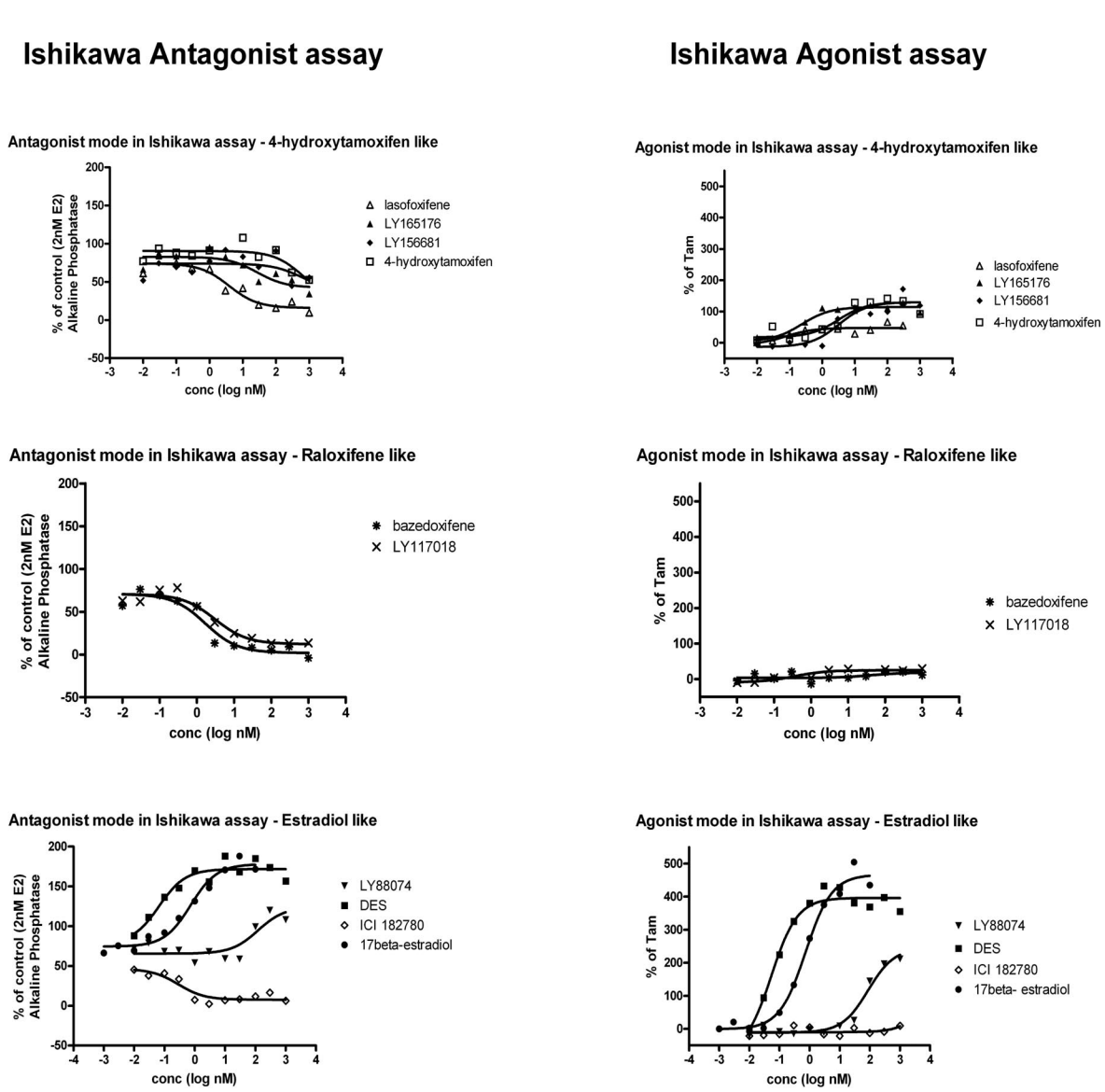

Fig. S4. Antagonist and agonist modes of ER ligands in Ishikawa cell assay. The curves are shown as 4-hydroxytamoxifen-like group, raloxifene-like group, and estradiol-like group individually which is correlated to the HDX classification in the study. In the antagonist mode assay, the percentage of inhibition is referred to blocking 2 nM of estradiol and in the agonist mode assay; the percentage of stimulation was compared with 4-hydroxytamoxifen.

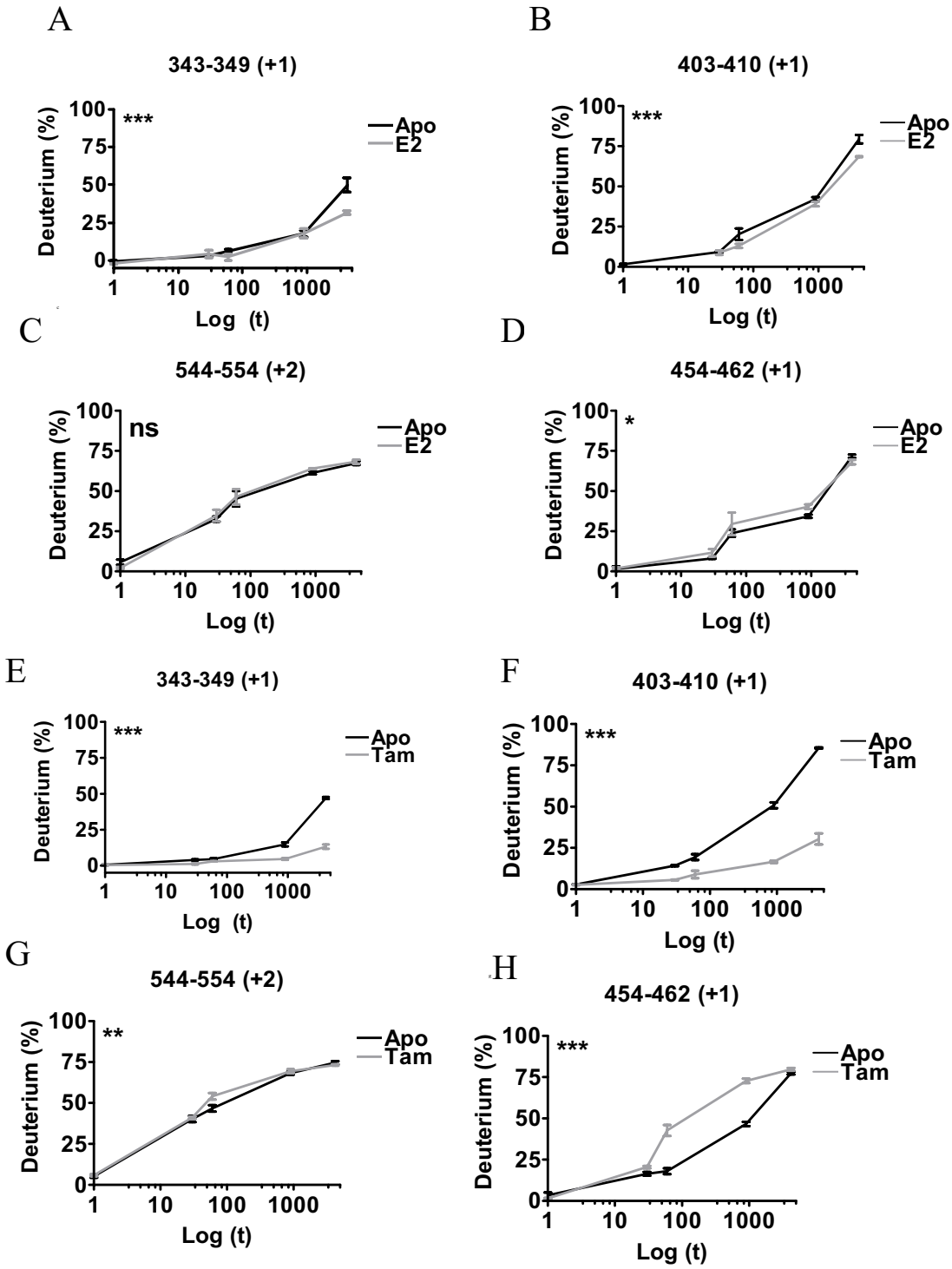

Fig. S5. Deuterium incorporation curves of four peptides. HDX data for the *apo* ER LBD is represented by the black line. The gray line represents the HDX data for the ligand bound ER LBD. Helix 3 (343–349) and β-sheet1/β-sheet2 (403–410) are protected from HDX when incubated with 4-hydroxytamoxifen (*E* and *F*) compared with estradiol (*A* and *B*) versus helix 12 (544–554) has no significant change (*C* and *G*). Helix 10 and the loop region (454–462) shows an increase in deuterium incorporation when incubated with 4-hydroxytamoxifen (*H*) compared with estradiol (*D*). Statistical summary from a two-way ANOVA between *apo* and ligand bound data. ***, $P < 0.001$; **, $P = 0.001$ to 0.01; *, P = 0.01 to 0.5; ns = not significant. The value in parentheses represents the charge state of the peptide ion.

Table S1. Data collection and refinement statistics (molecular replacement)

	Crystal 1 name	Crystal 2 name
Data collection	156,681	117,018
Space group	C2 2 21	C2 2 21
Cell dimensions		
a, *b*, *c*, Å	56.47, 102.761, 172.952	63.132, 98.737, 174.981
a, *b*, *c*, °	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Resolution, Å		
R_{sym} or R_{merge}	0.048 (0.219)	0.045 (0.324)
$I/\sigma I$	35.7 (4.2)	31 (2.0)
Completeness, %	92.2 (57.1)	91.3 (54.8)
Redundancy	6.3 (3.4)	4.5 (3.3)
Refinement		
Resolution, Å	2.00	2.00
No. of reflections	29,199	33,075
R_{work}/R_{free}	0.208/0.257	0.210/0.273
No. of atoms		
Protein	3,626	3,651
Ligand/ion	70	66
Water	104	150
Mean *B*-factors	40.144	42.581
R.m.s. deviations		
Bond lengths, Å	0.017	0.017
Bond angles, °	1.579	1.569

Table S2. Weights for each peptide in each group.

Variable	Group 1	Group 2	Group 3
Constant	−220,764,435	−1.79E + 09	−976,264,088
Loop	−46,395,965	−132,140,350	−97,570,816
S1S2	−29,950,674	−85,297,475	−62,978,767
H8	−8,424,706	−23,988,630	−17,708,508
H10_1	3,405,652	9,723,162	7,197,383
H10_2	2,245,260	6,364,138	4,675,888

A total of five peptides: the loop (320–327) S1S2 (403–410), H8 (421–428) and the two charge states of H10 (463–486) as listed in Table 2 were selected for the classification.