Modulation of Human Estrogen Receptor α Activity by Multivalent Estradiol-peptidomimetic Conjugates

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Supplementary Information

Synthesis and characterization of estradiol-peptidomimetic conjugates

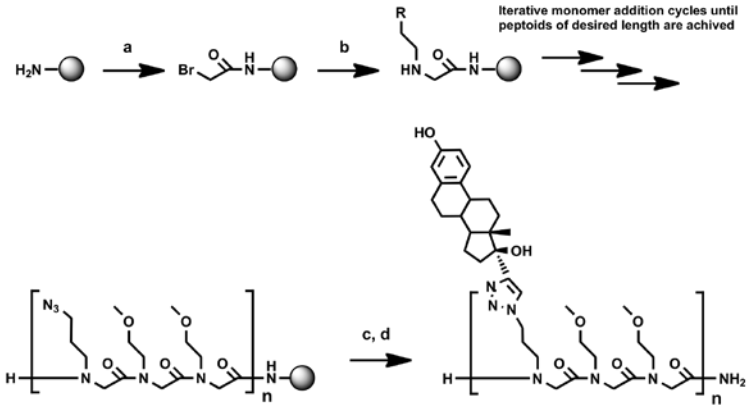
General synthesis of estradiol-peptidomimetic conjugates: Peptoid scaffolds including site-specifically positioned azidopropyl sidechains were synthesized using standard submonomer synthesis techniques described previously[1] (Scheme S1). Following oligomerization, linear resin-bound peptoids were suspended in 2-butanol:DMF:pyridine, 5:3:2, v/v/v (0.2 mL g^{-1} resin) and CuI, ascorbic acid and *N,N*-diisopropylethylamine (DIPEA) were added to the reaction mixture (see Scheme S1 and Table S1 for full reactant concentrations). The vessel was purged with nitrogen, capped and shaken at 45 °C for 18 hrs. The resin was then washed consecutively with DMF, copper scavenger (DMF/pyridine 6/5 v/v, ascorbic acid 0.02 g mL^{-1}) and DCM. The resin was then dried and a small amount was removed for characterization.

Purification of estradiol-peptidomimetic conjugates by RP-HPLC: Resin-bound EPCs were cleaved from solid support using 95% TFA in water (10 ml g^{-1} resin) for 10 min at rt. The cleavage cocktail was removed under reduced pressure and crude EPCs were resuspended in 80% water in acetonitrile, frozen and lyophilized to dryness. Crude EPC powder was redissolved in an appropriate volume of 50% water in acetonitrile and purified on a C18 preparatory column (Waters) on a System Gold HPLC system (Beckman Coulter). Linear gradients were conducted from 5% to 95% solvent B (0.1% TFA in acetonitrile) over solvent A (0.1% TFA in water) in 50 minutes with a flow rate of 5.0 mL min^{-1}. Product elution was monitored at 230 nm using a System Gold 166 detector (Beckman Coulter). Purified fractions were combined, frozen and lyophilized to dryness. Purified (>95%) EPCs were then dissolved at a final concentration of 10 mM in 100% EtOH.

Analytical Reversed-Phase High Pressure Liquid Chromatography (RP-HPLC): Peptoid oligomers were characterized by RP-HPLC using an analytical reversed-phase C18 column (Peeke Scientific) on a System Gold HPLC system (Beckman Coulter). All products were detected by UV absorbance at 214 nm. Linear gradients were conducted from 5% to 95% solvent B (0.1% TFA in acetonitrile) over solvent A (0.1% TFA

in water) in 10 min with a flow rate of 0.7 mL min^{-1}. Crude purities were estimated by RP-HPLC peak integration techniques described previously by Jang *et al*[2].

Mass Spectrometry: Additional characterization of peptoid oligomers was conducted by LC/MS. All estradiol peptidomimetic conjugates described herein (mass range: 684.84 to 4018.28 Da) were analyzed using an Agilent 1100 Series capillary LC system coupled to an ion trap mass spectrometer in positive ion mode. See Table S2 for full mass data.



Scheme S1. General scheme for the synthesis of multivalent estradiol peptidomimetic conjugates. a) Bromoacetic acid (1.2 M in DMF, 8.5 mL g^{-1} resin) and diisopropylcarbodiimide (2 mL g^{-1} resin), 20 min, rt. b) Submonomer amine (1.0 M in DMF, 10 mL g^{-1} resin), 20 min, rt. c) CuI, 17α-ethynylestradiol and ascorbic acid in 2-butanol/DMF/pyridine (5:3:2, v/v/v, 0.2 mL mg^{-1} resin), 18 h, 45 °C (see Table S1 for reactant concentrations). d) 95% TFA in water (40 mL g^{-1} resin), 10 min, rt.

Entry	Compound	Valency (n)	[EE2][a]	[CuI]	[AA][b]	[DIPEA]	Purity[c]
1	**1**	1	0.05 M	0.10 M	0.05 M	0.12 M	87%
2	**2**	2	0.125 M	0.24 M	0.125 M	0.30 M	69%
3	**3**	3	0.15 M	0.29 M	0.15 M	0.36 M	58%
4	**4**	6	0.15 M	0.29 M	0.15 M	0.36 M	54%
5	**5**	2	0.125 M	0.24 M	0.125 M	0.30 M	72%
6	**6**	2	0.125 M	0.24 M	0.125 M	0.30 M	70%
7	**7**	2	0.125 M	0.24 M	0.125 M	0.30 M	43%

Table S1. Reactant concentrations for solid-phase synthesis of multi- and divalent estradiol-peptidomimetic conjugates. [a]17α-ethynylestradiol; [b]ascorbic acid; [c]crude purity as determined by analytical RP-HPLC product peak integration (λ = 214 nm). All compounds were extensively purified (>95%) prior to biological assays (*vida supra).*

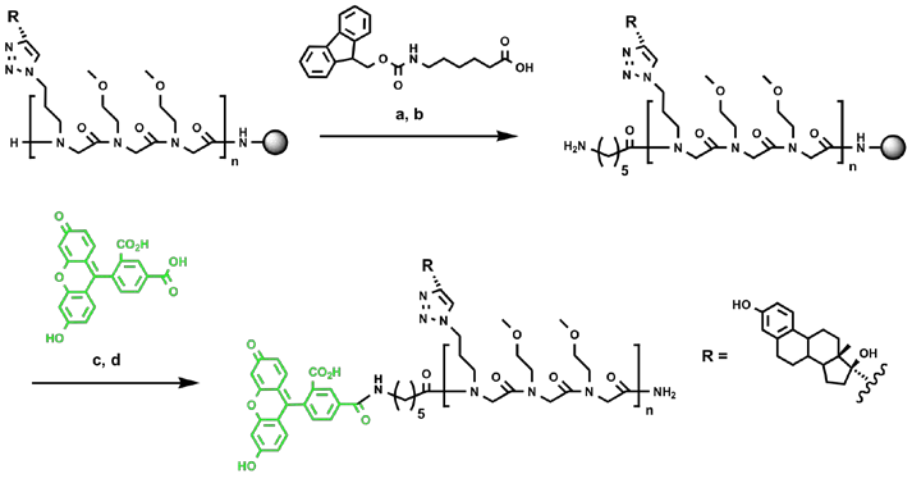
Entry	Compound	Charge[a]	Calc. *m/z*	Obs. *m/z*
1	**1**	+1	684.84	684.52
2	**2**	+1	1350.84	1350.91
3	**3**	+1	2018.45	2018.32
4	**4**	+2	2009.14	2009.46
5	**5**	+1	1695.97	1695.65
		+2	848.99	849.22
6	**6**	+1	2040.15	2039.35
		+2	1021.08	1021.65
7	**7**	+2	1367.27	1368.37
		+3	911.85	912.47
8	**8**	+1	1155.54	1156.71
		+2	578.77	578.24
9	**9**	+1	1822.21	1822.56
		+2	912.11	911.22
10	**10**	+2	1245.46	1244.47
		+3	830.64	831.01
11	**11**	+1	2167.50	2166.32
		+2	1084.75	1085.12
12	**12**	+2	1257.14	1258.70
		+3	838.43	835.13
13	**13**	+2	1602.83	1602.80
		+3	1068.89	1067.11
14	**14**	+1	1644.99	1643.24
		+2	823.49	824.62

Table S2. Mass spectrometry data for steroid-peptidomimetic conjugates. [a]All mass spectra data were generated in positive ion mode.

Synthesis of fluorescently labeled estradiol-peptidomimetic conjugates

All fluorescently labeled EPCs reported herein (compounds **8** – **13**) were synthesized under identical reaction conditions (Scheme S2). Fmoc-6-Ahx-OH (Novabiochem) was dissolved at a concentration of 0.25 M in a solution of DMF containing 0.25 M PyBOP and 1.0 M *N*-methylmorpholine. This solution (10 mL g^{-1} resin) was added to the resin-bound EPCs and the reaction was allowed to stir at room temperature for 2 h. Following completion, the reactants were washed from the solid-support with DMF and DCM, and the terminal amine was deprotected (20% piperidine in DMF, 15 mL g^{-1} resin) for 20 min at rt. 5,6 carboxyfluorescein (Sigma) was pre-activated by dissolving the dye at a concentration of 0.43 M in DMF

containing 0.43 M HBTU and 0.68 M *N*-methylmorpholine, and stirring in the dark for 30 min at rt. This solution was then added to the deprotected resin-bound EPCs (15 ml g^{-1} resin) and allowed to stir in the dark for 20 h at rt. Following completion, the reactants were washed from the resin with DMF and DCM, and the fluorescently labeled EPCs were cleaved and characterized as described above. Crude Flu-EPCs were then purified to >95% by RP-HPLC as described, frozen and lyophilized to dryness. The resultant Flu-EPC powder was dissolved in 100% EtOH to a final concentration of 10 mM.



Scheme S2. General scheme for the solid phase synthesis of fluorescently-labeled estradiol-peptidomimetic conjugates. a) 0.25 M Fmoc-6-Ahx-OH, 0.25 M PyBOP, 1.0 M *N*-methylmorpholine in DMF, 2 h, rt. b) 20% piperidine in DMF, 20 min, rt. c) 0.43 M 5,6 carboxyfluorescein, 0.43 M HBTU, 0.68 M *N*-methylmorpholine in DMF, 20 h, rt. d) 95% TFA in water, 10 min, rt.

Evaluation of emission intensities of fluorescently labeled EPCs

Emission spectra for all Flu-EPC constructs reported herein (compounds **8 – 13**) were generated under identical conditions (Figure S1). Fluorescently labeled EPCs (10 mM in EtOH) were dissolved in water to a final concentration of 3.0 μM. These solutions were transferred to a quartz cell (Starna) and emission spectra were generated on a fluorescence polarization spectrometer (Hitachi). Excitation wavelengths were set to 442 nm, corresponding to the predetermined maximum excitation wavelength for 5,6 carboxyfluorescein (3.0 μM in water). All scans were performed from 450 to 700 nm at rt.

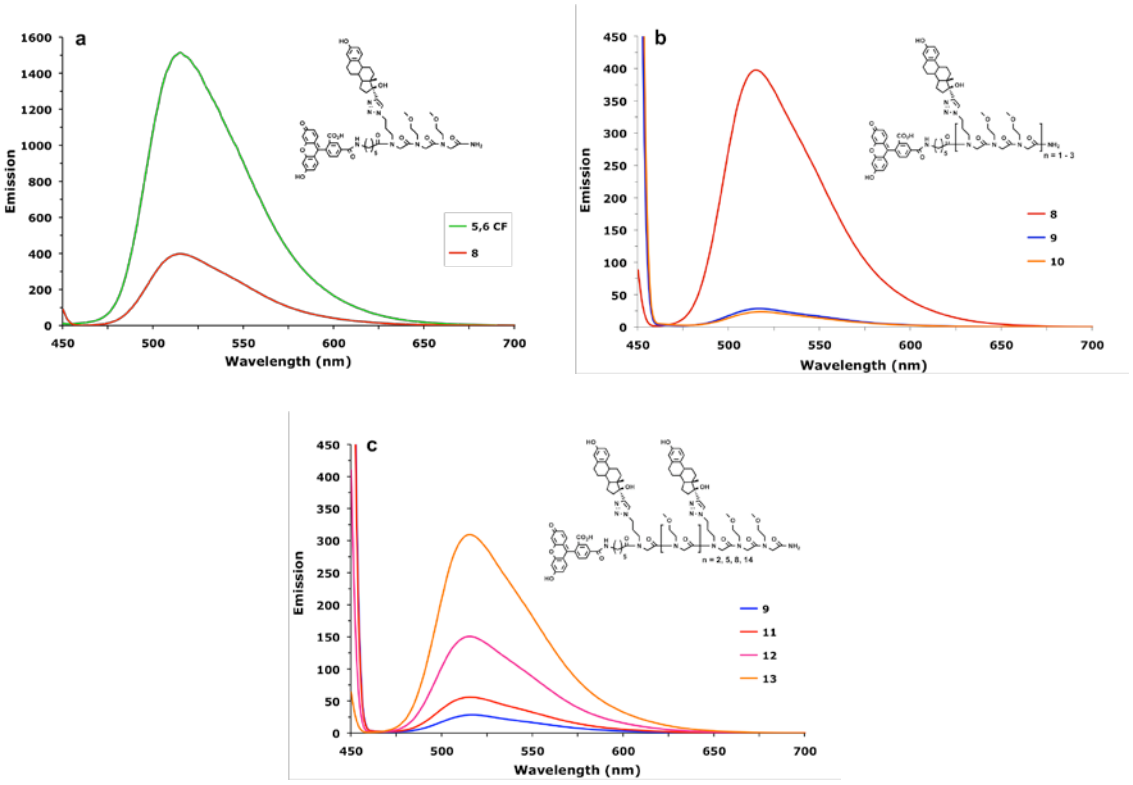


Figure S1. Fluorescence emission spectra of Flu-EPCs **8 – 13** (Ex_{λ} = 442 nm). a) Emission spectra of 5,6 carboxyfluorescein (5,6 CF) and Flu-EPC **8**, 3.0 μM in water. b) Fluorescence emission spectra of multivalent Flu-EPCs **8**, **9**, and **10**, 3.0 μM in water. c) Fluorescence emission spectra of divalent Flu-EPCs **9**, **11**, **12**, and **13**, 3.0 μM in water.

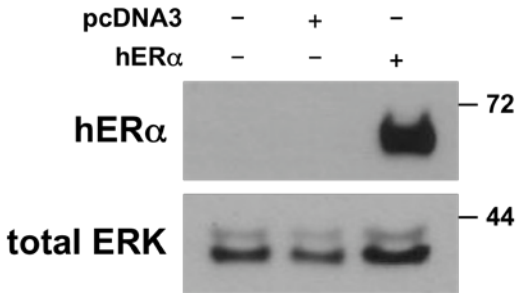


Figure S2. Expression of human estrogen receptor α in transfected HEK293 cells. HEK293 cells were transfected with plasmids containing hERα (hERα) or empty vector (pcDNA3). Following transfection, the cells were recovered and allowed to incubate for 48 h. Intracellular proteins were then extracted, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blot; 50 μg protein/lane. Size markers (kDa) are shown to the right of the blot.

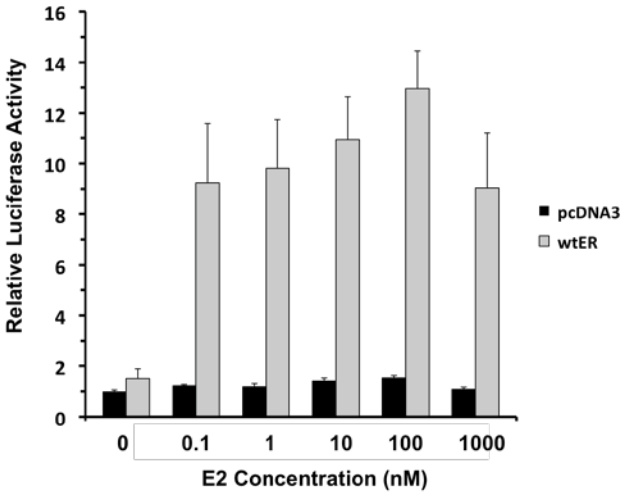


Figure S3. Activation of the hERα by 17β-estradiol (E2) is concentration dependant. HEK293 cells were stably transfected with XETL reporter plasmid, pcDNA3-hERα or empty vector, pCMV-LacZ, and pBluescript II SK. Cells were serum-starved for 48 h before treatments and E2 was administered to cells at concentrations indicated. Luciferase activities were quantified and normalized to vehicle treatment in pcDNA3-containing transfectants.

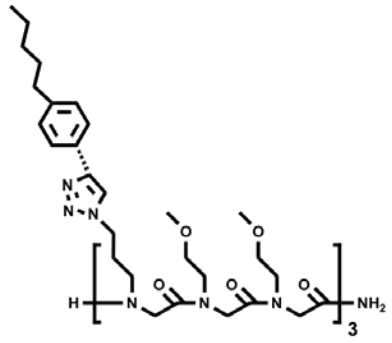


Figure S4. Non-steroidal, trivalent control peptidomimetic conjugate **14**. Synthesis was performed on solid-phase support as shown in Scheme S1 under the following conditions: 0.025 M 1-ethynyl 4-pentylbenzene (Sigma), 0.05 M CuI, 0.025 M AA, 0.06 M DIPEA, 18 h rt. Crude purity was found to be 43% by RP-HPLC. Peptoid **14** was purified and characterized as described above (see Table S2 for mass data).

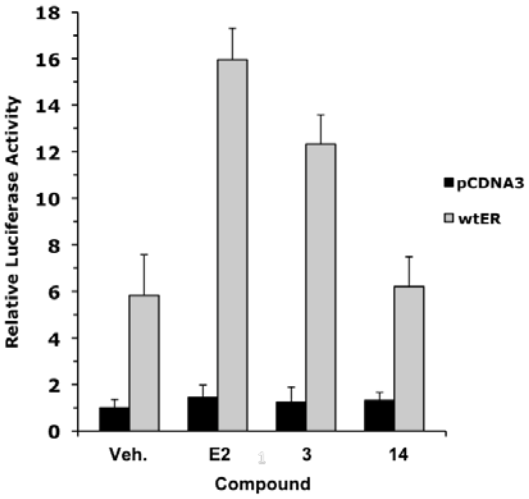


Figure S5. The hERα is not activated by non-steroidal peptoid conjugate **14**. HEK293 cells were stably transfected with XETL reporter plasmid, pcDNA3-hERα or empty vector, pCMV-LacZ, and pBluescript II SK. Cells were serum-starved for 48 h before treatments. All compounds were administered to cells at a concentration of 100 nM for 18 h. Luciferase activities were quantified and normalized to vehicle treatment in pcDNA3-containing transfectants. Veh. = vehicle (EtOH, 0.1% in media); E2 = 17β-estradiol.

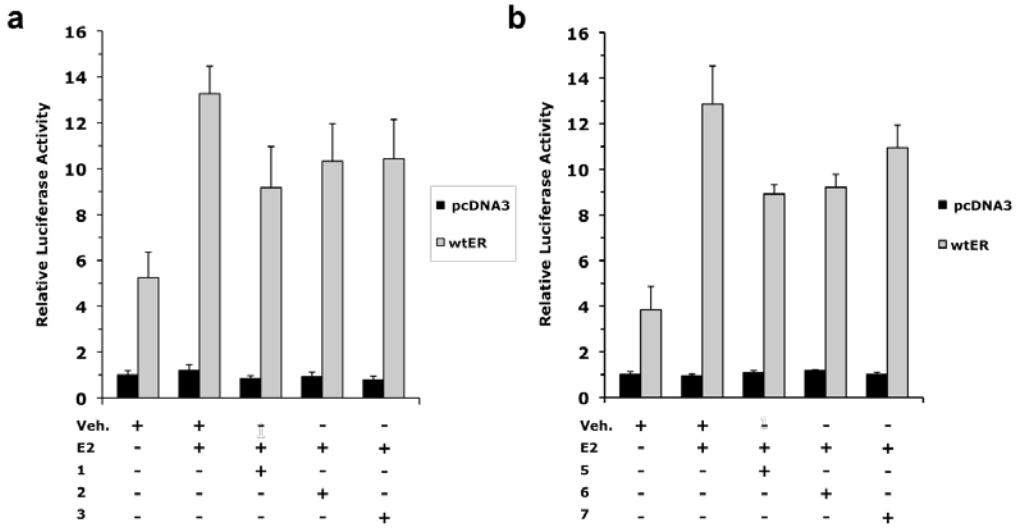


Figure S6. EPCs a) **1 – 3** or b) **5 – 7** do not block E2-mediated activation of the hERα. HEK293 cells were stably transfected with XETL, pcDNA3-hERα or empty vector, pCMV-LacZ, and pBluescript II SK. Cells were serum-starved for 48 h before treatments. Cells were treated with either vehicle or compounds **1 – 3**, **5 - 7** (100 nM) and incubated for 8 h. Following incubation, E2 (100 nM) was added to the pretreated cells. The cells were allowed to incubate for an additional 10 h. The final concentration of EtOH in the treatment media was 0.2%. Luciferase activities were quantified and normalized to vehicle treatment in pcDNA3

transfectants. Veh. = vehicle (EtOH, 0.2% in media); E2 = 17β-estradiol.

References

1. R. N. Zuckermann, J. M. Kerr, S. B. H. Kent, and W. H. Moos, *J. Am. Chem. Soc.*, 1992, **114**, 10646-10647.
2. H. Jang, A. Farfarman, J. M. Holub and K. Kirshenbaum, *Org. Lett.*, 2005, **7**, 1951-1954.