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Synthesis and evaluation of tamoxifen derivatives with a long alkyl side chain as selective estrogen receptor down-regulators

Takuji Shoda [a,*], Masashi Kato [a,b], Rintaro Harada [a], Takuma Fujisato [a,b], Keiichiro Okuhira [c], Yosuke Demizu [a], Hideshi Inoue [b], Mikihiko Naito [c], Masaaki Kurihara [a,d,*]

[a] Division of Organic Chemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[b] School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
[c] Division of Biochemistry and Molecular Biology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[d] Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama, Kanagawa 226-8501, Japan

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ABSTRACT

Estrogen receptors (ERs) play a major role in the growth of human breast cancer cells. An antagonist that acts as not only an inhibitor of ligand binding but also an inducer of the down-regulation of ER would be useful for the treatment for ER-positive breast cancer. We previously reported the design and synthesis of a selective estrogen receptor down-regulator (SERD), (*E*/*Z*)-4-(1-{4-[2-(dodecylamino)ethoxy]phenyl}-2-phenylbut-1-en-1-yl)phenol (C12), which is a tamoxifen derivative having a long alkyl chain on the amine moiety. This compound induced degradation of ERα via a proteasome-dependent pathway and showed an antagonistic effect in MCF-7 cells. With the aim of increasing the potency of SERDs, we designed and synthesized various tamoxifen derivatives that have various lengths and terminal groups of the long alkyl side chain. During the course of our investigation, C10F having a 10-fluorodecyl group on the amine moiety of 4-OHT was shown to be the most potent compound among the tamoxifen derivatives. Moreover, computational docking analysis suggested that the long alkyl chain interacted with the hydrophobic region on the surface of the ER, which is a binding site of helix 12 and coactivator. These results provide useful information to develop promising candidates as SERDs.

1. Introduction

Estrogen receptors (ERs) are a class of the nuclear receptor family divided into two subtypes, ERα and ERβ, which are derived from distinct genes but display a high degree of structural conservation in their DNA and ligand binding domains (LBDs).[1–5] These two ER isoforms are expressed at similar low levels in normal breast. At least 70% of breast cancer patients are categorized as having ER-positive cancer because growth of the tumor cells occurs by ERα activation.[6–8] ERs are activated by estrogen, such as 17β-estradiol (E2), which is produced by the ovaries. Once estrogen binds to the ER, the conformation of the ER is changed and coactivator can bind to it. The ER-coactivator complex binds to the promoter region of genes and transcriptional pathways are activated. Therefore, the proliferation of ER-positive breast cancer cells is promoted by estrogens.[9] Inhibition of ER activation is thus important for the development of anti-breast cancer drugs.[1–3]

Tamoxifen (Fig. 1) is the most widely used drug for hormonal therapy of ER-positive breast cancer. Tamoxifen is a triphenylethylene derivative that is metabolized by drug-metabolizing enzymes into 4-hydroxytamoxifen (4-OHT) and endoxifen (Fig. 1), which have 100 times more affinity for ER than tamoxifen itself.[10–13] The antagonistic mechanism of 4-OHT was suggested by the X-ray structure of 4-OHT and human ERα LBD.[14] The triphenylethylene core was shown to be buried in the ligand binding pocket and the dimethylamino group of 4-OHT projected out of the ligand binding pocket. Therefore, the conformation of helix 12 is changed to an antagonistic formation.[14]

Fulvestrant (ICI 182,780) and ICI 164,384 (Fig. 2), which are E2 derivatives with a long alkyl side chain from the 7α-position of E2, are known as pure antagonists. These compounds not only interfere with the binding of E2 to ER but also induce the rapid down-regulation of ER.[15,16] Owing to this latter property, these

Abbreviations: $(Boc)_2O$, di-*tert*-butyl dicarbonate; E2, 17β-estradiol; ER, estrogen receptor; DIEA, *N*,*N*-diisopropylethylamine; DMAP, *N*,*N*-dimethyl-4-aminopyridine; DMSO, dimethylsulfoxide; 4-OHT, 4-hydroxytamoxifen; SERD, selective estrogen receptor down-regulator; TBAF, tetra-*n*-butylammonium fluoride; THF, tetrahydrofuran; TsCl, *p*-toluenesulfonyl chloride.

* Corresponding authors. Tel.: +81 3 3700 1141; fax: +81 3 3707 6950.
E-mail addresses: tsho@nihs.go.jp (T. Shoda), masaaki@nihs.go.jp (M. Kurihara).

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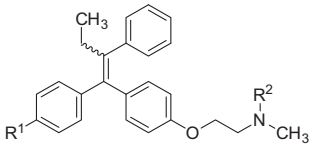
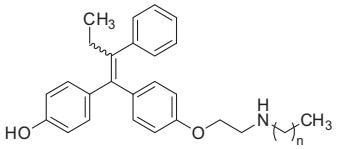


Tamoxifen (R^1 = H, R^2 = CH_3)
4-OHT (R^1 = OH, R^2 = CH_3)
Endoxifen (R^1 = OH, R^2 = H)

Figure 1. Structures of tamoxifen, 4-OHT and endoxifen.



C6 (n = 5)
C12 (n = 11)
C18 (n = 17)

Figure 3. Structures of tamoxifen derivatives.[18]

compounds are called selective estrogen receptor down-regulators (SERDs). The X-ray structure of ICI 164,384 and rat ERβ complex suggested the pure antagonistic mechanism of SERDs.[17] The alkyl side chain of ICI 164,384 was shown to protrude from the ligand binding pocket and the terminal of the alkyl side chain is thrust into a small hydrophobic pocket, which is formed by the side chains of Leu261, Met264, Ile265 and Leu286 in helices 3 and 5.[17] The protruding alkyl side chain of ICI 164,384 sterically prevents both the positioning of helix 12 and coactivator binding, resulting in destabilization of the ER.[17]

We previously reported the design and synthesis of tamoxifen derivatives with a long alkyl side chain on the amine moiety of 4-OHT (Fig. 3).[18] We synthesized triphenylethylene derivatives in which various lengths of alkyl chain had been introduced, such as hexyl (C6), dodecyl (C12) and octadecyl (C18) (Fig. 3). We expected that a long alkyl chain on the amine moiety might protrude from the ligand binding pocket and prevent helix 12 interaction and coactivator binding, resulting in destabilization of the ER. Among these compounds, C12 showed the ability to down-regulate the ER and an antagonistic effect in MCF-7 cells.[18]

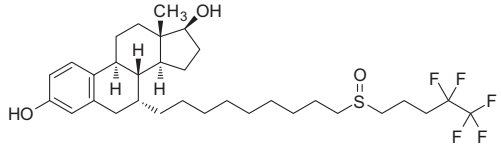
In the present study, in order to obtain more potent SERDs and to clarify the mechanism of down-regulation of ER, we sought to design and synthesize six tamoxifen derivatives by modification of the alkyl chain. We evaluated the ability of ER degradation in MCF-7 cells and the binding affinity for ER. After optimization of the length of the alkyl chain, we performed modification of its terminal. Furthermore, we performed a computational study to predict the binding mode of our compound to ER. The result indicated that the interaction between the alkyl chain and the hydrophobic surface of ER is important for the down-regulation of ER.

2. Results

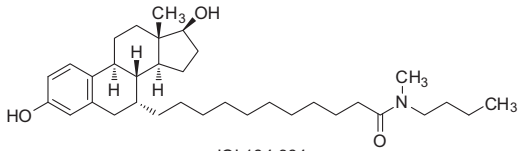
2.1. Chemistry

We designed six novel 4-OHT derivatives with octyl (C8), decyl (C10), tetradecyl (C14), hexadecyl (C16), 10-hydroxydecyl (C10OH) and 10-fluorodecyl (C10F) amines. The routes of synthesis of these compounds are summarized in Schemes 1 and 2. These compounds were prepared by using a microwave apparatus from (*E*/*Z*)-4-[1-{4-(2-chloroethoxy)phenyl}-2-phenylbut-1-en-1-yl]phenol (**1**)[18,19] and the corresponding alkyl amines with a base. We obtained all compounds at moderate yields.

The synthesis of C10F is depicted in Scheme 2. It was synthesized in 4 steps. The amine moiety of 10-amino-1-decanol was protected by the Boc group and the hydroxyl moiety was tosylated to obtain **7**. Treatment with TBAF followed by HCl resulted in **9**. Finally, C10F was obtained from **1** and **9** by using a microwave apparatus at a sufficient yield.



Fulvestrant (ICI 182,780)



ICI 164,384

Figure 2. Structures of fulvestrant and ICI 164,384.

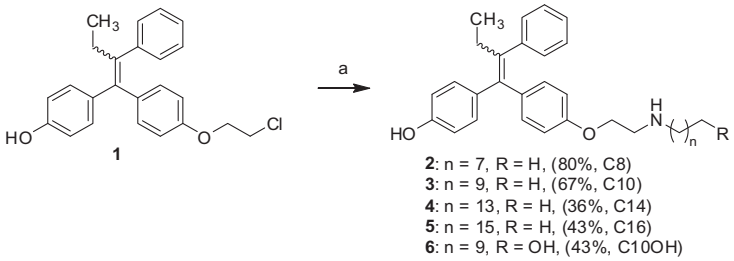
2.2. Down-regulation of ER

First, we examined the effects of the length of the long alkyl chain on reducing ERα protein levels in MCF-7 breast cancer cells. MCF-7 cells were treated with these compounds, whole protein was extracted and ERα protein levels were analyzed by Western blotting, as reported previously.[18,20] As shown in Figure 4, reductions of the ERα protein levels were observed in the cells treated with 10 μM C8, C10 and C12 (lanes 5–7) compared with the control (lane 1). C10 was the most effective compound among the long alkyl chain derivatives because the band of ERα had almost disappeared in association with it (lane 6). No significant differences in the treatment of C6, C14, C16 and C18 were observed under these conditions (lanes 4, 8, 9 and 10, respectively). The protein level of ERα was slightly increased by treatment with 10 μM 4-OHT and endoxifen (lanes 2 and 3), which was identical to findings reported previously.[21,22]

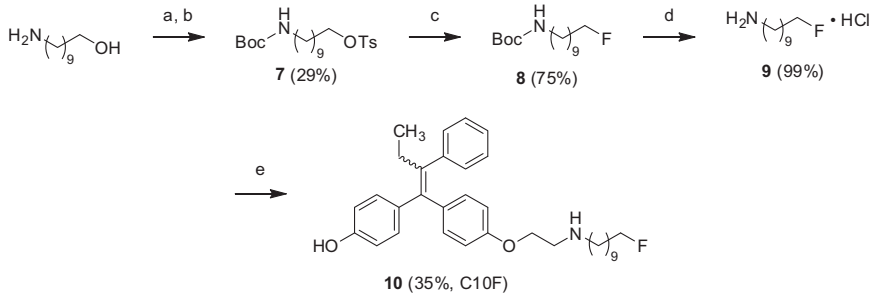
Next, we examined the effects of the terminal of the long alkyl chain in terms of reducing ERα protein levels in MCF-7 cells. C10F induced the reduction of ERα in the cells compared with C10 (Fig. 5, lanes 3 and 4). On the other hand, treatment with C10OH did not reduce the ERα protein level (lane 5). The reduction of ERα by C10F occurred in a dose-dependent manner (lanes 8–10) and was inhibited by a proteasome inhibitor, MG132 (lane 11). These results show that C10F has the ability to induce the proteasomal degradation of ERα in MCF-7 cells and that the length and the terminal group are important for the down-regulation of ER.

2.3. ER binding affinity

To evaluate the ability of compounds to bind to ERα, a fluorescence polarization-based competitive binding assay was conducted. The mean IC_{50} and RBA for the compounds tested in the ER competitive binding assay are presented in Table 1. The relative binding affinity (RBA) value for each competing compound was then calculated according to the IC_{50} of E2 (0.17 nM). The IC_{50} value of C18 was estimated to be over 100 μM because C18 had difficulty dissolving in the buffer solution and the binding curve of C18 did not saturate in the range that we examined. The IC_{50}s of C6 and C8 were 151 and 53 nM, respectively. The IC_{50}s of C10–C14 were in the range of 3.6–14 nM. Therefore, the binding affinities of C6,



Scheme 1. Synthesis of long alkyl chain derivatives of 4-OHT. Reagents and conditions: (a) $CH_3(CH_2)_nNH_2$ or 10-amino-1-decanol, triethylamine, methanol, microwave, 120 °C.



Scheme 2. Synthesis of C10F. Reagents and conditions: (a) $(Boc)_2O$, CH_2Cl_2, rt, 5 h, (b) TsCl, DMAP, pyridine, CH_2Cl_2, rt, 2 days, (c) TBAF, THF, reflux, 7 h, (d) HCl, 1,4-dioxane, rt, 2 h, (e) **1**, DIEA, CH_3OH, microwave, 120 °C, 6 h.

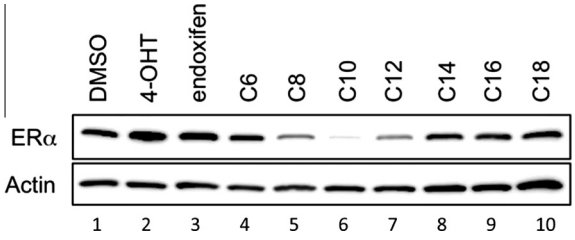


Figure 4. Reduction of endogenous ERα by 4-OHT, endoxifen and synthesized compounds (C6–C18). MCF-7 cells were incubated with 10 μM of the indicated compounds for 6 h. Shown are immunoblots of cell lysates stained with the indicated antibodies.

C8 and C18 are weaker than that of 4-OHT (5.6 nM), while those of C10–C16 are close to that of 4-OHT. The IC_{50} values of C10F and C10OH were 3.4 and 210 nM, respectively. The binding affinity of C10F was close to that of C10, whereas the binding affinity of C10OH was about 50-fold weaker than that of C10. These results indicate that the nature of the terminal of the alkyl chain plays an important role in the binding affinity for ER. Taking these findings together with the results of the Western blotting analysis

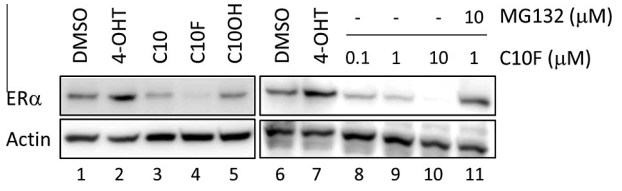


Figure 5. (Left) Reduction of endogenous ERα by 4-OHT, C10, C10F and C10OH. MCF-7 cells were incubated with 10 μM of the indicated compounds for 6 h. (Right) Dose-dependent response of ERα reduction induced by C10F and proteasome inhibitor MG132 inhibited ERα degradation. MCF-7 cells were incubated with the indicated concentrations of C10F for 6 h in the presence or absence of a proteasome inhibitor, MG132 (10 μM). Shown are immunoblots of cell lysates stained with antibodies mentioned in the Section 4.

Table 1
Estrogen receptor binding affinities of compounds[a]

	IC_{50} (nM)	RBA (%)
4-OHT	5.6	3.0
C6	151	0.11
C8	53	0.3
C10	3.6	4.7
C12	14	1.2
C14	11	1.5
C16	4.8	3.5
C18	>100,000	n.d.[b]
C10OH	210	0.08
C10F	3.4	5.0

[a] The IC_{50} value for each competing ligand was calculated according to the sigmoidal inhibition curve. The relative binding affinity (RBA) values were calculated against E2 by using the following equation: RBA (%) = 100 × (the IC_{50} for E2/the IC_{50} for the test compound).
[b] n.d. = not determined.

(Figs. 4 and 5), the length of the alkyl chain is important for binding and down-regulation of the ER and the introduction of a fluoro group at the terminal of the alkyl chain effectively increases the down-regulation without decreasing the binding activity of the compounds.

2.4. Computational modeling study

To predict the binding mode of the compound in the ER binding pocket, we conducted a computational modeling study. Figure 6 shows the docking models of C10 and human ERα LBD in which helix 12 is eliminated (PDB ID: 3ERT). In the calculated structure, the triphenylethylene core is located in the ligand binding pocket in a similar manner to 4-OHT. We also calculated the docking model of ICI 164,384 for ERα LBD. As shown in Figure 6, the side chains of both C10 and ICI 164,384 extend in the same direction and bind along the shallow groove that is formed by helices 3 and 5. The terminal of the alkyl chain reaches the hydrophobic region formed by the side chains of Leu354, Met357, Ile358,

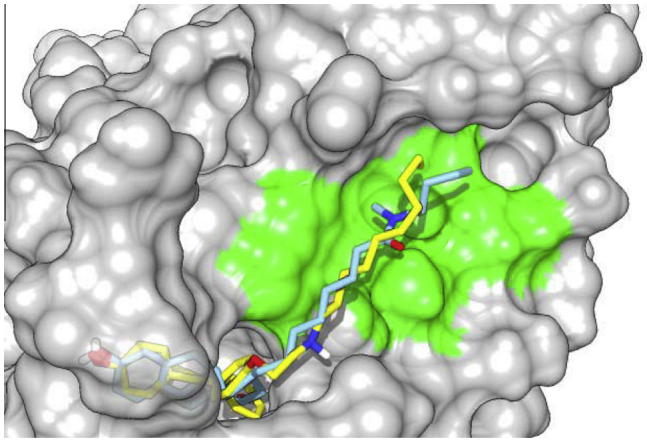


Figure 6. Computational model of human ERα LBD and C10. The hydrophobic region (formed by Leu354, Met357, Ile358, Leu379 and Trp383), C10 and ICI 164,384 are colored green, yellow and cyan, respectively.

Leu379 and Trp383 (Fig. 6, green). The positioning of the alkyl chain of our compound can sterically interfere with helix 12 interaction and the coactivator binding.

3. Discussion

SERD is a compound that can inhibit estrogen action by reducing ER protein levels in breast cancer cells. It is clear that this type of compound has particular promise for effective endocrine therapies in breast cancer. We previously reported that a long alkyl chain derivative of 4-OHT induced degradation of the ERα protein by a proteasome pathway and showed antagonistic activity in MCF-7 cells.[18] Herein, we performed optimization studies to obtain more potent compounds.

First, we synthesized various lengths of long alkyl chain derivatives of 4-OHT to clarify the optimal length of the alkyl chain. Western blotting analysis showed that C10, which has a decyl group on the amine moiety of 4-OHT, is more potent among derivatives that have a simple alkyl chain on the amine moiety (Fig. 4). The length of the alkyl chain is important for the down-regulation of ER.

Furthermore, we designed and synthesized C10F, which has a fluoro group at the terminal of the alkyl chain of C10. The design concept of this compound was based on the following findings: X-ray crystallographic analysis of the complex of rat ERβ and ICI 164,384 (PDB ID: 1HJ1),[17] the alkyl side chain of ICI 164,384 protrudes from the ligand binding pocket and the terminal of alkyl side chain is thrust into a small hydrophobic pocket, which is formed by the side chains of Leu261, Met264, Ile265, Leu286 and Trp290 (all residue numbering is based on 1HJ1; these residues are Leu354, Met357, Ile358, Leu379 and Trp383, respectively, in 3ERT), within the shallow hydrophobic groove that is formed by helices 3 and 5. Therefore, we expected that the modification of the terminal of alkyl group would perturb the interaction between the terminal alkyl group and ER protein surface and influence the ability to degrade ER. As we expected, the binding affinity of C10F for ERα was close to that of C10 (Table 1) and the reduction of ERα was more effective than for C10 (Fig. 5). In the case of C10OH, the hydrophilic substitute of C10, the ability to down-regulate the ERα protein level was decreased (Fig. 5). This is attributed to a decrease of the binding affinity of C10OH for ERα (Table 1).

Computational modeling study showed the predicted binding mode of C10 (Fig. 6). The alkyl side chains of C10 and ICI 164,384 interacted with the same hydrophobic surface that is covered with helix 12 and coactivator in the agonist conformation.[23] This alkyl side chain sterically inhibited the helix 12 interaction and coactivator binding, resulting in destabilization of the ERα. In the case of C6 or C16, which bound to ERα but did not induce down-regulation of ERα, too short as well as too long alkyl chains are unfavorable for the down-regulation of ERα.

In summary, we designed and synthesized new tamoxifen derivatives with a long alkyl chain, which down-regulate the ERα protein level in MCF-7 cells. The most effective length for down-regulation of the ER was determined by performing Western blotting and binding assay and introducing a fluoro group at the terminal of the alkyl chain that maintained high binding affinity for ERα and increased the potency for SERD activity. Computational modeling study showed the binding mode of our compound. The terminal alkyl group interacted with the hydrophobic surface of the ER and the position of the terminal alkyl chain sterically inhibited the helix 12 interaction and the coactivator binding, resulting in destabilization of the ER. Our findings provide useful information to design a promising candidate for SERDs.

4. Experimental section

4.1. Chemistry

4-Hydroxytamoxifen was purchased from Fluka. All other reagents and solvents were purchased from Sigma–Aldrich, Wako Pure Chemical and Tokyo Chemical Industry and were used without purification. Analytical TLC was carried out using Merck silica gel 60 F254 pre-coated plates and visualized using a 254 nm UV lamp, phosphomolybdic acid, *p*-anisaldehyde or ninhydrin staining. Column chromatography was performed with silica gel (spherical, neutral) purchased from Kanto Chemical. The microwave reactions were carried out using Biotage Initiator. ^{1}H NMR and ^{13}C NMR spectra were obtained on a Varian AS 400 Mercury spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). Chemical shifts are expressed as ppm downfield from a solvent residual peak or internal standard tetramethylsilane (TMS). FT-IR spectra were recorded on JASCO FT-IR 4100 equipped with an ATR unit as a sampling module and are expressed in ν (cm^{-1}). High-resolution mass spectra were obtained on a Shimadzu IT-TOF MS equipped with an electrospray ionization source. The syntheses of (*E*/*Z*)-4-(1-{4-[2-(hexylamino)ethoxy]phenyl}-2-phenylbut-1-en-1-yl)phenol (C6), (*E*/*Z*)-4-(1-{4-[2-(dodecylamino)ethoxy]phenyl}-2-phenylbut-1-en-1-yl)phenol (C12) and (*E*/*Z*)-4-(1-{4-[2-(octadecylamino)ethoxy]phenyl}-2-phenylbut-1-en-1-yl)phenol (C18) were as described in our previous report.

4.1.1. (*E*/*Z*)-4-(1-{4-[2-(Decylamino)ethoxy]phenyl}-2-phenylbut-1-en-1-yl)phenol (3, C10)

To a solution of decylamine (99.4 mg, 0.63 mmol, 3.2 equiv) in methanol (0.3 mL), (*E*/*Z*)-4-[1-{4-(2-chloroethoxy)phenyl}-2-phenylbut-1-en-1-yl]phenol[18] (**1**, 74.7 mg, 0.20 mmol, 1.0 equiv) and triethylamine (29.1 mg, 0.28 mmol, 1.5 equiv) were added. The mixture was stirred at 120 °C for 4 h by microwave-assisted synthesis (TLC monitoring: dichloromethane/MeOH, 9:1). The mixture was concentrated in a vacuum. The crude product was purified using a silica gel column (8 g, dichloromethane/MeOH, 9.5:0.5). The fractions containing the product were combined and concentrated under reduced pressure to give clear viscous C10. The NMR spectrum shows a nearly 1:1 mixture of *E* and *Z* isomers.

Yield = 65.6 mg, 67%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17–7.06 (12H, m), 7.00 (2H, d, J = 8.4 Hz), 6.81 (2H, d, J = 8.4 Hz), 6.74–6.72 (4H, m), 6.65 (2H, d, J = 8.4 Hz), 6.46 (2H, d, J = 8.4 Hz), 6.41 (2H, d, J = 8.4 Hz), 4.73 (2H, br), 4.09 (2H, t, J = 5.0 Hz), 3.93 (2H, t, J = 5.0 Hz), 3.04 (2H, t, J = 5.0 Hz), 2.94 (2H, t, J = 5.0 Hz), 2.71

(2H, t, J = 7.4 Hz), 2.65 (2H, t, J = 7.4 Hz), 2.51–2.44 (4H, m), 1.58–1.50 (4H, m), 1.29–1.25 (28H, m), 0.93–0.86 (12H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.5, 156.6, 155.8, 154.9, 143.0, 142.9, 141.0, 140.9, 138.2, 137.0, 136.5, 135.4, 135.1, 132.3, 132.2, 130.9, 130.8, 130.0, 128.1, 128.0, 126.1, 115.5, 114.8, 114.2, 113.5, 66.7, 66.5, 49.9, 48.8, 48.7, 32.1, 29.8, 29.7, 29.6, 29.3, 29.2, 27.5, 22.9, 14.4, 13.9; FT-IR (ATR) 3725, 3603, 2925, 2857, 1603, 1507, 1456, 1374, 1224, 1173, 1045, 830, 770, 688 cm^{-1}; HR-MS (ESI+) m/z calcd for $C_{34}H_{46}NO_2$ $[M+H]^+$ 500.3523, found 500.3529.

4.1.2. (*E*/*Z*)-4-(1-{4-[2-(Octylamino)ethoxy]phenyl}-2-phenylbut-1-en-1-yl)phenol (2, C8)

The same procedure as for C10 was used, starting from **1** and octylamine.

Yield = 74.4 mg, 80%. Clear viscous compound; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17–7.05 (12H, m), 7.00 (2H, d, J = 8.8 Hz), 6.81 (2H, d, J = 8.8 Hz), 6.74–6.72 (4H, m), 6.65 (2H, d, J = 8.8 Hz), 6.46 (2H, d, J = 8.8 Hz), 6.41 (2H, d, J = 8.8 Hz), 4.09 (2H, t, J = 5.0 Hz), 3.92 (2H, t, J = 5.0 Hz), 4.40–3.60 (2H, br), 3.03 (2H, t, J = 5.0 Hz), 2.93 (2H, t, J = 5.0 Hz), 2.71 (2H, t, J = 7.6 Hz), 2.65 (2H, t, J = 7.4 Hz), 2.51–2.44 (4H, m), 1.57–1.48 (4H, m), 1.31–1.27 (20H, m), 0.93–0.85 (12H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.5, 156.6, 155.8, 154.8, 143.0, 142.9, 141.0, 140.9, 138.2, 137.0, 136.5, 135.4, 135.2, 132.3, 132.2, 130.9, 130.8, 130.0, 128.1, 128.0, 126.1, 115.5, 114.8, 114.2, 113.5, 66.8, 66.5, 49.9, 48.9, 48.8, 32.0, 29.8, 29.7, 29.6, 29.5, 29.3, 29.2, 27.5, 22.9, 14.4, 13.9; FT-IR (ATR) 3729, 3597, 2925, 2859, 1603, 1506, 1458, 1372, 1237, 1174, 1109, 1044, 831, 769, 700, 607, 527, 514 cm^{-1}; HR-MS (ESI+) m/z calcd for $C_{32}H_{42}NO_2$ $[M+H]^+$ 472.3210, found 472.3215.

4.1.3. (*E*/*Z*)-4-(1-{4-[2-(Tetradecylamino)ethoxy]phenyl}-2-phenylbut-1-en-1-yl)phenol (4, C14)

The same procedure as for C10 was used, starting from **1** and tetradecylamine.

Yield = 38.0 mg, 36%. Clear viscous compound; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16–7.06 (12H, m), 7.00 (2H, d, J = 8.4 Hz), 6.81 (2H, d, J = 8.4 Hz), 6.74–6.72 (4H, m), 6.65 (2H, d, J = 8.4 Hz), 6.46 (2H, d, J = 8.4 Hz), 6.40 (2H, d, J = 8.4 Hz), 4.51 (2H, br), 4.09 (2H, t, J = 5.0 Hz), 3.93 (2H, t, J = 5.0 Hz), 3.03 (2H, t, J = 5.0 Hz), 2.94 (2H, t, J = 5.0 Hz), 2.71 (2H, t, J = 7.6 Hz), 2.65 (2H, t, J = 7.6 Hz), 2.51–2.44 (4H, m), 1.58–1.48 (4H, m), 1.30–1.26 (44H, m), 0.93–0.86 (12H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.5, 156.6, 155.8, 154.8, 143.0, 142.9, 141.0, 140.9, 138.2, 137.0, 136.5, 135.5, 135.2, 132.3, 132.2, 130.9, 130.8, 130.0, 128.1, 128.0, 126.1, 115.5, 114.8, 114.2, 113.5, 66.8, 66.5, 50.0, 49.9, 48.9, 48.8, 32.2, 29.9, 29.8, 29.7, 29.6, 29.3, 29.2, 27.5, 22.9, 14.4, 13.9; FT-IR (ATR) 3729, 3597, 2925, 2855, 1604, 1507, 1459, 1372, 1226, 1174, 1108, 1046, 830, 771, 694, 528, 516, 513 cm^{-1}; HR-MS (ESI+): m/z calcd for $C_{38}H_{54}NO_2$ $[M+H]^+$ 556.4149, found 556.4121.

4.1.4. (*E*/*Z*)-4-(1-{4-[2-(Hexadecylamino)ethoxy]phenyl}-2-phenylbut-1-en-1-yl)phenol (5, C16)

The same procedure as for C10 was used, starting from **1** and hexadecylamine.

Yield = 45.3 mg, 43%. Clear viscous compound; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17–7.08 (26H, m), 7.01 (2H, d, J = 8.4 Hz), 6.82 (2H, d, J = 8.4 Hz), 6.73 (4H, d, J = 8.4 Hz), 6.65 (2H, d, J = 8.4 Hz), 6.47 (2H, d, J = 8.4 Hz), 6.41 (2H, d, J = 8.4 Hz), 4.09 (2H, t, J = 5.0 Hz), 3.93 (2H, t, J = 5.0 Hz), 4.13–3.91 (2H, br), 3.03 (2H, t, J = 5.0 Hz), 2.93 (2H, t, J = 5.0 Hz), 2.71 (2H, t, J = 7.4 Hz), 2.65 (2H, t, J = 7.4 Hz), 2.50–2.46 (4H, m), 1.58–1.50 (4H, m), 1.32–1.26 (52H, m), 0.93–0.88 (12H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.5, 156.6, 155.8, 154.8, 143.0, 142.9, 141.0, 140.9, 138.2, 137.0, 136.5, 135.5, 135.2, 132.3, 132.2, 130.9, 130.8, 130.0, 128.1, 128.0, 126.1, 115.5, 114.8, 114.2, 113.5, 66.8, 66.5, 51.0, 49.9, 48.8, 48.7, 32.2, 30.0, 29.9, 29.8, 29.7, 29.6, 29.3, 29.2, 27.5, 22.9, 14.4, 13.9; FT-IR (ATR) 3729, 3597, 2924, 2854, 1603, 1507, 1458, 1372, 1224, 1172, 1108, 1045, 830, 771, 691, 606, 546, 528, 518, 513 cm^{-1}; HR-MS (ESI+): m/z calcd for $C_{40}H_{58}NO_2$ $[M+H]^+$ 584.4462, found 584.4402.

4.1.5. (*E*/*Z*)-4-[1-(4-{2-[(10-Hydroxydecyl)amino]ethoxy}phenyl)-2-phenylbut-1-en-1-yl]phenol (6, C10OH)

The same procedure as for C10 was used, starting from **1** and 10-amino-1-decanol.

Yield = 30.6 mg, 43%. Viscous compound. ^{1}H NMR (400 MHz, CD_3OD) δ 7.20–7.06 (12H, m), 7.03 (2H, d, J = 8.5 Hz), 6.85 (2H, d, J = 8.7 Hz), 6.78 (2H, d, J = 8.6 Hz), 6.74 (2H, d, J = 8.7 Hz), 6.67 (2H, d, J = 8.5 Hz), 6.50 (2H, d, J = 8.9 Hz), 6.45 (2H, d, J = 8.7 Hz), 4.17 (2H, t, J = 5.0 Hz), 4.00 (2H, t, J = 5.0 Hz), 3.67–3.59 (4H, m), 3.12 (2H, t, J = 5.0 Hz), 3.02 (2H, t, J = 5.0 Hz), 2.91–2.70 (4H, m), 2.52–2.42 (4H, m), 1.75–1.10 (32H, m), 0.91 (6H, t, J = 7.4 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 157.70, 156.20, 155.46, 154.51, 142.88, 142.83, 141.20, 138.01, 137.20, 136.74, 135.32, 132.27, 132.23, 130.89, 130.86, 129.93, 128.09, 128.05, 126.12, 115.40, 114.69, 114.28, 113.50, 63.17, 49.25, 47.89, 41.11, 37.77, 35.71, 32.91, 32.16, 29.41, 28.25, 27.13, 25.89, 24.38, 15.41, 13.94, 13.88; FT-IR (ATR) 3643, 3471, 2927, 2857, 1726, 1671, 1603, 1508, 1455, 1372, 1239, 1173, 1105, 1046, 911, 830, 731 cm^{-1}; HR-MS (ESI+) m/z calcd for $C_{34}H_{46}NO_3$ $[M+H]^+$ 516.3472, found 516.3448.

4.1.6. 10-[(*tert*-Butoxycarbonyl)amino]decyl 4-methylbenzenesulfonate (7)

Di-*t*-butyl dicarbonate (2.5 g, 12 mmol, 1.0 equiv) was added to a solution of 10-amino-1-decanol (2.0 g, 12 mmol, 1.0 equiv) in CH_2Cl_2 (40 ml) at room temperature. After stirring for 5 h, the CH_2Cl_2 was removed in vacuo. The crude product was dissolved in CH_2Cl_2 (16 ml) and pyridine (4.5 g, 57 mmol, 5.0 equiv). TsCl (4.5 g, 24 mmol, 2.0 equiv) and DMAP (141 mg, 1.2 mmol, 0.1 equiv) were added and stirred for 6 h at room temperature. The reaction mixture was washed with brine, dried with sodium sulfate and then evaporated. The residue was purified by column chromatography (EtOAc/hexane, 1:4). Yield = 1.41 g, 29%. Colorless oil. ^{1}H NMR (400 MHz, CD_3OD) δ 7.79 (2H, d, J = 8.0 Hz), 7.35 (2H, d, J = 8.0 Hz), 4.53 (1H, s), 4.01 (2H, t, J = 6.0 Hz), 3.10–3.07 (2H, m), 2.45 (3H, s), 1.65–1.59 (2H, m), 1.44 (9H, s), 1.26–1.22 (14H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 156.25, 144.86, 133.83, 130.03, 128.11, 79.02, 70.93, 40.81, 30.26, 29.59, 29.50, 29.43, 29.10, 29.01, 28.65, 26.98, 25.52, 21.88; FT-IR (ATR) 3406, 2928, 2858, 1703, 1516, 1456, 1361, 1220, 1174, 952, 772 cm^{-1}; MS (ESI+) m/z calcd for $C_{22}H_{37}NNaO_5S$ $[M+Na]^+$ 450, found 450.

4.1.7. *tert*-Butyl (10-fluorodecyl)carbamate (8)

Compound **7** (278 mg, 0.65 mmol, 1.0 equiv) was dissolved in THF (5 ml). A TBAF solution (1.0 M in THF, 1.5 ml, 1.5 mmol, 2.3 equiv) was added dropwise to the reaction mixture at room temperature and refluxed for 7 h. The solvent was evaporated and the residue was purified by column chromatography (EtOAc/hexane, 1:6). Yield = 134 mg, 75%. Colorless oil. ^{1}H NMR (400 MHz, CD_3OD) δ 4.53 (1H, br), 4.44 (2H, dt, J = 47.2, 6.4 Hz), 3.13–3.08 (2H, m), 1.76–1.62 (2H, m), 1.44 (9H, s), 1.40–1.28 (14H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 156.2, 84.50, 45.43, 40.81, 30.71, 30.51, 30.26, 29.64, 29.42, 28.64, 27.00, 25.78, 25.33; FT-IR (ATR) 3357, 2929, 2855, 1700, 1518, 1458, 1364, 1249, 1173, 1040, 1002, 913, 868, 791, 744 cm^{-1}.

4.1.8. 10-Fluorodecan-1-amine hydrochloride (9)

Compound **8** (142 mg, 0.52 mmol) was dissolved in a HCl solution (ca. 4 M in 1,4-dioxane) and stirred for 2 h at room temperature. The solvent was evaporated to give compound **9**. Yield = 111 mg, 99%. Colorless viscous compound. ^{1}H NMR (400 MHz, CD_3OD) δ 4.43 (2H, dt, J = 47.6, 6.0 Hz), 3.23 (2H, br), 2.70 (2H, br), 1.75–1.63 (2H, m), 1.52–1.25 (14H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 84.45, 45.40, 40.30, 30.61, 29.61, 29.50, 29.40, 29.19, 26.75, 25.36; HR-MS (ESI+) m/z calcd for $C_{10}H_{23}FN$ $[M+H]^+$ 176.1736, found 176.1767.

4.1.9. (*E*/*Z*)-4-[1-(4-{2-[(10-Fluorodecyl)amino]ethoxy}phenyl)-2-phenylbut-1-en-1-yl]phenol (10, C10F)

The same procedure as for C10 was used, starting from **1** and **9**. Yield = 24 mg, 35%. Colorless viscous compound. ^{1}H NMR (400 MHz, CD_3OD) δ 7.21–7.05 (12H, m), 7.01 (2H, d, J = 8.3 Hz), 7.01 (2H, d, J = 8.3 Hz), 6.77–6.70 (4H, m), 6.66 (2H, d, J = 8.5 Hz), 6.47 (2H, d, J = 8.6 Hz), 6.41 (2H, d, J = 8.5 Hz), 4.43 (2H, dt, J = 47.4, 6.2 Hz,), 4.09 (2H, t, J = 5.0 Hz,), 3.93 (2H, t, J = 5.0 Hz), 3.04 (2H, t, J = 5.0 Hz), 2.94 (2H, t, J = 5.0 Hz), 2.77–2.61 (4H, m), 2.55–2.39 (4H, m), 1.82–1.61 (4H, m), 1.59–1.47 (4H, m), 1.46–1.20 (24H, m), 0.99–0.86 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 158.60, 157.74, 157.05, 156.07, 144.17, 144.13, 142.14, 142.01, 139.37, 138.20, 137.69, 133.52, 132.36, 131.15, 129.27, 127.26, 116.70, 116.70, 116.03, 115.38, 114.61, 86.51, 84.89, 67.85, 67.55, 51.08, 51.04, 49.99, 49.91, 40.11, 31.92, 31.73, 30.85, 30.64, 30.52, 30.42, 30.34, 28.67, 28.63, 26.60, 26.54, 25.14, 24.42, 15.52, 15.13, 15.09; FT-IR (ATR) 3779, 3549, 3047, 2926, 2857, 1727, 1602, 1505, 1457, 1379, 1240, 1172, 1120, 1043, 910, 824, 738 cm^{-1}; HR-MS (ESI+) m/z calcd for $C_{34}H_{45}NO_2F$ $[M+H]^+$ 518.3429, found 518.3407.

4.2. Cell culture

Human breast cancer MCF-7 was maintained in RPMI 1640 medium containing 10% FBS and 60 μg/ml kanamycin. Cells were then treated with the indicated concentration of compounds for the indicated times.

4.3. Western blot analysis

MCF-7 cells were treated with the compounds at the indicated concentrations in the presence or absence of 10 μM MG132 (purchased from Peptide Institute) for 6 h, and then the cells were collected and extracted with lysis buffer (1% SDS, 0.1 M Tris–HCl, pH 7.0, 10% glycerol) and boiled for 10 min. Protein concentrations were determined by the BCA method, and equal amounts of protein lysate were separated by SDS–PAGE, transferred to a PVDF membrane and subjected to Western blotting using the following antibodies: anti-human ERα mouse monoclonal antibody (Santa Cruz) and anti-β-actin mouse monoclonal antibody (Sigma).

4.4. Fluorescence polarization assay

Test compounds were dissolved in DMSO to prepare a stock solution (10 mM). Fluorescent polarization-based competition binding assays were conducted to determine the relative binding affinity of compounds for ERα using commercially available kits (P2698, Life Technologies) according to the manufacturer's instructions. Fluorescence polarization signals (mP values) were then measured using EnVision multiple plate reader (Perkin Elmer) with a 470 nm excitation filter and a 535 nm emission filter. The fraction of compound bound to ERα was correlated to the mP value and plotted against values of competitor concentrations. The IC_{50} values were obtained by fitting the data using GraphPad Prism 6 J. Relative binding affinity (RBA) was calculated by dividing the IC_{50} of E2 (0.17 nM) by the IC_{50} of the compound and is expressed as a percent (E2 = 100).

4.5. Computational modeling studies

The crystal structure of the complex of human ERα and 4-OHT (PDB code 3ERT), from which helix 12 was deleted, was used as a template for the computational study. Molecular modeling and graphics manipulations were performed using the Molecular Operating Environment (MOE, Chemical Computing Group) and UCSF-Chimera software packages.[24]

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