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Synthesis and evaluation of raloxifene derivatives as a selective estrogen receptor down-regulator

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ABSTRACT

Estrogen receptors (ERs) play a major role in the growth of human breast cancer cells. A selective estrogen receptor down-regulator (SERD) that acts as not only an inhibitor of ligand binding, but also induces the down-regulation of ER, would be useful for the treatment for ER-positive breast cancer. We previously reported that tamoxifen derivatives, which have a long alkyl chain, had the ability to down-regulate ERα. With the aim of expanding range of the currently available SERDs, we designed and synthesized raloxifene derivatives, which had various lengths of the long alkyl chains, and evaluated their SERD activities. All compounds were able to bind ERα, and RC10, which has a decyl group on the amine moiety of raloxifene, was shown to be the most potent compound. Our findings suggest that the ligand core was replaceable, and that the alkyl length was important for controlling SERD activity. Moreover, RC10 showed antagonistic activity and its potency was superior to that of 4,4′-(heptane-4,4-diyl)bis(2-methylphenol) (**18**), a competitive antagonist of ER without SERD activity. These results provide information that will be useful for the development of promising SERDs candidates.

1. Introduction

Selective estrogen receptor down-regulators (SERDs) are a class of pure antagonists that not only interfere with the binding of estradiol to estrogen receptors (ERs), but also induce the rapid down-regulation of ER.[1–5] Compared with selective estrogen receptor modulators (SERMs), which act as either agonists or antagonists, depending on the tissue, such as tamoxifen and its active metabolite, 4-OHT (Fig. 1), SERDs showed no agonistic activity in any tissue and are often used as second-line agent for tamoxifen-resistant breast cancer. Fulvestrant[6,7] and GW7604[8] are two well-known SERDs (Fig. 1). Fulvestrant is an estradiol derivative with an 4,4,5,5,5-pentafluoropenthylsulfinyl alkyl group at the 7α position of estradiol. Fulvestrant is an effective therapeutic option for postmenopausal women with ER-positive breast cancers.[9] GW7604 has a triphenylethylene ligand core and an acrylic acid unit. X-ray crystallography of the ERα-GW7604 complex indicates that the carboxylic acid group plays a key role. The direct interaction between the carboxylic acid group and the N terminus of helix 12 of the receptor induced a conformational change and destabilization of the receptor.[10] This result promoted extensive studies into the synthesis and evaluation of compounds with ligand cores and acrylic acid.[11–15] These reports indicate that introducing an acrylic acid to a ligand core is an effective way to develop SERDs. Our group has previously reported the design and synthesis of tamoxifen derivative SERDs, which have a long alkyl chain on the amine moiety.[16,17] The compound with a decyl group, termed C10 (Fig. 1), had the highest ability to induce the down-regulation of ERα among the simple alkyl chain derivatives. Next, we investigated the relation between the ligand core and the SERD activity of the compound. We chose raloxifene (Fig. 1) as the ligand core, which is also known as a non-steroidal SERM that binds effectively to ERα and ERβ subtypes.[18,19] In this manuscript, we describe the synthesis of raloxifene derivatives having a long alkyl chain and evaluate their activities. Our findings suggest that the ligand core is replaceable with other structures, and that an appropriate length of the long alkyl chain of the ligand is necessary for the ability to down-regulate ER.

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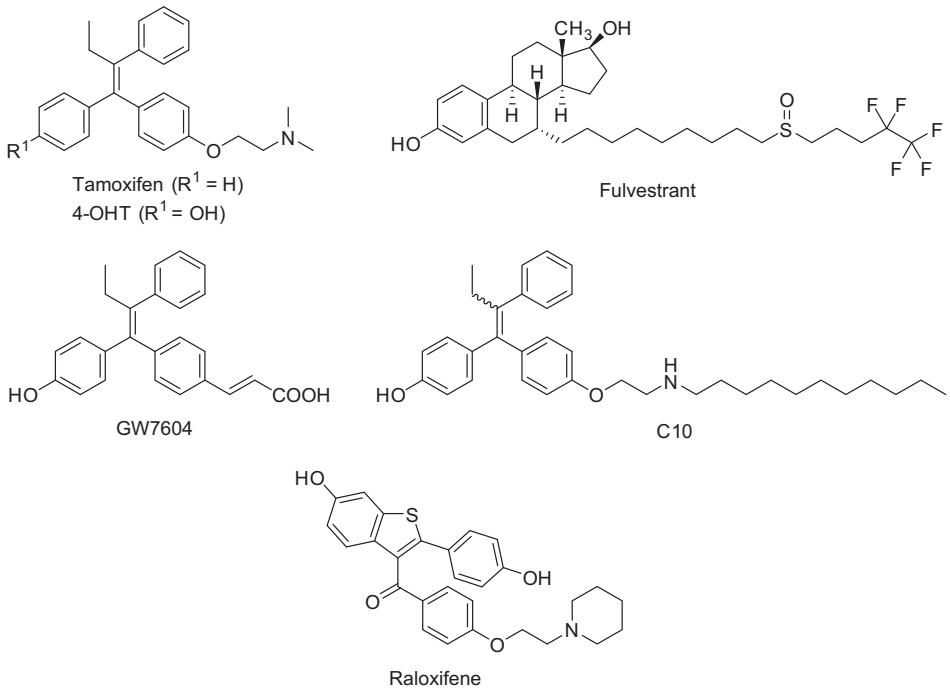

Figure 1. Structures of tamoxifen, 4-OHT, fulvestrant, GW7604, C10, and raloxifene.

2. Results

2.1. Chemistry

Raloxifene, a benzothiophene derivative tethered to a basic side chain, is well known as a SERM that binds effectively to ERα and ERβ subtypes. The binding affinity[18,19] and the binding mode[20] of raloxifene is almost identical to that of tamoxifen.

We designed seven novel raloxifene derivatives with long alkyl chains on the amine moiety. Synthetic route of compounds are summarized in Scheme 1. Firstly, we synthesized [4-(2-chloroethoxy)phenyl][6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophen-3-yl]methanone (**4**) according to the patent,[21] with slight modification. Commercially available methyl 4-hydroxybenzoate was reacted with 1,2-dichloroethane in the presence of K_2CO_3 as a base to afford **1** (93% yield), which was then hydrolyzed to afford **2** (91% yield). The crude acid chloride **3** was obtained by refluxing in $SOCl_2$ as solvent, and used without further purification. Acid chloride **3** was then reacted with 6-methoxy-2-(4-methoxyphenyl)benzo[*b*]thiophene in the presence of BCl_3 to afford the demethylated compound **4** (94% yield). The chloride group was converted to iodide to afford **5**. Then, compounds **6**–**12** were synthesized from corresponding amines in moderate yields, as indicated in Scheme 1.

2.2. Down-regulation of ERα

We first examined the effects of the length of the long alkyl chain on raloxifene for 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 previously reported.[16,22] As shown in Figure 2, reduction of the ER protein level was observed in the cells treated with 10 μM RC10 and RC12 (lanes 5, 6), compared with raloxifene and other compounds (lane 2–4 and 7–9). RC10 was the most potent of all compounds examined. Next, we examined the dose-dependency of RC10 and RC12. After treatment with RC10 and RC12, ERα protein level decreased in a dose-dependent manner (Fig. 3, lane 3–5 and 7–9, respectively). These reductions were inhibited by addition of a proteasome inhibitor, MG132 (lane 6 and 10), suggesting that RC10 and RC12 have the ability to induce proteasomal degradation of ERα in MCF-7 cells.

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} values of the compounds tested in the binding assay are summarized in Table 1. The IC_{50} values of all synthesized compounds were between 1.8–61 nM, and the IC_{50} of raloxifene was 0.47 nM. These findings suggest that the binding affinity for ERα was decreased by the introduction of a long alkyl chain to the amine moiety of the ligand core, and that the length of the alkyl chain does not significantly affect the binding affinity. Moreover, no correlation was observed between binding affinity and ER degradation. A similar pattern of results was found with the corresponding tamoxifen derivatives.[17] We also measured binding affinity of compound **18**, 4,4′-(heptane-4,4-diyl)bis(2-methylphenol), a non-steroidal antagonist developed by Maruyama et al.,[23] that has an IC_{50} value of 2.4 nM. The binding affinity of compound **18** was similar to that of our compounds.

2.4. ER antagonistic activity

Finally, we examined the antagonistic effects of RC10 and RC12, both of which had the ability to down-regulate ERα. The ER-antagonistic activities were evaluated using previously established methods.[23–27] Transcriptional activity was activated by 0.3 nM estradiol, and the IC_{50} values were calculated using the luciferase activity value divided by the β-galactosidase activity value. These compounds showed dose-dependent inhibition of transcriptional activity, suggesting that RC10 and RC12 exhibit antagonistic effects. IC_{50} values are summarized in Table 2.

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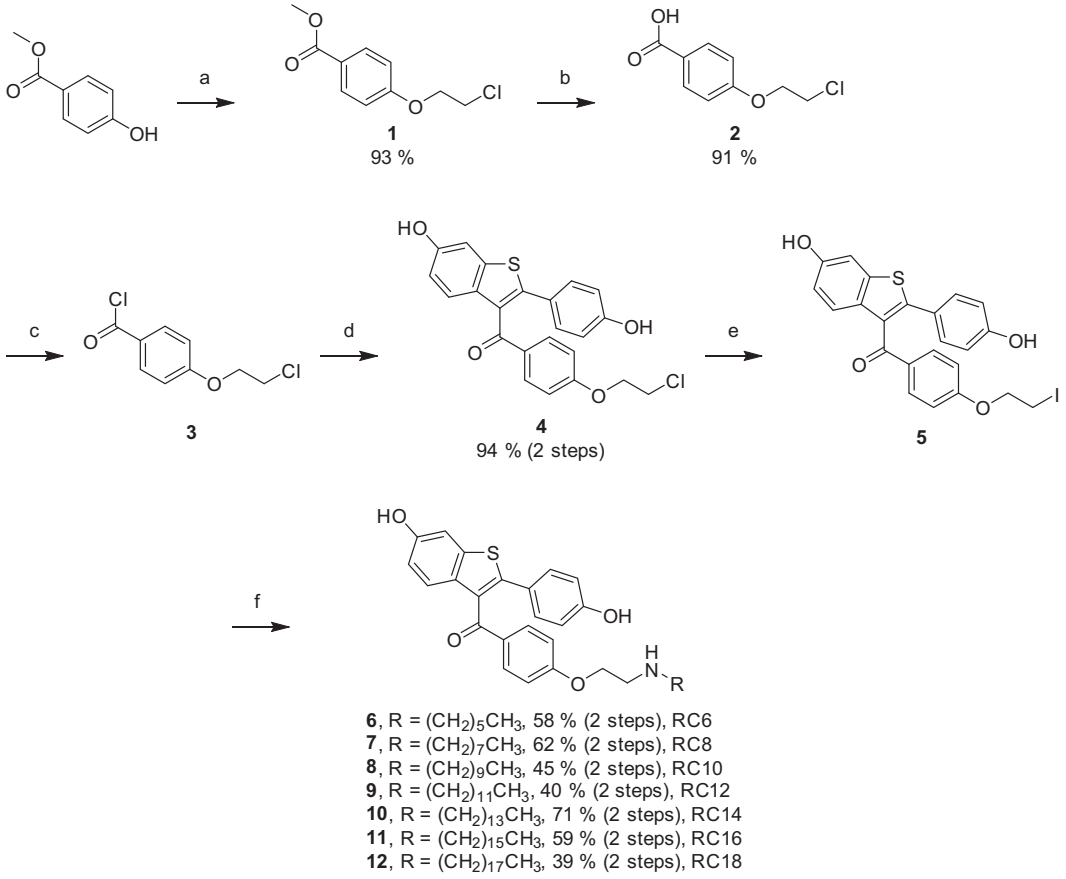

Scheme 1. Synthesis of raloxifene derivatives. Reagents and conditions: (a) 1,2-dichloroethane, K_2CO_3, DMSO, 2.5 h; (b) aqueous NaOH, methanol, reflux, 2 h; (c) $SOCl_2$, reflux, 1.5 h; (d) 6-methoxy-2-(4-methoxyphenyl)benzo[*b*]thiophene, BCl_3, 1,2-dichloroethane, 0–30 °C, 2 days; (e) NaI, acetone, microwave 56 °C, 24 h; (f) RNH_2, triethylamine, methanol, microwave, 120 °C, 1 h.

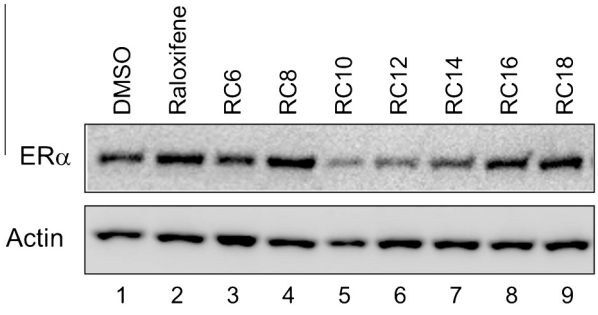

Figure 2. Reduction of endogenous ERα by raloxifene and synthesized compounds. MCF-7 cells were incubated with 10 μM of indicated compounds for 6 h. Examples of immunoblots for cell lysates stained with the indicated antibodies are shown.

3. Discussion

SERDs are a class of compounds that can inhibit estrogen action by reducing ER protein levels in breast cancer cells. At least 70% of breast cancer patients are diagnosed with ER-positive breast cancer, and SERD is a promising agent for the treatment this type of breast cancer. Previously we have described the design and synthesis of tamoxifen derivatives with long alkyl chains, and have reported that these compounds show SERD activity.[16,17] In this manuscript, we investigated replacement of the ligand core in the development of new SERDs. We synthesized seven raloxifene derivatives with various lengths of long alkyl chains at the amine moiety. Although all compounds could bind ERα with high affinity, only RC10 and RC12, which have decyl and dodecyl groups, respectively, showed SERD activity. Interestingly, the most effective length of alkyl chain of all the raloxifene derivatives was identical to that of the tamoxifen derivative.[17] These findings strongly suggest that the ligand core is replaceable, and that the length of the alkyl chain is an important factor in controlling SERD activity. Furthermore, both compounds showed higher antagonistic activity than compound **18** (IC_{50} = 4.9 nM[23]), an antagonist which does not down-regulate ER.[28]

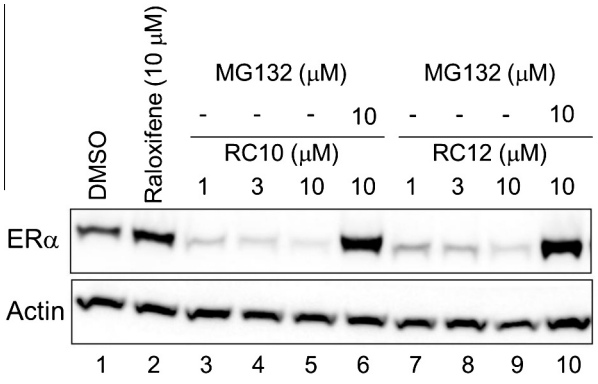

Figure 3. Dose-dependent response of ERα reduction induced by RC10 and RC12. MCF-7 cells were incubated with the indicated concentrations of the compound for 6 h. Examples of immunoblots for cell lysates stained with indicated antibodies are shown.

Table 1
Estrogen receptor binding affinities and ERα degradation activities of compounds

Compound	IC_{50}[a] (nM)
RC6	61
RC8	49
RC10	40
RC12	5.9
RC14	28
RC16	11
RC18	1.8
Raloxifene	0.47
Compound **18**[b]	2.4

[a] The IC_{50} values for each competing ligand were calculated according to the sigmoidal inhibition curves.
[b] 4,4′-(Heptane-4,4-diyl)bis(2-methylphenol).[23]

4. Conclusion

In summary, we report the design and synthesis of raloxifene derivatives with long alkyl chains at the amine moiety, as potential SERD candidates. All seven compounds were able to bind ERα, but only two had the ability to down-regulate ERα. These findings suggest that the ligand core is replaceable, and that introducing the appropriate length of alkyl chain on the amine moiety of the ligand core produces efficacious SERDs. Furthermore, these compounds showed antagonistic activities, and the potency of our compounds was superior to that of an antagonist that binds ER with high affinity, but is not able to down-regulate ER. These results provide useful information for the development of promising SERD candidates. As a result of these findings, we are currently working on another type of ligand core to further investigate promising SERD candidates.

5. Experimental

5.1. Chemistry

All reagents and solvents were purchased from Sigma–Aldrich, Wako Pure Chemical, and Tokyo Chemical Industry and were used without purification. Analytical TLC was conducted using Merck silica gel 60 F254 pre-coated plates and visualized using a 254 nm UV lamp, phosphomolybdic acid, *p*-anisaldehyde, or ninhydrin stains. Column chromatography was performed using silica gel (spherical, neutral) purchased from Kanto Chemical. Microwave reactions were conducted using a Biotage Initiater. ^{1}H NMR and ^{13}C NMR spectra were measured using a Varian AS 400 Mercury spectrometer or Varian 600 MHz NMR spectrometer. Chemical shifts are expressed in ppm downfield from a solvent residual peak or internal standard tetramethylsilane (TMS). FT-IR spectra were measured using a JASCO FT-IR 4100 equipped with an ATR unit as a sampling module, and were expressed in ν (cm^{-1}). High-resolution mass spectra were measured using a Shimadzu IT-TOF MS equipped with an electrospray ionization source. Compounds **1**–**4** were prepared according to a previously reported method.[21]

Table 2
The IC_{50} values of compounds

Compound	IC_{50} (nM)
RC10	0.68
RC12	0.09
18	4.9[23]

5.1.1. {4-[2-(Decylamino)ethoxy]phenyl}[6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophen-3-yl]methanone (8, RC10)

Compound **4** (49.6 mg, 0.117 mmol) and sodium iodide (353.4 mg, 2.36 mmol) were dissolved in 1 ml acetone. Reaction was conducted using microwave irradiation at 56 °C for 24 h. The solvent was evaporated, AcOEt was added, and the organic layer was washed with water and brine. The solvent was evaporated and the residue was dissolved in methanol to transfer to the reaction vial. Then, decylamine (21.9 mg, 0.14 mmol) and triethylamine (31.5 mg, 0.31 mmol) were added. The mixture was stirred and heated in microwave apparatus (Initiater, Biotage) at 120 °C until the reaction was completed. The mixture was evaporated in vacuo and the products were purified by silica gel chromatography (MeOH/DCM = 8/92). Yield = 28.8 mg, 45%. Pale yellow viscous compound; IR: 3284, 2925, 2850, 1594, 1459, 1250, 1166, 1026, 823, 617, 524, 510, 462, 435, 401 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 7.70 (2H, d, J = 8.8 Hz), 7.41 (1H, d, J = 8.8 Hz), 7.26 (1H, d, J = 2.0 Hz), 7.17 (2H, d, J = 8.8 Hz), 6.83–6.87 (3H, m), 6.61 (2H, d, J = 8.4 Hz), 4.08 (2H, t, J = 5.0 Hz), 2.97 (2H, t, J = 5.2 Hz), 2.64 (2H, t, J = 7.6 Hz), 1.52 (2H, m), 1.25–1.30 (14H, m), 0.89 (3H, t, J = 7.0 Hz); ^{13}C NMR (150 MHz, CD_3OD) δ 195.53, 164.49, 159.29, 156.79, 143.96, 141.38, 134.22, 133.42, 131.80, 131.32, 131.13, 125.94, 124.64, 116.44, 116.02, 115.27, 107.84, 67.84, 32.99, 30.70, 30.62, 30.52, 30.38, 30.03, 28.22, 23.67, 14.37; HRMS (ESI+) m/z calcd for $C_{33}H_{40}NO_4S^+$ $[M+H]^+$ 546.2673, found 546.2648.

5.1.2. {4-[2-(Hexylamino)ethoxy]phenyl}[6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophen-3-yl]methanone (6, RC6)

The same procedure as for RC10 was used, but starting from hexylamine.

Yield = 13.7 mg, 58%. Pale yellow viscous compound; IR: 3332, 2927, 2857, 1595, 1253, 1034, 814, 754, 492, 489, 485, 445, 435 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 7.73–7.75 (2H, m), 7.43 (1H, d, J = 8.8 Hz), 7.26 (1H, d, J = 2.0 Hz), 7.17–7.19 (2H, m), 6.91–6.93 (2H, m), 6.85–6.88 (1H, m), 6.59–6.62 (2H, m), 4.27 (2H, t, J = 5.0 Hz), 3.43 (2H, t, J = 4.8 Hz), 3.05 (2H, t, J = 8.0 Hz), 1.66–1.73 (2H, m), 1.42–1.28 (6H, m), 0.92 (3H, t, J = 6.8 Hz); ^{13}C NMR (150 MHz, CD_3OD) δ 193.98, 162.00, 157.84, 155.43, 142.87, 140.06, 132.05, 130.01, 123.24, 115.01, 114.66, 113.94, 106.45, 105.00, 63.27, 30.95, 25.80, 25.69, 22.01, 12.82; HRMS (ESI+) m/z calcd for $C_{29}H_{32}NO_4S^+$ $[M+H]^+$ 490.2047, found 490.2028.

5.1.3. [6-Hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophen-3-yl]{4-[2-(octylamino)ethoxy]phenyl}methanone (7, RC8)

The same procedure as for RC10 was used, but starting from octylamine.

Yield = 36.1 mg, 62%. Pale yellow viscous compound; IR: 3296, 2928, 2853, 1599, 1257, 1171, 1033, 830, 533, 499, 488, 435 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 7.71–7.73 (2H, m), 7.43 (1H, d, J = 8.8 Hz), 7.27(1H, d, J = 2.4 Hz), 7.16–7.18 (2H, m), 6.85–6.91 (3H, m), 6.60–6.62 (2H, m), 4.22 (2H, t, J = 4.8 Hz), 3.29–3.32 (2H, m), 2.94 (2H, t, J = 7.8 Hz), 1.61–1.67 (2H, m), 1.29–1.35 (10H, m), 0.90 (3H, t, J = 6.8 Hz); ^{13}C NMR (150 MHz, CD_3OD) δ 195.42, 163.57, 159.20, 156.77, 144.22, 141.42, 134.19, 133.43, 132.42, 131.38, 131.30, 131.00, 125.98, 124.64, 116.42, 116.05, 115.35, 107.87, 65.18, 32.83, 30.16, 30.12, 27.61, 27.56, 23.65, 23.61, 14.35; HRMS (ESI+) m/z calcd for $C_{31}H_{36}NO_4S^+$ $[M+H]^+$ 518.2360, found 518.2321.

5.1.4. (4-(2-(Dodecylamino)ethoxy)phenyl)(6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophen-3-yl)methanone (9, RC12)

The same procedure as for RC10 was used, but starting from dodecylamine.

Yield = 24.1 mg, 40%. Pale yellow viscous compound; IR: 3318, 2926, 2856, 1602, 1460, 1258, 1172, 1035, 825, 658, 491, 460,

428 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 7.73 (2H, d, J = 8.8 Hz), 7.43 (1H, d, J = 8.8 Hz), 7.27 (1H, d, J = 2.0 Hz), 7.18 (2H, d, J = 8.8 Hz), 6.86–6.92 (3H, m), 6.61 (2H, d, J = 8.4 Hz), 4.23 (2H, t, J = 5.0 Hz), 3.31 (2H, m), 2.94 (2H, t, J = 7.6 Hz), 1.65 (2H, m), 1.24–1.36 (18H, m), 0.89 (3H, t, J = 6.8 Hz); ^{13}C NMR (150 MHz, CD_3OD) δ 132.05, 130.01, 115.08, 114.66, 113.92, 106.45, 105.00, 31.62, 29.30, 29.19, 29.04, 29.02, 28.77, 26.12, 22.29, 12.98; HRMS (ESI+) m/z calcd for $C_{35}H_{44}NO_4S^+$ $[M+H]^+$ 574.2986, found 574.2968.

5.1.5. [6-Hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophen-3-yl]{4-[2-(tetradecylamino)ethoxy]phenyl}methanone (10, RC14)

The same procedure as for RC10 was used, but starting from tetradecylamine.

Yield = 38.4 mg, 71%. Pale yellow viscous compound; IR: 3294, 2924, 2854, 1597, 1461, 1353, 1255, 1169, 831, 655, 530 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 7.73 (2H, d, J = 8.8 Hz), 7.423 (1H, d, J = 8.8 Hz), 7.26 (1H, d, J = 2.0 Hz), 7.18 (2H, d, J = 8.8 Hz), 6.85–6.91 (3H, m), 6.61 (2H, d, J = 8.8 Hz), 4.23 (2H, t, J = 4.8 Hz), 3.31 (2H, m), 2.95 (2H, t, J = 7.6 Hz), 1.65 (2H, m), 1.23–1.35 (22H, m), 0.89 (3H, t, J = 6.8 Hz); ^{13}C NMR (150 MHz, CD_3OD) δ 162.09, 142.87, 140.05, 132.80, 132.06, 130.01, 115.02, 114.66, 113.94, 106.46, 105.00, 63.53, 31.64, 29.36, 29.33, 29.21, 29.04, 28.80, 26.17, 25.95, 22.31, 13.01; HRMS (ESI+) m/z calcd for $C_{37}H_{48}NO_4S^+$ $[M+H]^+$ 602.3299, found 602.3260.

5.1.6. {4-[2-(Hexadecylamino)ethoxy]phenyl}[6-hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophen-3-yl]methanone (11, RC16)

The same procedure as for RC10 was used, but starting from hexadecylamine.

Yield = 32.2 mg, 59%. Pale yellow viscous compound; IR: 3311, 2925, 2855, 1600, 1463, 1354, 1258, 1171, 1034, 808, 668, 532, 489, 485 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 7.74 (2H, d, J = 8.8 Hz), 7.43 (1H, d, J = 8.8 Hz), 7.27 (1H, d, J = 2.4 Hz), 7.18 (2H, d, J = 8.8 Hz), 6.86–6.92 (3H, m), 6.61 (2H, d, J = 8.4 Hz), 4.24 (2H, t, J = 5.0 Hz), 3.36 (2H, t, J = 4.8 Hz), 2.99 (2H, t, J = 8.0 Hz), 1.67 (2H, m), 1.25–1.36 (26H, m), 0.89 (3H, t, J = 6.8 Hz); ^{13}C NMR (150 MHz, CD_3OD) δ 157.85, 155.44, 142.88, 140.05, 132.78, 132.06, 130.02, 129.92, 124.60, 123.25, 115.00, 114.66, 113.92, 106.44, 105.00, 31.64, 29.35, 29.34, 29.33, 29.30, 29.21, 29.05, 28.77, 26.12, 22.31, 13.01; HRMS (ESI+) m/z calcd for $C_{39}H_{52}NO_4S^+$ $[M+H]^+$ 630.3612, found 630.3595.

5.1.7. [6-Hydroxy-2-(4-hydroxyphenyl)benzo[*b*]thiophen-3-yl]{4-[2-(octadecylamino)ethoxy]phenyl}methanone (12, RC18)

The same procedure as for RC10 was used, but starting from octadecylamine.

Yield = 30.7 mg, 39%. Pale yellow viscous compound; IR: 3263, 2924, 2853, 1598, 1462, 1353, 1256, 1033, 835, 720, 680, 647, 583, 538, 510 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 7.73 (2H, d, J = 9.2 Hz), 7.43 (1H, d, J = 8.8 Hz), 7.26 (1H, d, J = 2.4 Hz), 7.18 (2H, d, J = 8.4 Hz), 6.85–6.90 (3H, m), 6.61 (2H, d, J = 8.4 Hz), 4.21 (2H, t, J = 4.8 Hz), 3.27 (2H, t, J = 4.8 Hz), 2.91 (2H, t, J = 7.6 Hz), 1.64 (2H, m), 1.23–1.35 (30H, m), 0.89 (3H, t, J = 7.0 Hz); ^{13}C NMR (150 MHz, CD_3OD) δ 157.85, 155.42, 140.04, 132.80, 132.06, 130.01, 124.59, 123.27, 115.02, 114.66, 113.93, 106.46, 105.00, 31.65, 29.36, 29.34, 29.33, 29.32, 29.22, 29.09, 29.05, 28.83, 22.31, 13.02; HRMS (ESI+) m/z calcd for $C_{41}H_{56}NO_4S^+$ $[M+H]^+$ 658.3925, found 658.3915.

5.2. Cell culture

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

5.3. Western blot analysis

MCF-7 cells were treated with compounds at the indicated concentrations in the presence or absence of 10 μM of MG132 (purchased from Peptide Institute) for 6 h. Cells were then 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 blot analysis using the following antibodies: anti-human ER α mouse monoclonal antibody (Santa Cruz) and anti-β-actin mouse monoclonal antibody (Sigma). Band was visualized using an LAS-3000 imaging system.

5.4. Fluorescence polarization assay

Test compounds were dissolved in DMSO to prepare a stock solution (10 mM). Fluorescence polarization (FP) competition binding assays were conducted to determine the relative binding affinity of compounds for ER α, using commercially available kits (P2698, Life Technologies) and according to the manufacturer's instruction. FP signals (mP values) were then measured using an EnVision multiple plate reader (Perkin Elmer) with a 470 nm excitation filter and a 535 nm emission filter. The fraction of compound bound to ER α was correlated to the mP value and plotted against values of competitor concentrations. The IC_{50} values were calculated using GraphPad Prism 6J software.

5.5. Reporter gene assay

ER-antagonistic activity of compounds was evaluated using a previously reported method.[23–27] Antagonist activity was measured in the presence of 0.3 nM 17β-estradiol, and test compounds were added 8 h after transfection. Cells were harvested 16–20 h after the treatment, and luciferase and β-galactosidase activities were assayed using a luminometer and a microplate reader. The relative light unit (RLU) value of each compound was normalized to an internal β-galactosidase control, and the means of triplicate assays was used for the final value.

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