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Bioorganic & Medicinal Chemistry 13 (2005) 1819–1828
Bioorganic & Medicinal Chemistry

In vitro evaluation of the anti-estrogenic activity of hydroxyl substituted diphenylnaphthyl alkene ligands for the estrogen receptor☆

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Received 27 August 2004; revised 21 November 2004; accepted 24 November 2004
Available online 22 December 2004

Abstract—There is still a need for additional scaffolds to further explore tissue selectivity and improving efficacy of selective estrogen receptor modulators (SERMs). A series of hydroxyl substituted diphenylnaphthyl alkene ligands for the two estrogen receptors are described that arose from an initial de novo designed diphenylnaphthyl propylene ligand **1**. All compounds gave K_is under 10 nM when assayed in the presence of ERα. Generally these compounds had very high affinity for both ER isotypes. Moving the hydroxyl group on naphthalene from the 6- to the 5-position of the α-naphthalene attached compounds (**6b** and **6e** vs **6c** and **6f**) had little affect on ER binding nor did altering the position of the naphthalene attachment (α or β) to the alkene moiety. In transfection assays none of the compounds displayed agonistic activity in the absence of E_2. In MCF-7 proliferation assays **6a–d**, **6f** and **12a–c** successfully abrogated E_2 stimulation and resulted in greater than 50% inhibition at 1 μM, a level of efficacy similar to that obtained when the cells were treated with raloxifene. Our results show that this new class of SERMs are good candidates for further study as therapeutic agents for the treatment of breast cancer and osteoporosis.

Keywords: Diphenylnaphthyl alkene ligands; Estrogen receptor; SERM.

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doi:10.1016/j.bmc.2004.11.051

1. Introduction

Stimulation of the estrogen receptor (ER) by endogenous estrogens plays an important role in both male and female physiology. Estrogens are involved in the regulation of cholesterol and lipid levels, and the functioning of skeletal, central nervous and reproductive systems.[1,2] Estradiol (E_2) stimulation, however, is also implicated in the development of breast cancer.[3] Consequently, many ER ligands are being developed with the aim of preventing E_2-mediated tumour growth. Tamoxifen, which was originally developed as an ER antagonist, is currently the hormonal treatment of choice for both pre-and post-menopausal women with breast carcinoma. It is now known that tamoxifen and other selective estrogen receptor modulators (SERMs) display a broad range of agonist and antagonist activity dependent on the tissue and species being evaluated, and that several factors are thought to contribute to this phenomenon.[4–7] With the discovery of a new ER subtype designated ERβ,[8] experiments based on the tissue

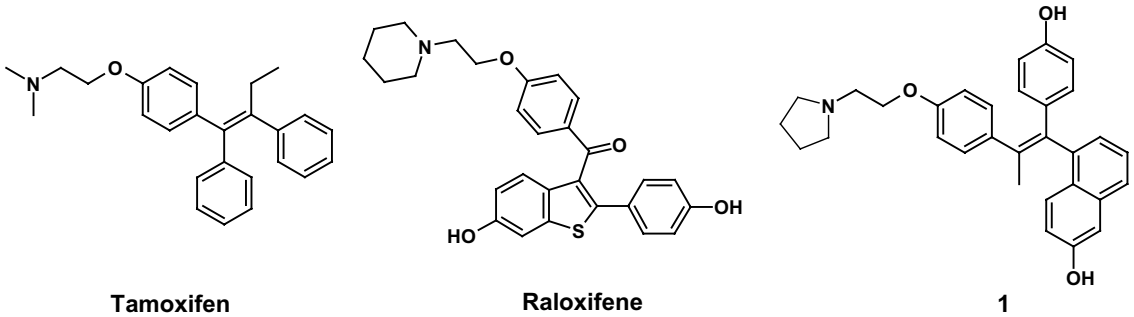


Figure 1.

distribution and pharmacology of ERα and ERβ suggest that the tissue selectivity of certain estrogens may be related, at least in part, to their different effects at the ERα and ER subtypes.[6,9,10] Other factors that may contribute to selectivity include the tissue-specific presence of co-repressors and co-activators, and the tissue/species presence of different DNA-response elements.[9,11]

Since the tissue selective effects of tamoxifen were discovered other SERMs with improved selectivity profiles have been developed. The most notable is the benzothiophene, raloxifene that is marketed for the prevention and treatment of osteoporosis. Although raloxifene displays improved selectivity compared with tamoxifen, optimal tissue selectivity has not yet been demonstrated. As a result there remains a strong need for additional diversity and new chemical scaffolds to allow for the exploration of improved tissue selectivity.

We have previously reported the favourable in vitro characteristics of a diphenylnaphthyl propylene ligand **1** (Fig. 1) for ERα that arose from our de novo design technology.[12] We subsequently embarked on a program to further explore analogues around this ligand. Specifically we were interested in substituting an ethyl group in place of the methyl group on the alkene moiety of **1**, since ethyl substitution is found in tamoxifen. In addition we determined the effect of removing the methyl group on the alkene moiety. The effect of altering the position of attachment (β vs α) of the naphthalene moiety to the alkene was also evaluated. Since the antagonist potency tends to increase when the piperidinoethoxy side chain replaces the pyrrolidinoethoxy,[13,14] all analogues were prepared with the latter group.

2. Chemistry

2.1. Synthesis

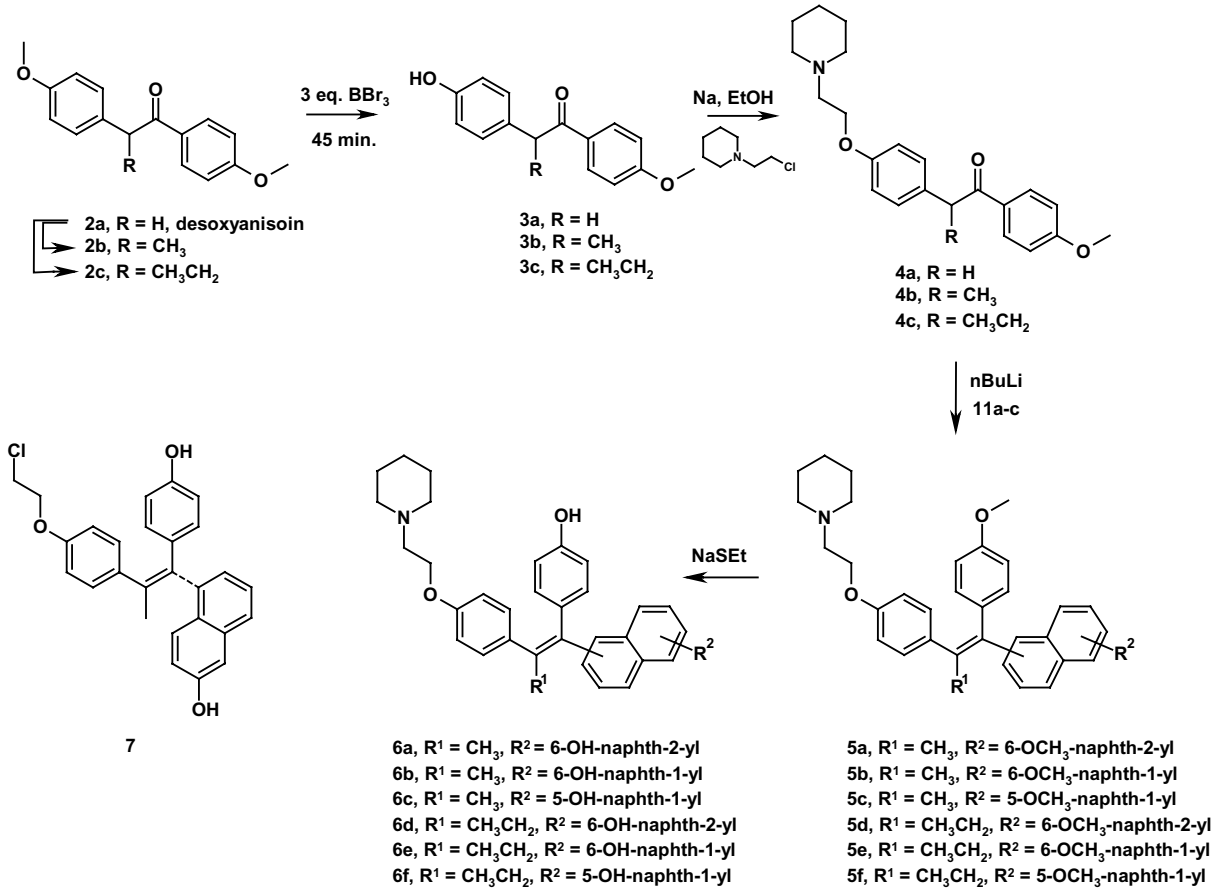
Since we were interested in determining the effects of ethyl and hydrogen substitution on the alkene portion of **1**, in addition to methyl substitution, it was more efficient to take advantage of the well known α-alkylation of desoxyanisoin followed by regioselective demethylation with a Lewis acid.[15,16] Hence as shown in Scheme 1, desoxyanisoin **2a**, was alkylated using NaH and CH_3I or EtBr to give **2b**,**c**, regioselectively demethylated using BBr_3 to give the monomethyl ethers **3a**–**c** and alkylated with 1-(2-chloroethyl)piperidine to give the piperidines **4a**–**c**. Two of these, **4b**,**c**, were alkylated with a variety of halogenated naphthalenes using halogen–lithium exchange conditions generating tertiary alcohols that were dehydrated using HCl in ethanol to give the methoxy ethers **5a**–**f**. These were demethylated using sodium ethyl thiolate to give **6a**–**f**. Unfortunately all attempts to alkylate the hydrogen analogue **4a** with halogenated naphthalenes were unsuccessful.

At the same time we were pursuing tamoxifen analogues whereby the phenyl ring geminal to the ethyl group in tamoxifen was replaced by our naphthalene moieties and the ethyl group shortened to hydrogen. As presented in Scheme 2, we prepared the simple alkene derivatives whereby 4,4′-dihydroxybenzophenone was alkylated with 1-(2-chloroethyl)piperidine to give the monoalkylated piperidine **8**. The hydroxynaphthaldehydes were prepared from the corresponding halogenated naphthalenes **9a**–**c** by halogen–lithium exchange conditions followed by formylation with DMF[17] generating **10a**–**c**. Demethylation with NaSEt in DMF afforded the requisite naphthaldehydes **11a**–**c**. Using McMurry coupling conditions[18–20] **8** was treated with **11a**–**c** giving the diphenylnaphthyl ethylenes **12a**–**c**.

The double bond configuration of the final compounds (*E*/*Z* isomerization) was determined from the chemical shift and integral of the piperidine basic side chain O-methylene protons in the NMR spectra.[19,21] The shifts of these protons for the *E*-isomers as noted in the literature[19,21] are further downfield than for the *Z*-isomers and the isomeric composition is given in Table 1. No real pattern has emerged regarding the final *E*/*Z* composition of the double bond, whether using metal–halogen exchange conditions followed by NaSEt demethylation or McMurray coupling although the *E*-isomer was the major isomer formed with the exception of **6d** and **12c**.

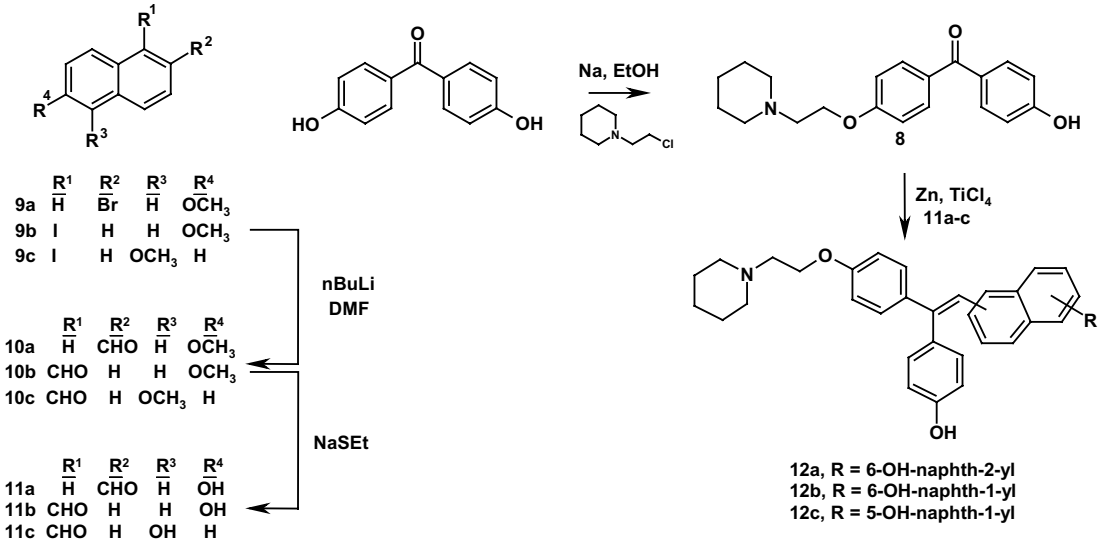
2.2. Computational studies

We had previously conducted relatively detailed modelling studies of **1** and out of those studies arose the suggestion that altering the position of the hydroxyl group on the naphthalene moiety from the 6- to the 5-position



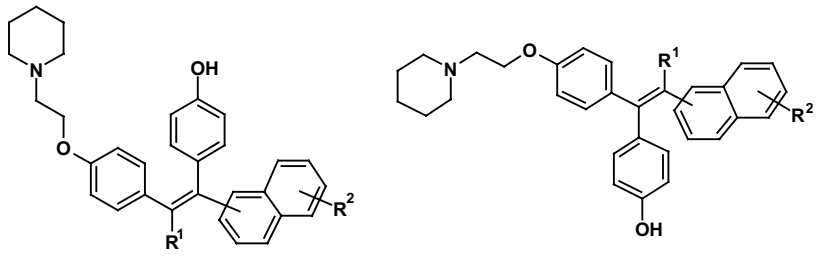
Scheme 1.

could improve binding,[12] by enabling the establishment of an additional potential hydrogen bond with His524 of ERα. To further evaluate this hypothesis and guide our synthetic efforts, a variety of virtual structures, **v13**–**v19** and two subsequently synthesized compounds, **6e**,**f** (Scheme 3) were docked into ERα using the GOLD package. GOLD is known to reproduce experimental binding positions well[22] and we have confirmed this for different receptor systems including ERα.[23] The base docking speed (times 1) was applied as it provided the best precision from all predefined GA settings. A particular problem in establishing the binding modes of these ligands arises because of the existence of two tautomeric states of His524 that are in equilibrium. Since the donor and



Scheme 2.

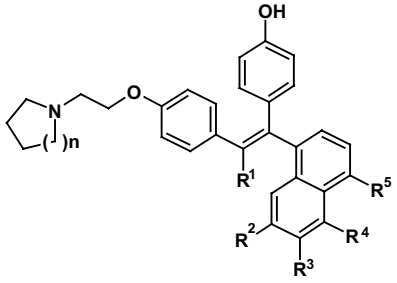
Table 1. ERα and ERβ binding, receptor activity and MCF-7 inhibition of diphenylnaphthyl alkenes



Compound	*E*/*Z* Ratio[a]	Scaffold	R^1	R^2	ER binding (K_i, nM)		Receptor activity[b]	MCF-7 growth inhibition[c]	Agonism[d]
					ERα	ERβ			
1	9:1				6.02 ± 1.97[e]	37.9 ± 13.8	++	++	No
6a	2:1	A	CH_3	6-OH	1.06 ± 0.49	0.99 ± 0.49	+++	++	No
6b	7:1	A	CH_3	6-OH	4.61 ± 1.66	59.4 ± 18.3	+++	++	No
6c	4:1	A	CH_3	5-OH	4.33 ± 1.89	20.8 ± 8.50	++	++	No
6d	1:2	A	CH_3CH_2	6-OH	0.82 ± 0.47	0.45 ± 0.56	+++	++	No
6e	8.5:1	A	CH_3CH_2	6-OH	4.11 ± 1.08	39.7 ± 16.3	+++	+	No
6f	8.5:1	A	CH_3CH_2	5-OH	4.45 ± 1.46	9.01 ± 3.51	+++	++	No
7[f]					10.1 ± 5.36	48.9 ± 28.1	+++	—	No
12a	19:1	B	H	6-OH	1.16 ± 0.51	2.43 ± 1.42	+++	++	No
12b	1:1	B	H	6-OH	0.9 ± 0.38	1.37 ± 0.82	+++	++	No
12c	1:1.8	B	H	5-OH	1.57 ± 0.82	2.66 ± 0.81	+++	++	No
Ral[g]					0.57 ± 0.20	2.41 ± 1.14	+++	++	Nd[h]

[a] *E*/*Z* Ratio determined from the integral of the respective OCH_2 triplets in the NMR spectra.
[b] Cos-1 cells transfected with human ERα and an ERE-bLuc reporter in the presence of 10 nM estradiol and increasing SERM ('+++', EC_{50} > 50 nM; '++', EC_{50} > 200 nM; '+', EC_{50} > 200 nM).
[c] MCF-7 cells stimulated with 10 nM estradiol for 72 h in the presence of 1 μM SERM ('++', >50% inhibition; '+' >50% inhibition; '—', no inhibition).
[d] HeLa cells transfected with human ERα and an ERE-bLuc reporter in the presence of increasing SERM ('no', no induction of reporter gene; 'yes', measurable reporter gene activity).
[e] 3.7 nM.[12]
[f] Chlorine in place of pyrrolidine.
[g] Raloxifene.
[h] Not determined.

acceptor positions of His are switched in the two states, this can strongly affect ligand orientations. Therefore in order to account for this effect, the ligands were docked to two forms of the receptor, each containing one of the His tautomers.



	n	R^1	R^2	R^3	R^4	R^5
1	1	CH_3	H	OH	H	H
v13	1	CH_3	OH	H	H	H
v14	1	CH_3	H	H	OH	H
v15	1	CH_3	H	H	H	OH
v16	1	CH_3	H	H	OH	OH
6e	2	CH_3CH_2	H	OH	H	H
v17	2	CH_3CH_2	OH	H	H	H
6f	2	CH_3CH_2	H	H	OH	H
v18	2	CH_3CH_2	H	H	H	OH
v19	2	CH_3CH_2	H	H	OH	OH

Scheme 3.

For the virtual analogues of **1**, in addition to moving the hydroxyl group on the naphthalene from the 6- to the 5-position (**v13**) we also docked compounds with the hydroxyl group at the 7- (**v14**) and 4-positions (**v15**) as well as the 4,5-dihydroxynaphthalene (**v16**). The corresponding analogues containing the ethyl group and with piperidine in the basic side chain as opposed to the pyrrolidine group, **v17**–**v19**, **6e**,**f** were also docked.

From the results of these GOLD docking studies two compounds, the 5-OH naphthalene **v14** (corroborating our previous modelling studies[12]) and the 4,5-dihydroxynaphthalene **v16** were predicted to establish hydrogen bonds with His524. Interestingly, the corresponding ethyl analogues (including the two ultimately synthesized, **6e**,**f**) did not appear to establish interactions with His524.

3. Biology

For the compounds in this study the questions we wished to answer pertained to their relative affinities

for both ER isotypes, the ability of these molecules to act as anti-estrogens in cellular transfection assays, their efficacy in inhibiting the E_2-stimulated growth of the human breast cancer cell line, MCF-7, and assessment as to whether these molecules behaved as partial agonist/antagonists (SERMs) or as pure anti-estrogens.

Several different assays were used to address these questions. These included ER binding studies using in vitro transcribed–translated ERs, receptor transcriptional activity via reporter genes under the control of estrogen response elements (EREs), and inhibition of estrogen stimulated MCF-7 cells, with all results presented in Table 1.

3.1. ER binding

The compounds were assayed as mixtures of the *E*/*Z* isomers. With the exception of **6a**, **6d**, **12b** and **12c** all compounds contained the *E*-isomer at a minimum of 80%. All of the compounds tested, except for **7**[12] gave K_is under 10 nM when assayed in the presence of ERα. When assayed with ERβ, six compounds (**1**, **6b**,**c** and **7**) had calculated K_is greater than 10 nM. Of the compounds that displayed better binding to ERβ (**6a** and **6d**) neither had greater than a two-fold preference for this ER isotype. These binding results suggest that some of these compounds had very high affinity for both ER isotypes and there was a modest preference for ERα.

Moving the hydroxyl group on naphthalene from the 6- to the 5-position of the α-naphthalene attached compounds (**6b** and **6e** vs **6c** and **6f**) had little affect on binding. The hydroxyl group is positioned a number of different ways in the various crystal structures of ERα with diethylstilbesterol, raloxifene and is even absent with 4-hydroxytamoxifen.[24] There are indications that a variety of lipophilic substituents (e.g., CH_3, F) can be substituted for the OH group of raloxifene[25] that can interact with His524, so perhaps we were too optimistic in our additional binding expectations from the modelling studies. Unfortunately we were unable to prepare the hydrogen-substituted compounds, which may have provided additional insight.

3.2. Receptor activity

These assays were designed to measure receptor transcriptional activity via reporter genes under the control of estrogen response elements (EREs) in order to determine the mechanistic influences of the compounds on receptor activity.

There are many permutations of the transfection assay that can be used to address the stimulation or repression of receptor activity. For example, under conditions where the receptor is stimulated by E_2, we observed that all compounds efficiently abrogated ERα transcriptional activity with EC_{50} values that correlated, for the most part, with receptor affinity. Data presented in Table 1 was obtained using Cos-1 cells, but we observed very similar results in HeLa and HepG2 cells (Data not shown). In addition, although this data was obtained using a reporter under the control of a synthetic ERE, a natural E_2-dependent promoter, pS2, also produced similar results (data not shown). We also observed anti-estrogen activity in the presence of ligand-stimulated ERβ, albeit with reduced efficacy as predicted by the overall reduction in affinity of these compounds for this ER isotype.

Other experiments were carried out to gauge the SERM potential of these compounds. For example, although these compounds were shown to be very good inhibitors of ER activity in the presence of E_2, we wished to determine if any of these molecules exhibited inherent estrogenic activity. We measured this activity in a similar fashion to the assay described above but utilized a basal luciferase reporter plasmid with one copy of an ERE to minimize background transcription. As shown in Table 1, none of the compounds displayed agonistic activity in the absence of E_2.

3.3. Inhibition of MCF-7 cells

In vitro profiling demonstrated that these molecules have a very high affinity for ER, with a modest preference for the ERα isotype, and results from the transfection assays indicated several behaved as potent anti-estrogens. We therefore wished to determine if these molecules could effectively inhibit the proliferation of ER+ human breast cancer MCF-7 cells. We routinely obtained approximately a twofold stimulation in proliferation in the presence of E_2 over the untreated cells indicating that the ER^+ MCF-7 cells were responding to hormone. Treatment of these stimulated cells with the test compounds resulted in a wide range of responses. Compound **7**, which lacks a basic side chain was unable to inhibit the proliferation of MCF-7 cells. In contrast compounds **1**, **6a**–**d**, **6f** and **12a**–**c**, which all contain the basic side chain, successfully abrogated E_2 stimulation and resulted in greater than 50% inhibition at 1 μM, a level of efficacy similar to that obtained when the cells were treated with raloxifene. This confirms the correlation of effective inhibition of breast cancer cell proliferation with the presence of a basic side chain in the molecule, presumably as a result of interaction with Asp351.[26] Overall, these results demonstrate that several of the compounds were quite effective in this assay and could inhibit proliferation to a level that was comparable to raloxifene.

The biological profiling of compounds of this nature (mixtures of *E*/*Z* isomers) have been reported previously.[19,21,27] It has been suggested, however, that it is the *Z*-isomer of tamoxifen that is anti-estrogenic[27] whilst the *E*-isomer acts as an estrogen agonist. Moreover it is known that facile isomerization with these types of compounds frequently occurs and it proves difficult to separate the isomers chromatographically.[20] Therefore, although the compounds in this study were tested as *E*/*Z* mixtures and have thus far generated appropriate in vitro SERM activity, due to the possibility of the two isomers displaying different activity further work would be required to determine the biological effects of the individual isomers. Nonetheless in terms of potency

the data indicates that at least one of the two *E*/*Z* isomers is at least as potent as the mixture. It is also unlikely that either isomer has substantial estrogenic activity (although the higher affinity compound may mask this).

4. Conclusion

In conclusion, we have carried out the in vitro characterization of several members of the novel hydroxyl substituted diphenylnaphthyl alkene family of nonsteroidal potential SERMs containing the piperidine basic side chain. Minor structural modifications/changes such as extending the alkyl portion on the alkene moiety, altering the position of attachment of the naphthalene moiety (α to β) to the alkene or moving the hydroxyl group on the naphthalene moiety produced minor effects in terms of binding affinity and functional effects. Our results clearly show that these compounds (as *E*/*Z* mixtures) have very high affinity for the α and β ERs and display effective SERM-like activity in cell-based assays. Most abrogate transcriptional activity of E_2-stimulated ERs and can efficiently prevent the proliferation of human MCF-7 breast cancer cells. At the same time cell transfection assays in the presence of the TGFβ promoter in HeLa cells indicate that our compounds may behave like raloxifene, a known SERM, and not like the pure anti-estrogen ICI182,780.[28] Our results show that these new naphthalene SERMs as a class are good candidates for further study as therapeutic agents for the treatment of breast cancer and osteoporosis, or perhaps in hormone replacement therapy.

5. Experimental

5.1. General

All solvents were reagent grade and dried appropriately prior to their use. NMR spectra were obtained using a Gemini 200 or Bruker 400 MHz spectrometer using $CDCl_3$, DMSO-d_6, acetone-d_6 or methanol-d_4 as solvents. Silica gel (200–430 mesh) was used for flash column chromatography.

5.1.1. 2-(4-Hydroxyphenyl)-1-(4-methoxyphenyl)propan-1-one, 3b. Prepared from **2b**[15] following the method given for **3c**. Yield (88%). 400 MHz ^{1}H NMR ($CDCl_3$) δ 7.93–7.95 (d, 2H, ArH); 7.12–7.14 (d, 2H, ArH); 6.85–6.87 (d, 2H, ArH); 6.75–6.77 (d, 2H, ArH); 4.59 (q, 1H, CH); 3.82 (s, 3H, OCH_3); 1.48 (d, 3H, C*H*$_3$CH).

5.1.2. 2-(4-Hydroxyphenyl)-1-(4-methoxyphenyl)butan-1-one, 3c. Compound **2c**[15] (2.85 g, 0.01 mol) was dissolved in CH_2Cl_2 (50 mL) and placed in a CO_2/acetone bath for 15 min. BBr_3 (1 M in CH_2Cl_2, 30 mL, 0.03 mol) was slowly added and the reaction left in the CO_2/acetone bath for 15 min. The reaction was allowed to come to room temperature and after an additional 30 min TLC indicated the reaction was complete. The reaction was added dropwise to satd $NaHCO_3$. The organic layer was separated and the aqueous layer washed with CH_2Cl_2. The combined organic layers were washed with water then brine, dried and concentrated to give a purple oil that was purified by flash column chromatography to give 2.6 g (95%) of **3c** as white crystals. 400 MHz ^{1}H NMR ($CDCl_3$) δ 7.94–7.96 (d, 2H, ArH); 7.13–7.16 (d, 2H, ArH); 6.85–6.87 (d, 2H, ArH); 6.75–6.77 (d, 2H, ArH); 5.70 (br s, 1H, OH); 4.34 (t, 1H, CH); 3.81 (s, 3H, OCH_3); 2.13 (m, 1H, CH_3CH_2); 1.81 (m, 1H, CH_3CH_2); 0.88 (t, 3H, CH_3CH_2).

5.1.3. 1-(4-Methoxyphenyl)-2-[4-(2-piperidin-1-yl-ethoxy)phenyl]ethan-1-one, 4a. To a solution of **3a**[16] (0.7 g, 2.9 mmol) in absolute ethanol (35 mL) that was refluxing vigorously was added Na (0.13 g, 5.8 mmol). After further refluxing for 30 min 1-(2-chloroethyl)piperidine hydrochloride (0.59 g, 3.2 mmol) was added. After refluxing for 1 h the reaction was filtered, washed with acetone and concentrated. The residue was purified by silica gel flash chromatography using a gradient of 35% ethyl acetate in hexanes to 40–50% acetone in hexanes to give 0.16 g (26%) of **4a**. 400 MHz ^{1}H NMR ($CDCl_3$) δ 7.98–8.00 (d, 2H, ArH); 7.15–7.17 (d, 2H, ArH); 6.91–6.93 (d, 2H, ArH); 6.85–6.87 (d, 2H, ArH); 4.16 (2, 2H, CH_2); 4.07 (t, 2H, OCH_2); 3.86 (s, 3H, OCH_3); 2.75 (t, 2H, NCH_2); 2.49 (br s, 4H, CH_2NCH_2); 1.60 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.44 (m, 2H, $NCH_2CH_2CH_2$).

5.1.4. 1-(4-Methoxyphenyl)-2-[4-(2-piperidin-1-yl-ethoxy)phenyl]propan-1-one, 4b. Prepared following the method for **4a** using **3b**. Yield 0.88 g (45%). 200 MHz ^{1}H NMR ($CDCl_3$) δ 7.92–7.96 (d, 2H, ArH); 7.15–7.20 (d, 2H, ArH); 6.81–6.88 (d, 4H, ArH); 4.60 (q, 1H, CH); 4.06 (t, 2H, OCH_2); 3.82 (s, 3H, OCH_3); 2.76 (t, 2H, NCH_2); 2.51 (br s, 4H, CH_2NCH_2); 1.60 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.48 (d, 3H, CH_3); 1.44 (m, 2H, $NCH_2CH_2CH_2$).

5.1.5. 1-(4-Methoxyphenyl)-2-[4-(2-piperidin-1-yl-ethoxy)phenyl]butan-1-one, 4c. Prepared following the method for **4a** using **3c**. Yield 2.9 g (53%). 200 MHz ^{1}H NMR ($CDCl_3$) δ 7.93–7.97 (d, 2H, ArH); 7.18–7.21 (d, 2H, ArH); 6.79–6.88 (d, 4H, ArH); 4.33 (q, 1H, CH); 4.04 (t, 2H, OCH_2); 3.81 (s, 3H, OCH_3); 2.73 (t, 2H, NCH_2); 2.48 (br s, 4H, CH_2NCH_2); 2.14 (m, 1H, CH_2); 1.83 (m, 1H, CH_2); 1.58 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.43 (m, 2H, $NCH_2CH_2CH_2$); 0.87 (t, 3H, CH_3).

5.1.6. (*E*/*Z*)-1-(4-Methoxyphenyl)-2-(4-(2-piperidin-1-yl)-ethoxy)phenyl-1-(6-methoxynaphthalen-2-yl)propene, 5a. 2-Bromo-6-methoxynaphthalene (406 mg, 1.71 mmol) was dissolved in anhydrous THF (20 mL) and cooled to −78 °C. *n*-BuLi (2 M in cyclohexane, 0.86 mL, 1.71 mmol) was added and after 5 min **4b** (210 mg, 0.57 mmol) dissolved in THF was added. The reaction was removed from the cooling bath and allowed to come to rt. After 1 h the reaction was quenched with methanol and concentrated. The residue was dissolved in 10% CH_3OH in $CHCl_3$ and the tertiary alcohol was partially purified using silica gel chromatography using 10% CH_3OH in $CHCl_3$. After concentrating the partially

purified tertiary alcohol was dissolved in abs ethanol (15 mL). Concd HCl (1.5 mL) was added and the reaction refluxed for 1 h. After evaporating the solvent the residue was dissolved in CH_2Cl_2, water was added, the mixture adjusted to pH 8 with satd $NaHCO_3$ and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with 1 N NaOH, water, brine, dried, concentrated and purified by silica gel flash chromatography using a gradient of $CHCl_3$–hexanes (1/1) to $CHCl_3$ to 2% CH_3OH in $CHCl_3$ to give 0.21 g (72%) of **5a**. 200 MHz ^{1}H NMR ($CDCl_3$) δ 6.62–7.80 (m, 14H, ArH); 4.00–4.17 (t, 2H, OCH_2); 3.73–3.95 (s, 6H, OCH_3); 2.70–2.86 (t, 2H, NCH_2); 2.54 (m, 4H, CH_2NCH_2); 2.19–2.22 (s, 3H, CH_3); 1.64 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.49 (m, 2H, $NCH_2CH_2CH_2$).

5.1.7. (*E*/*Z*)-1-(4-Methoxyphenyl)-2-(4-(2-piperidin-1-yl)-ethoxy)phenyl-1-(6-methoxynaphthalen-1-yl)propene, 5b. Prepared following the method for **5a** using **4b** and 1-iodo-6-methoxynaphthalene.[12] Yield 0.185 g (67%). 200 MHz ^{1}H NMR ($CDCl_3$) δ 6.47–8.00 (m, 14H, ArH); 4.09 (t, 2H, OCH_2); 3.67–3.93 (s, 6H, OCH_3); 2.77 (t, 2H, NCH_2); 2.53 (m, 4H, CH_2NCH_2); 1.84 (s, 3H, CH_3); 1.63 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.48 (m, 2H, $NCH_2CH_2CH_2$).

5.1.8. (*E*/*Z*)-1-(4-Methoxyphenyl)-2-(4-(2-piperidin-1-yl)-ethoxy)phenyl-1-(5-methoxynaphthalen-1-yl)propene, 5c. Prepared following the method for **5a** using **4b** and 1-iodo-5-methoxynaphthalene (see method for 1-iodo-6-methoxynaphthalene[12]). Yield 0.142 g (52%). 200 MHz ^{1}H NMR ($CDCl_3$) δ 6.50–8.27 (m, 14H, ArH); 4.11 (t, 2H, OCH_2); 3.67–4.02 (s, 6H, OCH_3); 2.80 (t, 2H, NCH_2); 2.56 (m, 4H, CH_2NCH_2); 1.85 (s, 3H, CH_3); 1.64 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.49 (m, 2H, $NCH_2CH_2CH_2$).

5.1.9. (*E*/*Z*)-1-(4-Methoxyphenyl)-2-(4-(2-piperidin-1-yl)-ethoxy)phenyl-1-(6-methoxynaphthalen-2-yl)butene, 5d. Prepared following the method for **5a** using **4c** and 2-bromo-6-methoxynaphthalene. Yield 0.263 g (91%). 200 MHz ^{1}H NMR ($CDCl_3$) δ 6.52–7.76 (m, 14H, ArH); 3.95–4.13 (t, 2H, OCH_2); 3.68–3.93 (s, 6H, OCH_3); 2.65–2.80 (t, 2H, NCH_2); 2.47 (m, 6H, $CH_2NCH_2 + CH_2$); 1.60 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.45 (m, 2H, $NCH_2CH_2CH_2$); 0.97 (t, 3H, CH_3).

5.1.10. (*E*/*Z*)-1-(4-Methoxyphenyl)-2-(4-(2-piperidin-1-yl)-ethoxy)phenyl-1-(6-methoxynaphthalen-1-yl)butene, 5e. Prepared following the method for **5a** using **4c** and 1-iodo-6-methoxynaphthalene. Yield 0.185 g (85%). 200 MHz ^{1}H NMR ($CDCl_3$) δ 6.50–7.99 (m, 14H, ArH); 4.10 (t, 2H, OCH_2); 3.66–3.92 (s, 6H, OCH_3); 2.79 (t, 2H, NCH_2); 2.53 (m, 4H, CH_2NCH_2); 2.20 (q, 2H, CH_2); 1.62 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.46 (m, 2H, $NCH_2CH_2CH_2$); 0.77 (t, 3H, CH_3).

5.1.11. (*E*/*Z*)-1-(4-Methoxyphenyl)-2-(4-(2-piperidin-1-yl)-ethoxy)phenyl-1-(5-methoxynaphthalen-2-yl)butene, 5f. Prepared following the method for **5a** using **4c** and 1-iodo-5-methoxynaphthalene. Yield 0.238 g (83%). 200 MHz ^{1}H NMR ($CDCl_3$) δ 6.48–8.25 (m, 14H, ArH); 4.09 (t, 2H, OCH_2); 3.64–4.00 (s, 6H, OCH_3); 2.78 (t, 2H, NCH_2); 2.53 (m, 4H, CH_2NCH_2); 2.18 (q, 2H, CH_2); 1.61 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.46 (m, 2H, $NCH_2CH_2CH_2$); 0.76 (t, 3H, CH_3).

5.1.12. (*E*/*Z*)-1-(4-Hydroxyphenyl)-2-(4-(2-piperidin-1-yl)ethoxy)phenyl-1-(6-hydroxynaphthalen-2-yl)propene, 6a. To **5a** (208 mg, 0.41 mmol) dissolved in DMF (10 mL) was added NaSEt (80%, 0.86 g, 10.2 mmol) and refluxed for 1.5 h. Water was added, the mixture adjusted to pH 9 with satd $NaHCO_3$, extracted with EtOAc, washed with water, brine, dried and concentrated. The residue was purified by silica gel flash chromatography using a gradient of $CHCl_3$–hexanes (1/1) to $CHCl_3$ to 3–8% CH_3OH in $CHCl_3$ to give 0.144 g (73%) of **6a**. 400 MHz ^{1}H NMR (DMSO-d_6) δ 9.11–9.63 (s, 2H, OH); 6.37–7.66 (m, 14H, ArH); 3.83–3.93 (t, 2H, OCH_2); 2.40–2.60 (t, 2H, NCH_2); 2.32 (m, 4H, CH_2NCH_2); 1.96–1.99 (s, 3H, CH_3); 1.40 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.28 (m, 2H, $NCH_2CH_2CH_2$).

5.1.13. (*E*/*Z*)-1-(4-Hydroxyphenyl)-2-(4-(2-piperidin-1-yl)ethoxy)phenyl-1-(6-hydroxynaphthalen-1-yl)propene, 6b. Prepared following the method for **6a**. Yield 0.03 g (51%). 200 MHz ^{1}H NMR (acetone-d_6) δ 6.46–8.00 (m, 14H, ArH); 3.85–4.09 (t, 2H, OCH_2); 2.72 (t, 2H, NCH_2); 2.50 (m, 4H, CH_2NCH_2); 1.82–2.21 (s, 3H, CH_3); 1.53 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.44 (m, 2H, $NCH_2CH_2CH_2$).

5.1.14. (*E*/*Z*)-1-(4-Hydroxyphenyl)-2-(4-(2-piperidin-1-yl)ethoxy)phenyl-1-(5-hydroxynaphthalen-1-yl)propene, 6c. Prepared following the method for **6a**. Yield 0.06 g (45%). 400 MHz ^{1}H NMR (acetone-d_6) δ 6.38–8.07 (m, 14H, ArH); 3.75–4.01 (t, 2H, OCH_2); 2.50–2.62 (t, 2H, NCH_2); 2.41 (m, 4H, CH_2NCH_2); 1.73 (s, 3H, CH_3); 1.47 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.34 (m, 2H, $NCH_2CH_2CH_2$).

5.1.15. (*E*/*Z*)-1-(4-Hydroxyphenyl)-2-(4-(2-piperidin-1-yl)ethoxy)phenyl-1-(6-hydroxynaphthalen-2-yl)butene, 6d. Prepared following the method for **6a**. Yield 0.124 g (51%). 400 MHz ^{1}H NMR (methanol-d_4) δ 6.41–7.65 (m, 14H, ArH); 3.97–4.05 (t, 2H, OCH_2); 2.65–2.75 (t, 2H, NCH_2); 2.40–2.58 (m, 4H, $CH_2NCH_2 + CH_2$); 1.55 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.43 (m, 2H, $NCH_2CH_2CH_2$); 0.90 (m, 3H, CH_3).

5.1.16. (*E*/*Z*)-1-(4-Hydroxyphenyl)-2-(4-(2-piperidin-1-yl)ethoxy)phenyl-1-(6-hydroxynaphthalen-1-yl)butene, 6e. Prepared following the method for **6a**. Yield 0.124 g (51%). 400 MHz ^{1}H NMR (methanol-d_4) δ 6.31–7.83 (m, 14H, ArH); 3.99 (t, 2H, OCH_2); 2.69 (t, 2H, NCH_2); 2.47 (m, 4H, CH_2NCH_2); 2.07 (m, 2H, CH_2); 1.54 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.40 (m, 2H, $NCH_2CH_2CH_2$); 0.66 (m, 3H, CH_3).

5.1.17. (*E*/*Z*)-1-(4-Hydroxyphenyl)-2-(4-(2-piperidin-1-yl)ethoxy)phenyl-1-(5-hydroxynaphthalen-1-yl)butene, 6f. Prepared following the method for **6a**. Yield 0.111 g (69%). 400 MHz ^{1}H NMR (DMSO-d_6) δ 10.04 (s, 1H, OH); 9.08 (s, 1H, OH); 6.28–8.23 (m, 14H, ArH); 3.94 (t, 2H, OCH_2); 2.57 (t, 2H, NCH_2); 2.35 (m, 4H,

CH_2NCH_2); 2.00 (m, 2H, CH_2); 1.41 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.30 (m, 2H, $NCH_2CH_2CH_2$) 0.60 (m, 3H, CH_3).

5.1.18. 4-(2-Piperidin-1-yl)ethoxy-4′-hydroxybenzophenone, 8. Na (0.23 g, 10 mmol) was added to refluxing abs ethanol (150 mL). After 5 min at reflux, 4,4′-dihydroxybenzophenone (1 g, 4.7 mmol) was added and the reaction refluxed for 0.5 h. 1-(2-Chloroethyl)piperidine hydrochloride (0.95 g, 5.1 mmol) was added as the free base by treatment with K_2CO_3 (1.4 g, 10.2 mmol) in abs ethanol and continued refluxing for 1 h. The solvent was evaporated and the residue dissolved in acetone, filtered, concentrated and purified by silica gel flash chromatography using a gradient of 3–4.5% CH_3OH in $CHCl_3$ to give 0.73 g (48%) of **8**. 400 MHz ^{1}H NMR (DMSO-d_6) δ 6.80–7.75 (m, 8H, ArH); 4.18 (m, 2H, OCH_2); 2.70 (m, 2H, NCH_2); 2.44 (m, 4H, CH_2NCH_2); 1.50 (m, 4H, $CH_2CH_2NCH_2CH_2$); 1.41 (m, 2H, $NCH_2CH_2CH_2$).

5.1.19. 6-Methoxy-2-naphthaledhyde, 10a. To a solution of 2-bromo-6-methoxynaphthaldehyde (0.5 g, 2.1 mmol) in anhydrous THF (20 mL) cooled to −78 °C under Ar was added *n*-BuLi (2 M in cyclohexane, 2.6 mL, 5.3 mmol) and stirred for 15 min. Anhydrous DMF (1.0 mL, 12.7 mmol) was added and the reaction stirred for a further 15 min at −78 °C. The reaction was poured into 3 N HCl (15 mL), extracted with ether, washed with water, satd $NaHCO_3$, brine, dried and concentrated. The residue was purified by silica gel flash chromatography using 5% acetone in hexanes to give 0.29 g (74%). 400 MHz ^{1}H NMR ($CDCl_3$) δ 10.10 (s, 1H, CHO); 7.80–7.94 (m, 3H, ArH); 7.16–7.25 (m, 2H, ArH); 3.94 (s, 3H, OCH_3).

5.1.20. 6-Methoxy-1-naphthaledhyde, 10b. Prepared following the method for **10a** from 1-iodo-6-methoxynaphthalene.[12] Yield 0.26 g (76%). 200 MHz ^{1}H NMR ($CDCl_3$) δ 10.34 (s, 1H, CHO); 9.18 (d, 1H, ArH); 8.00 (d, 1H, ArH); 7.84 (m, 1H, ArH); 7.60 (dd, 1H, ArH); 7.35 (dd, 1H, ArH); 7.22 (dd, 1H, ArH); 3.94 (s, 3H, OCH_3).

5.1.21. 5-Methoxy-1-naphthaledhyde, 10c. Prepared following the method for **10a** from 1-iodo-5-methoxynaphthalene. Yield 0.18 g (69%). 200 MHz ^{1}H NMR ($CDCl_3$) δ 10.42 (s, 1H, CHO); 8.80 (d, 1H, ArH); 8.62 (d, 1H, ArH); 8.02 (m, 1H, ArH); 7.61 (m, 2H, ArH); 6.95 (d, 1H, ArH); 3.97 (s, 3H, OCH_3).

5.1.22. 6-Hydroxy-2-naphthaldehyde, 11a. To **10a** (0.25 g, 1.3 mmol) and NaSEt (80%, 0.45 g, 5.4 mmol) was added anhydrous DMF (15 mL) and refluxed for 20 min. After cooling the reaction was poured into 3 N HCl (50 mL), extracted with EtOAc, washed with water, brine, dried and concentrated. The residue was purified by silica gel flash chromatography using a gradient of hexanes to 50% ether in hexanes to give 0.13 g (59%). 200 MHz ^{1}H NMR (DMSO-d_6) δ 10.35 (br s, 1H, OH); 10.06 (s, 1H, CHO); 8.42 (d, 1H, ArH); 8.03 (d, 1H, ArH); 8.69 (m, 2H, ArH); 7.20 (m, 2H, ArH).

5.1.23. 6-Hydroxy-1-naphthaldehyde, 11b. Prepared following **11a** using **10b**. Yield 0.085 g (39%). 400 MHz ^{1}H NMR (acetone-d_6) δ 10.35 (s, 1H, CHO); 9.16 (d, 1H, ArH); 8.93 (br s, 1H, OH); 8.05 (d, 1H, ArH); 7.94 (d, 1H, ArH); 7.65 (dd, 1H, ArH); 7.35 (m, 2H, ArH).

5.1.24. 5-Hydroxy-1-naphthaldehyde, 11c. Prepared following **11a** using **10c**. Yield 0.06 g (35%). 400 MHz ^{1}H NMR (acetone-d_6) δ 10.43 (s, 1H, CHO); 9.40 (br s, 1H, OH); 8.75 (d, 1H, ArH); 8.62 (d, 1H, ArH); 8.15 (d, 1H, ArH); 7.70 (dd, 1H, ArH); 7.53 (dd, 1H, ArH); 7.07 (d, 1H, ArH).

5.1.25. (*E*/*Z*)-1-(4-Hydroxyphenyl)-1-(4-(2-piperidin-1-yl)ethoxy)phenyl-2-(6-hydroxynaphthalen-2-yl)ethene, 12a. To a suspension of Zn (0.1 g, 1.5 mmol) in anhydrous THF (4 mL) under Ar at −10 °C was added $TiCl_4$ (0.068 mL, 0.62 mmol) slowly. The cooling bath was removed and the reaction gently refluxed for 2 h. The heat was removed and a solution of **8** (0.1 g, 0.31 mmol) and **11a** (0.08 g, 0.46 mmol) in anhydrous THF (22 mL) was added. The reaction mixture was then refluxed for a further 2.5 h. Additional **17a** (0.06 g, 0.35 mmol) was added in anhydrous THF (7 mL) and refluxed for a further 40 min. The reaction mixture was cooled, poured into 10% K_2CO_3 and EtOAc and stirred overnight. After separating, the EtOAc layer was washed with brine, dried, concentrated and purified by silica gel flash chromatography using a gradient of EtOAc–hexanes (1/1) to 4% CH_3OH/36% hexanes in EtOAc to give 0.05 g (35%). 400 MHz ^{1}H NMR (acetone-d_6) δ 9.71 (br s, 1H, OH); 9.55 (br s, 1H, OH); 6.72–7.51 (m, 14H, ArH); 4.04 (m, 2H, OCH_2); 2.66 (m, 2H, NCH_2); 2.44 (br s, 4H, CH_2NCH_2); 1.49 (br s, 4H, $CH_2CH_2NCH_2CH_2$); 1.38 (br s, 2H, $NCH_2CH_2CH_2$).

5.1.26. (*E*/*Z*)-1-(4-Hydroxyphenyl)-1-(4-(2-piperidin-1-yl)ethoxy)phenyl-2-(6-hydroxynaphthalen-1-yl)ethene, 12b. Prepared following the method for **12a** using 6-hydroxy-1-naphthaldehyde. Yield 0.025 g (66%). 400 MHz ^{1}H NMR (DMSO-d_6) δ 9.71 (br s, 1H, OH); 9.58 (br s, 1H, OH); 6.56–7.94 (m, 14H, ArH); 4.09 (m, 2H, OCH_2); 2.68 (m, 2H, NCH_2); 2.42 (br s, 4H, CH_2NCH_2); 1.50 (br s, 4H, $CH_2CH_2NCH_2CH_2$); 1.38 (br s, 2H, $NCH_2CH_2CH_2$).

5.1.27. (*E*/*Z*)-1-(4-Hydroxyphenyl)-1-(4-(2-piperidin-1-yl)ethoxy)phenyl-2-(5-hydroxynaphthalen-1-yl)ethene, 12c. Prepared following the method for **12a** using 5-hydroxy-1-naphthaldehyde. Yield 0.025 g (60%). 400 MHz ^{1}H NMR (DMSO-d_6) δ 10.12 (br s, 1H, OH); 9.59 (br s, 1H, OH); 6.55–8.00 (m, 14H, ArH); 4.09 (m, 2H, OCH_2); 2.68 (m, 2H, NCH_2); 2.42 (br s, 4H, CH_2NCH_2); 1.50 (br s, 4H, $CH_2CH_2NCH_2CH_2$); 1.38 (br s, 2H, $NCH_2CH_2CH_2$).

5.2. ER binding

Human cDNAs encoding ERα and ERβ were used as templates to express receptor proteins in vitro. The proteins were produced with rabbit reticulocyte lysates as supplied by Promega (TNT kit) that couples transcrip-

tion and translation in a single reaction. The amount of template used in each reaction was determined empirically and expression was monitored in parallel reactions where [^{35}S]methionine was incorporated into the receptor followed by gel electrophoresis and exposure to film.

Binding reactions were carried out in 100 μL final volumes in TEG buffer (10 mM tris, pH 7.5, 1.5 mM EDTA, 10% glycerol). Five microlitres of in vitro transcribed–translated receptor was used in each binding reaction in the presence of 0.5 nM [^{3}H]estradiol (E_2). All compounds were routinely tested from 10^{-11} to 10^{-6} M and diluted in ethanol. The reactions were incubated at 4 °C overnight and bound E_2 was quantified by adding 200 μL dextran-coated charcoal. After a 15 min rotation at 4 °C, the tubes were centrifuged for 10 min and 150 μL of the supernatant was added to 5 mL scintillation cocktail for determination of cpms by liquid scintillation counting.

Controls for background were included in each experiment using 5 μL unprogrammed rabbit reticulocyte lysate. This value, typically 10–15% of the maximal counts was subtracted from all values. The maximum binding was determined by competing bound E_2 with only the ethanol vehicle. This value was set to 100% (maximal E_2 binding). Values for percent inhibition were calculated based on the maximal E_2 binding. The data were plotted and K_i values calculated using the Prism Software.

5.3. Receptor activity

Typically cells are seeded in 12-well plates and allowed to grow until 60–80% confluence. Transfection of DNA into cells is carried out using a liposome-based delivery system from Qiagen, Polfect reagent. During transfection experiments, the cells were maintained in phenol-red free medium in the presence of 10% fetal calf serum that had been treated with dextran-coated charcoal to remove steroids. Under standard conditions, 100 ng of pCMX-hERα or pCMX-hERβ, 1000 ng ERE-driven luciferase reporter plasmid, and 500 ng pCMX-β-galactosidase was transfected in each well. Twenty-four hours after transfection, the medium was removed and the cells were treated with the vehicle (0.1% ethanol), 10 nM E_2, or 10 nM E_2 in the presence of increasing concentrations of test compound. The cells were harvested 24–48 h later by lysing in 10 mM potassium phosphate buffer, pH 7.8 and 1% triton X-100 for 15 min at 4 °C. Luciferase and β-galactosidase activity were determined by standard methods with 25 μL of cell lysate. Luciferase values were normalized with the corresponding β-galactosidase activity. Receptor activity was expressed as the percentage of the maximal E_2 stimulation in the absence of any compound and this value was set to 100%.

5.4. Inhibition of MCF-7 cells

The ability of the test compounds to inhibit the proliferation of human breast cancer cells in vitro was assessed using MCF-7 cells (ATCC, Manassas, VA). These cells contain ERα and have been shown to be responsive to treatment with E_2. Briefly, MCF-7 cells were seeded in 48-well plates in Iscove's phenol red-free medium containing 10% FBS and 10 μg/mL insulin. The next day, the cells were starved for 72 h in medium containing 10% dextran-coated charcoal treated serum in the absence of insulin. The cells were treated with 10 nM E_2 in the absence or presence of increasing concentration of raloxifene or test compound. Cell number was determined after 96 h using the MTS assay as described by the manufacturer (Promega Corp.).

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