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Synthesis of 4,4′-Diaminotriphenylmethanes with Potential Selective Estrogen Receptor Modulator (SERM)-like Activity

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Dedicated to Prof. Ángel G. Ravelo on the occasion of his recent retirement.

In this study, a series of new 4,4′-diaminotriphenylmethanes was efficiently synthesized from aromatic aldehydes and 2,5-dimethoxybenzenamine under microwave irradiation in the presence of $Sc(OTf)_3$ as a catalyst. Antiproliferative activity was assessed by using the MCF-7 estrogen receptor (ER)-positive breast cancer cell line, and antagonist/agonist transcriptional activities were determined. Docking studies and competition studies of triphenylmethanes and radiolabeled estradiol determined that these compounds do not bind the ER, indicating that triphenylmethane-induced changes in proliferative and transcriptional activities differ from conventional mechanisms of action triggered by other selective ER modulators.

Introduction

Triarylmethanes are an important group of organic compounds, useful as precursors for the preparation of printing inks and in the dyeing of ceramics, leather, and polyacrylonitrile fibers.[1–6] They have been widely used in analytical fields, for example, detection of hydrogen peroxide in medical diagnostic kits, biotechnology process control, analyses of biological fluids, and wastewater treatment.[7] The triarylmethine moiety can also be found in bioactive natural products such as cassigarol B,[8] mohsenone, and chamaechromone.[9–11] Furthermore, these compounds are attractive targets because of their applications in medicinal chemistry as promising antitumor,[12–15] antibacterial,[16] and anti-HIV agents.[17,18]

On the other hand, estrogen receptors (ERs) regulate mammalian hormonal and physiological processes through binding with their common endogenous steroid hormone, 17β-estradiol (E2). This natural estrogen controls a number of physiological processes within, but not limited to, the reproductive organs in both females and males.[19–21] There are two known subtypes of ERs, namely ERα and ERβ, each with distinct tissue expression.[22] ERα is mainly distributed in breast, uterus, and bone, while ERβ is widely expressed in prostate, lungs, the central nervous system, and the cardiovascular system. It has been identified that ERs are responsible of a variety of estrogen-related diseases, including osteoporosis, breast cancer, prostate cancer, and inflammation.[23] In addition to the main clinical application of breast cancer therapy, ER modulators are also used in hormone replacement therapy (HRT) to treat postmenopausal diseases. However, clinical studies suggest that long-term usage of these steroid mimics could increase patient risks of developing breast and uterine cancer.[24]

Selective estrogen receptor modulators (SERMs) refer to a class of compound showing tissue-specific estrogenic activity, thereby granting the possibility to selectively exert agonistic or antagonistic estrogen-like action in various tissues.[25] These tissue-specific estrogenic actions could be beneficial for clinical application. Raloxifene, tamoxifen, and ormeloxifene are examples of marketed ER modulators.[25] Although these marketed SERMs have achieved remarkable success in clinical therapies, insufficient subtype selectivity causing adverse effects and drug resistance has appeared in the last decades. Therefore, discovery of new SERMs is of great importance for the clinical treatment of breast cancer.

Taking into account the aforementioned evidence, together with the antecedent that triarylmethanes are endowed with estrogenic activity,[14,15] we describe herein the preparation and assessment of estrogenic activity of a set of new 4,4′-diaminotriphenylmethanes. These compounds were obtained by the reaction of aromatic aldehydes with 2,5-dimethoxybenzenamine under microwave (MW) irradiation in the presence of $Sc(OTf)_3$ as a catalyst. The results showed that some of these novel compounds displayed mixed SERM-like activities, although their mechanisms of action might differ from those reported for other established SERMs[26] in that they do not directly interact with the ligand-binding domain of ER.

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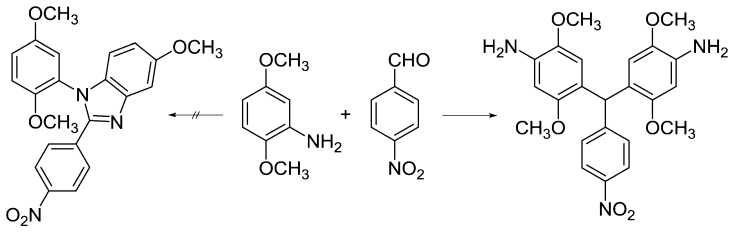
Results and Discussion

Chemistry

One of the simplest and most straightforward approaches for the synthesis of 4,4′-diaminotriarylmethanes is the Baeyer condensation, which involves the direct reaction of arylaldehydes with *N,N*-dimethylaniline. A variety of reagents, such as *p*-toluenesulfonic acid,[27] aniline hydrochloride,[28] polymer-supported sulfonic acid,[29] montmorillonite K-10,[30] $ZrOCl_2$,[31] *n*-butylpyridinium chloroaluminate,[32] $NbCl_5$,[33] and atomized sodium/THF under sonic conditions[34] have been employed to accomplish this transformation. However, some of the reported methods suffer from serious drawbacks like longer reaction times, drastic reaction conditions, unsatisfactory yields, or the use of toxic solvents. Thus, there is a need for new versatile and simple protocols for the preparation of these compounds.

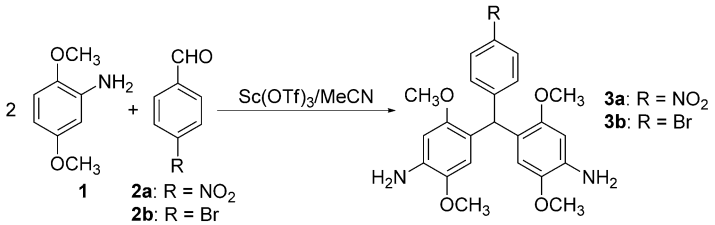
We recently published the preparation of complex disubstituted naphthoimidazoles from 1,4-dimethoxynaphthalen-2-amine, imines obtained from this amine, and aromatic aldehydes in the presence of $Sc(OTf)_3$.[35] When we carried out the reaction of 4-nitrobenzaldehyde with 2,5-dimethoxybenzeneamine, we found that a triarylmethane was obtained via an ABB′ domino reaction, instead of the expected benzoimidazole (Scheme 1).



Scheme 1. Formation of triarylmethanes.

Thus, we decided to study and to optimize this reaction, which was carried out with anilines instead of *N,N*-dialkyl aniline and without an excess of this reagent, as is employed in most of the mentioned procedures. Table 1 shows the results obtained in the reaction of 2,5-dimethoxybenzeneamine (**1**) with 4-nitro- or 4-bromobenzaldehyde under different conditions. The best yield was obtained using 5 mol% of $Yb(OTf)_3$ under MW irradiation at 80 °C for 10 min (entries 15 and 16).

Table 1. Reaction conditions for the formation of 4,4′-diaminetriphenylmethanes.



1 **2a**: R = NO_2 **2b**: R = Br **3a**: R = NO_2 **3b**: R = Br

Entry	R	Cat. [mol%]	Conditions	Compd	Yield [%][a]
1	NO_2	100	RT, 180 min	**3a**	94
2	NO_2	100	MW, 100 °C, 30 min	**3a**	84
3	NO_2	100	MW, 100 °C, 15 min	**3a**	82
4	NO_2	20	MW, 100 °C, 30 min	**3a**	84
5	NO_2	10	MW, 100 °C, 30 min	**3a**	92
6	NO_2	10	MW, 100 °C, 15 min	**3a**	92
7	NO_2	10	MW, 100 °C, 5 min	**3a**	92
8[b]	NO_2	1	RT, 240 min	**3a**	66
9	NO_2	5	MW, 100 °C, 5 min	**3a**	91
10	NO_2	1	MW, 100 °C, 5 min	**3a**	56
11	NO_2	–	MW, 100 °C, 30 min	**3a**	0
12	NO_2	5	MW,80 °C, 5 min	**3a**	90
13	Br	5	MW, 80 °C, 5 min	**3b**	82
14	NO_2	5	MW, 60 °C, 5 min	**3a**	87
15	NO_2	5	MW, 80 °C, 10 min	**3a**	98
16	Br	5	MW, 80 °C, 10 min	**3b**	95

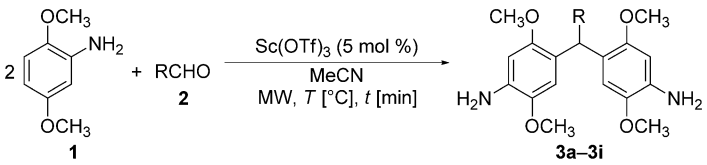
[a] Isolated yields. [b] $Yb(OTf)_3$ was used following the same reaction conditions described previously.[36]

We also employed the reaction conditions used by Genovese et al.[36] with methoxybenzene and aromatic aldehydes (entry 8, neat, 1 mol% $Yb(OTf)_3$), but only a 66% yield was obtained.

Higher percentage loading of the catalyst lowered the reaction time but did not increase the yield. Thus, we selected the conditions of entry 15 in Table 1 in order to analyze the formation of 4,4′-diaminotriphenylmethanes using other aldehydes. Various mono- and di-substituted aromatic aldehydes containing both electron-withdrawing and electron-donating substituents were used, and the results are shown in Table 2. Good yields (93–98%) were achieved with aldehydes having electron-withdrawing groups or with benzaldehyde (entries 1–6). In the case of aldehydes with electron-donating groups (entries 7–8) or the heteroaromatic aldehyde pyridine-4-carbaldehyde (entry 9), higher temperatures (120–160 °C) and a longer reaction time (30 min) were necessary to obtain good yields. From a mechanistic point of view, this may be due to the high oxophilicity of Sc^{III}, which coordinates the oxygen atom of the aldehyde, thereby enhancing the electrophilicity of the carbonyl group and facilitating attack of the π-electron-rich arene. The triarylmethanes were regioselectively obtained, with the new carbon–carbon bond in a *para* position to the amino group as determined by ^1H NMR analysis.

Due to the antecedents of estrogenic activity of some triarylmethanes,[14,15] and because our triarylmethanes have hydrogen bond donors (–NH_2) that resemble phenolic groups pres-

Table 2. Scope of the reaction with aldehydes **2**.



Entry	R	T [°C]	t [min]	Compd	Yield [%][a]
1	4-NO_2-C_6H_4	80	10	**3a**	98
2	4-Br-C_6H_4	80	10	**3b**	95
3	4-F-C_6H_4	80	10	**3c**	88
4	3-F-C_6H_4	80	10	**3d**	99
5	4-Cl-C_6H_4	80	10	**3e**	98
6	phenyl	80	10	**3f**	94
7	4-OCH_3-C_6H_4	120	30	**3g**	93
8	3,4-methylenedioxyphenyl	120	30	**3h**	70
9	3-pyridyl	160	30	**3i**	76

[a] Isolated yields.

ent in many estrogenic compounds, such as 4-hydroxytamoxifen, we decided to test them for their potential SERM-like activity.

Biological activity

In vitro antiproliferative activity

MCF-7 cells were exposed at triphenylmethane concentrations ranging from 0.01 μM to 10 μM in the absence or presence of E2 (0.1 nM), followed by dynamic monitoring of changes in exponentially growing cells for a further 4 days. The triphenylmethane derivatives shown in Figure 1A (**3a**, **3b**, **3d**, and **3e**) and Figure 1B (**3f**, **3g**, **3h**, and **3i**) led to a significant (25–75%) decrease in basal (vehicle)-treated MCF-7 cell growth at concentrations as low as 0.01 μM. In contrast, triphenylmethane derivative **3c** (Figure 1A) was able to increase cell proliferation above basal cell growth in a dose-dependent manner, reaching a maximal effect (E_{max}) similar to 0.1 nM E2. Figure 1C also shows that **3a**, **3b**, and **3e** counteracted the increase in E2-induced MCF-7 cell growth in a dose-dependent manner, with IC_{50} values of 0.013 ± 0.002, 2.8 ± 0.1, and 0.7 ± 0.09 μM, respectively. In contrast, neither **3c** nor **3d** inhibited E2-induced cell proliferation (Figure 1C). Taken together, these results indicated agonist activity of **3c**, whereas **3b** and **3e** had antagonistic activity toward MCF-7 cell growth. Notably, the anti-proliferative effects of **3a**, **3b**, and **3e** reached vehicle values. These effects were not attributed to compound-induced cellular toxicity, because we did not detect significant changes in cell morphology, membrane permeability (as assessed by YOYO DNA staining), or MTT cell metabolism, suggesting that their anti-proliferative effects are associated with antagonism of ER-mediated MCF-7 cell growth.

Figure 1D shows that **3f**, **3g**, **3h**, and **3i** did not markedly counteract the increase in E2-induced cell proliferation, suggesting that these agents displayed antagonism toward ER-mediated cell growth that could be reversed by E2. Interestingly, **3f** (Figure 1D) enhanced E2-induced cell growth at concentrations as low as 0.01 μM, suggesting that very low concentrations of triphenylmethane could cause cell growth, but higher concentrations of this agent were inhibitory.

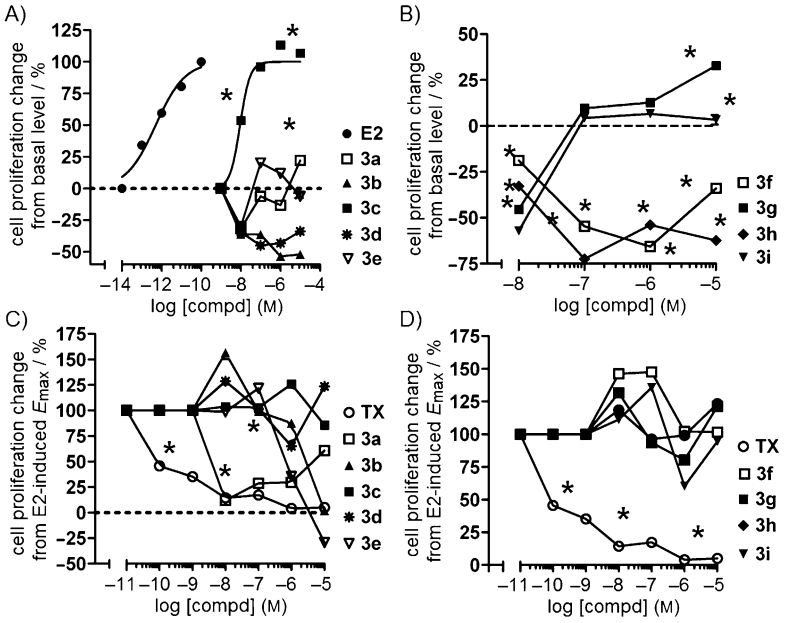


Figure 1. Effects of triphenylmethanes on MCF-7 cell proliferation. MCF-7 cells were incubated for 4 days with increased concentrations (0.01 μM–10 μM) of triphenylmethanes alone (A and B, estrogenic approach) or pretreated with triphenylmethanes for 2 h before addition of 0.1 nM E2 (C and D, anti-estrogenic approach). Data are presented as the mean ± SEM of cell proliferation change from vehicle-treated cells (basal level, A and B) or from E2-induced maximal cell proliferation (E_{max}, C and D) of three assays for agonist or antagonist experiments, respectively. At least three replicated assays were used in all experiments; *$p < 0.05$ vs. basal level or E_{max}.

Transcriptional activation assay

Agonistic and antagonistic ER-mediated transcriptional activity was assayed using stably transfected T47D-kbluc cells, which contain an estrogen response element coupled to a luciferase reporter gene.[37] Treatment of T47D-kbluc cells with E2 led to increased transcriptional activity in a dose-dependent manner ($EC_{50} = 14 \pm 1.1$ pM), and the agonist activity of E2 could be abolished by co-incubation with the ER antagonist ICI-182780 ($EC_{50} = 0.0002 \pm 0.00008$ μM) or 4OH-TX ($EC_{50} = 0.38 \pm 0.009$ μM). Instead of ICI, the agonistic approach showed that 4OH-TX caused a slight, yet significant, incremental increase in activity (2–3 fold) above vehicle-treated T47D-KBluc cells.

Next, the synthesized triphenylmethanes were tested at different doses for the assessment of estrogenic and anti-estrogenic responses. Figures 2A and 2B show that **3a**, **3b**, **3c**, **3e**, or **3i** caused a moderate yet significant increase (20–40%) in luciferase transcriptional activity above vehicle-treated T47D-KBluc cells. To assess anti-estrogenic activity, compounds were assayed against 0.1 nM E2, the lowest concentration that produced a maximal estrogenic response. Figures 2C and 2D show that **3b**, **3c**, **3d**, or **3i** counteracted the increase in E2-induced transcription, suggesting that these agents displayed partial antagonism on ER-induced transcription. The decreasing effects in luciferase expression were not attributed to compound-induced cellular toxicity, because we did not detect reduction in the amount of total protein or altered MTT metabolism in T47D-KBluc cells, but rather indicates that they inhibited ER-mediated transcriptional processes. In contrast, agents such as **3f**, **3g**, or **3h** did not counteract the increase in E2-induced transcription transcriptional activity (Figure 2D). Finally, neither 4OH-TX nor synthesized triphenylmethanes displayed cross-activation of luciferase expression under the control of androgen or glucocorticoid receptors, as demonstrated in the hormone-responsive MDA-kb2 cell line (data not shown).

From these results, we were able to observe how the type of substituent on the non-amino aromatic ring modulates the estrogenic/anti-estrogenic activity. Thus, compound **3f** (without a substituent) was inactive, while compounds **3a** (with a nitro group) and **3i** (with a pyridine ring) behaved as moderate agonists. The presence of an electron-donating group led to weak agonists **3g** and **3h.** The type and position of the halogen determined whether each compound exhibited antagonistic or dual behavior. Compounds **3b** and **3c** (with halogens (Br and F) in the *para* position) act as antagonists, and compounds **3d** (with a chlorine substituent in the *para* position) and **3e** (with a fluorine in the *meta* position) have dual profiles.

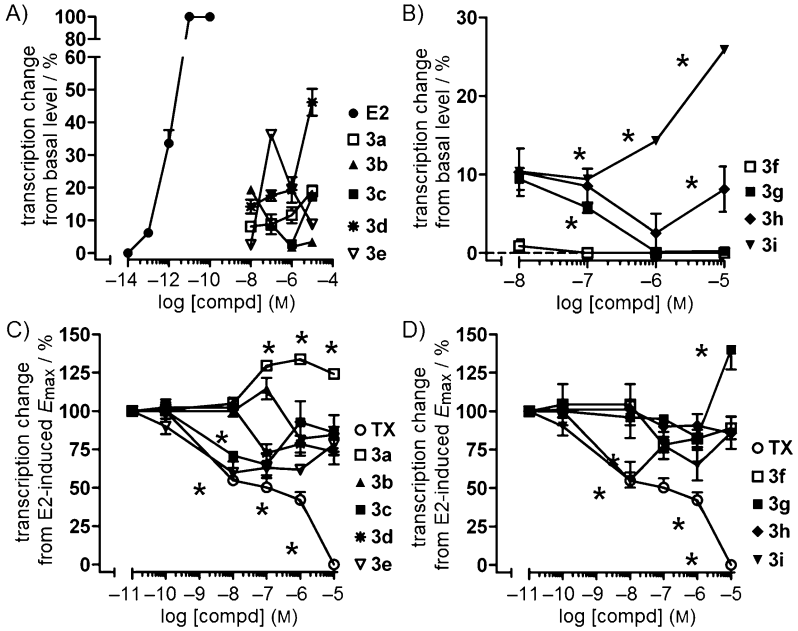


Figure 2. Transcriptional activities of triphenylmethanes on transfected T47D-KBluc cells with an estrogen response element coupled to a luciferase reporter gene. Dose–response curves for agonism (A and B) and antagonism (C and D) of ER-mediated transcriptional activities were determined. Data are presented as the mean ± SEM of ER-mediated transcriptional activity change from vehicle-treated cells (basal level, A and B) or from E2-induced maximal transcription (E_{max}, C and D) of three assays for agonist or antagonist experiments, respectively. At least three replicated assays were used in all experiments; $^*p < 0.05$ vs. basal level or E_{max}.

Docking studies

Given the effects of synthesized triphenylmethanes on cellular proliferation and ER-mediated transcriptional activation, we next used a docking computational approach to assess the potential interaction of different triphenylmethanes behaving as agonists and/or antagonists with the ligand binding domain of ERα.

When we tried to carry out flexible docking simulations studies with the Glide software,[38] we found that the 4,4′-diaminotriphenylmethanes **3a–3i** were too large to fit into the usual binding site of ERα complexed with genistein, as the size of the binding cavity is estimated to be 490 Å^3.[39] Thus, we studied the binding mode of our compounds against both conformations of the ERα and ERβ ligand-binding domains (LBD) using the following PDB codes: 1A52, 1ERR, 1X7J, 1X7R, and 3ERT. We obtained docking results only in the cases of 1A52 for agonist conformation (ERα LBD co-crystallized with estradiol (E2)) and 3ERT for antagonist conformation (ERα LBD co-crystallized with 4-hydroxytamoxifen (4OHT)), but low XP Gscores were achieved (i.e., 3ERT Gscores in kcal mol^{-1}: **3a**

−3.40, **3b** −3.74, **3c** −5.67, **3d** −5.55, **3e** −4.40, **3f** −4.92, **3g** −3.92, **3h** −4.50, **3i** −5.28, TX −10.28, 4OH-TX −12.58).

Competition studies on ER

Competition experiments were performed using rat uterus cytosol, which upon ovariectomy upregulates ER expression.[40] The extracts rich in ER were initially saturated with 5 nM of labeled E2 in the presence of increasing concentrations (1 pM–10 μM) of unlabeled E2 or candidate competitors **3b** and **3c**. As can be seen in Figure 3A, left panel, the presence of E2 competed off the binding of [^{3}H]E2 in a dose-dependent manner, with an IC_{50} value of ~1.1 nM. Conversely, compounds **3b** and **3c** failed to compete the [^{3}H]E2 binding to ER in the whole range of concentrations, indicating that these triphenylmethane derivatives do not bind to ER (Figure 3A). As positive control of competition, we used tamoxifen (5 μM), which indeed was able to competitively displace the binding of [^{3}H]E2 from uterine ER (Figure 3A, right panel).

These results encouraged us to modify the assay conditions by decreasing the amount of [^{3}H]E2 down to 0.5 nM (half the IC_{50} determined above), in order to facilitate the potential competition by triphenylmethane derivatives. The results shown in Figure 3B demonstrate that, with the exception of unlabeled E2 or TX, neither compound was capable to compete off the binding of radioactive estradiol to ER.

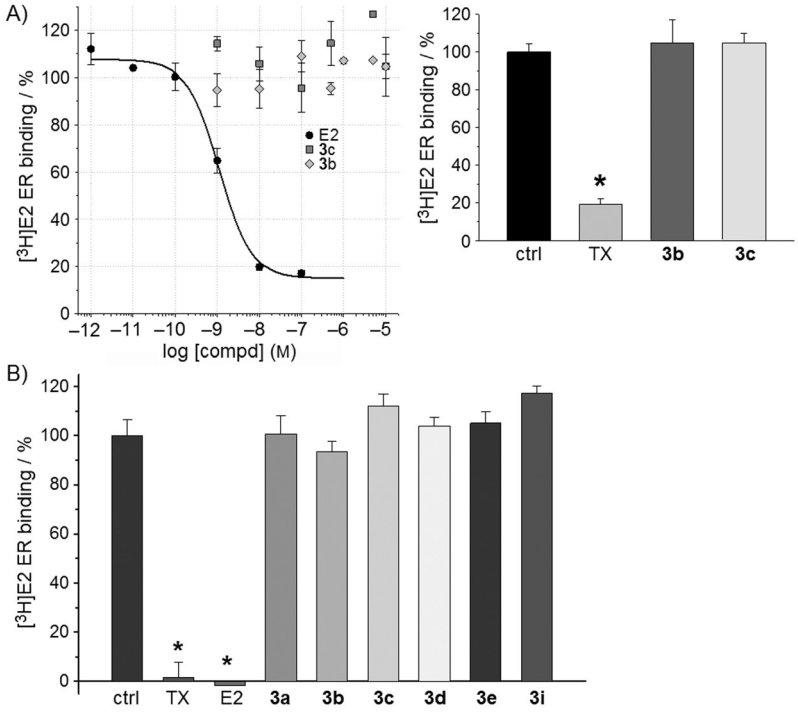


Figure 3. ER binding competition assays. A) Left: Uterine cytosolic extracts were saturated with 5 nM of ^{3}H-labeled estradiol in the presence of increasing concentrations of unlabeled competitors (1 pM–10 μM) for 18 h at 4 °C. Right: Summary of the effects of **3b** and **3c** (10 μM) and TX (5 μM) in the presence of 5 nM of [^{3}H]estradiol (control). B) Effects of compounds **3a**, **3b**, **3c**, **3d**, **3e**, **3i** (10 μM), TX (5 μM), and E2 (100 nM) on ER binding in the presence of 0.5 nM of [^{3}H]E2 (control). Data are presented as the mean ± SEM for three different assays for each compound and concentration; $^*p < 0.01$ statistically different from control value.

These data strongly indicate that the effects observed for triphenylmethane on MCF-7 cell proliferation and ER-dependent transcriptional activation are not due to direct binding of these compounds to the ligand binding pocket of ER, which in part agrees with the results from docking studies, but rather to non-canonical modulation of ER signaling. Such unconventional modulation of ER has been observed in different preparations and cell models and different ligand-independent pathways to modulate ERs have been described.[26,41,42] For instance, activation of signaling leading to stimulation of kinases, that is, mitogen-activated protein kinase or cyclin A/Cdk2 complex, phosphorylate different domains in ER (AF-1, AF-2, or even hinge domain) and have been reported to activate ERs in the absence of ligand.[41–43] Interestingly, these growth factor activated pathways are thought to significantly contribute to hormone-independent growth in some tumors.[44,45] Other possibilities include regulation of coactivators and corepressors that exist in multifunctional protein complexes associated to ER signaling,[26] especially those involving domain interactions outside LBD that modify the ER coactivators (such as the p160 family) interaction or those that alter interactions with the CoRNR box.[26,44] Interestingly, disruption of the ER–SRC protein–protein interaction has been reported to explain the anti-estrogenic effects of novel synthetic anti-estrogens ER5 and ER7, which do not bind the LBD of ER.[45] The potential involvement of any of these mechanisms in the response to triphenylmethane compounds analyzed here requires further investigation.

Conclusions

A set of new 4,4′-diaminotriphenylmethanes has been synthesized through an efficient protocol using aromatic aldehydes and 2,5-dimethoxybenzenamine under MW irradiation in the presence of $Sc(OTf)_3$ as a catalyst. These compounds were tested for antiproliferative activity against ER-positive MCF-7 cell lines, and the T47D-Kb-luc transcriptional activation activity was also determined. From the results obtained, the type of substituent on the non-amino aromatic ring seems to modulate the estrogenic/anti-estrogenic activity. Docking studies indicated that 4,4′-diaminotriphenylmethanes **3a**–**3i** are too large to fit into the usual binding cavity of ERα complexed

with genistein. In agreement with this, we observed that these derivatives failed to displace the cognate ligand, 17β-estradiol, from the LBD of ER, indicating that their effects on cell proliferation and ER-dependent transcriptional activity involve unconventional, yet unidentified, modulation of ER signaling. The compounds used in this study might serve as starting points for the development of novel SERMs.

Experimental Section

General methods: NMR spectra were recorded in $CDCl_3$ or C_6D_6 at 400 MHz for ^1H NMR and 100 or 150 MHz for ^{13}C NMR. Chemical shifts are given in (δ) parts per million and coupling constants (J) in hertz (Hz). ^1H and ^{13}C spectra were referenced using the solvent signal as an internal standard. Microwave (MW) reactions were conducted in sealed glass vessels (capacity 10 mL) using a Biotage microwave reactor. EIMS and HREIMS were recorded using an ion trap mass spectrometer. Polygram-SIL G/UV254 analytical thin-layer chromatography plates were used. Flash column chromatography was carried out using Merck silica gel 60 (particle size less than 0.020 mm), using an appropriate mixtures of ethyl acetate and hexanes. All solvents and reagents were purified by standard techniques reported[46] or used as supplied from commercial sources. All compounds were named using the ACD40 Name-Pro program, which is based on IUPAC rules. 2,5-Dimethoxybenzeneamine was synthesized following the procedure described in the literature.[35] 17β-Estradiol (E2), testosterone (T), genestein (GEN), 4-hydroxy-tamoxifen (OHTX), dexamethasone (DEX), and testosterone were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). ICI 182,780 (ICI) was purchased from Tocris Bioscience (Bristol, UK).

General procedure for the preparation of 4,4′-diaminotriphenylmethanes (3a–3i): A solution of aldehyde (30.0 mg, 0.1 mmol), 2,5-dimethoxybenzeneamine (2.0 equiv), and 5 mol% of $Yb(OTf)_3$ in CH_3CN (2 mL) was placed in a microwave-special closed vial, and the solution was irradiated in a single-mode microwave oven. The reaction mixture was then cooled to room temperature. After removing the solvent under reduced pressure, the product was purified by flash chromatography.

4-((4-Amino-2,5-dimethoxyphenyl)(4-nitrophenyl)methyl)-2,5-dimethoxybenzene amine (3a): Following the general procedure described above, a solution of 2,5-dimethoxybenzenamine (50.2 mg, 0.33 mmol), 4-nitrobenzaldehyde (24.8 mg, 0.16 mol), and $Sc(OTf)_3$ (4.0 mg) in dry MeCN (2 mL) was irradiated for 10 min at 80 °C. After elimination of the solvent, the residue was purified by flash chromatography using hexane/EtOAc (1:1) to yield 70.6 mg (98%) of compound **3a** as an amorphous red solid: mp: 180–182 °C; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 3.60$ (s, 6H, 2′-MeO, 2″-MeO), 3.61 (s, 6H, 5′-MeO, 5″-MeO), 3.78 (bs, 4H, 4′-NH_2, 4″-NH_2), 6.02 (s, 1H, 1-H), 6.30 (s, 2H, 6′-H, 6″-H), 6.35 (s, 2H, 3′-H, 3″-H), 7.20 (d, $J = 8.5$ Hz, 2H, 2‴-H, 6‴-H), 8.06 ppm (d, $J = 8.5$ Hz, 2H, 3‴-H, 5‴-H);^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 43.3$ (CH, C-1), 56.5 (4 CH_3, 2′-MeO, 2″-MeO, 5′-MeO, 5″-MeO), 100.5 (2 CH, C-3′, C-3″), 113.8 (2 CH, C-6′, C-6″), 120.7 (2 C, C-1′, C-1″), 123.2 (2 CH, C-3‴, C-5‴), 129.7 (2 CH, C-2‴, C-6‴), 135.7 (2 C, C-4′, C-4″), 141.3 (2 C, C-5′, C-5″), 146.1 (C, C-4‴), 152.0 (2 C, C-2′, C-2″), 154.2 ppm (C, C-1‴); ESIMS m/z (%): 440 (26) $[M+1]^+$, 439 (100) $[M]^+$, 424 (12), 408 (19), 317 (26), 271 (14), 153 (36), 138 (71); IR (CH_2Cl_2): $\tilde{\nu} = 3465$, 3374, 3205, 3074, 3000, 2941, 2838, 1626, 1601, 1518, 1468, 1419, 1348, 1252, 1213, 1045, 835, 740, 710 cm^{-1}; HRESIMS: 439.1746 (calcd for $C_{23}H_{25}N_3O_6$ $[M]^+$ 439.1740); Anal. calcd for $C_{23}H_{25}N_3O_6$: C 62.86, H 5.73, N 9.56, found: C 62.44, H 5.61, N 9.27.

4-((4-Amino-2,5-dimethoxyphenyl)(4-nitrophenyl)methyl)-2,5-dimethoxybenzene amine (3b): Following the general procedure described above, a solution of 2,5-dimethoxybenzenamine (50.2 mg, 0.33 mmol), 4-bromobenzaldehyde (30.3 mg, 0.16 mol), and $Sc(OTf)_3$ (4.0 mg) in dry MeCN (2 mL) was irradiated for 10 min at 80 °C. After elimination of the solvent, the residue was purified by flash chromatography using hexane/EtOAc (2:3) to yield 73.8 mg (95%) of compound **3b** as an amorphous white solid: mp: 170–171 °C; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 3.59$ (s, 6H, 2′-MeO, 2″-MeO), 3.61 (s, 6H, 5′-MeO, 5″-MeO), 3.71 (bs, 4H, 4′-NH_2, 4″-NH_2), 5.93 (s, 1H, 1-H), 6.31 (s, 2H, 6′-H, 6″-H), 6.33 (s, 2H, 3′-H, 3″-H), 6.93 (d, $J = 8.2$ Hz, 2H, 2‴-H, 6‴-H), 7.32 ppm (d, $J = 8.2$ Hz, 2H, 3‴-H, 5‴-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 42.3$ (CH, C-1), 56.5 (2 CH_3), 56.7 (2 CH_3), 100.8 (2 CH, C-3′, C-3″), 113.9 (2 CH, C-6′, C-6″), 119.3 (C, C-4‴), 122.1 (2 C, C-1′, C-1″), 130.9 (4 CH, C-2‴, C-3‴, C-5‴, C-6‴), 135.2 (2 C, C-4′, C-4″), 141.2 (2 C, C-5′, C-5″), 144.6 (C, C-1‴), 152.0 ppm (2 C, C-2′, C-2″); HRESIMS 497.0880 (calcd for $C_{23}H_{25}N_2O_4Na^{81}Br$ $[M]^+$ 497.0875), 495.0884 (calcd for $C_{23}H_{25}N_2O_4Na^{79}Br$ $[M]^+$ 495.0895); Anal. calcd for $C_{23}H_{25}BrN_2O_4$: C 58.36, H 5.32, N 5.92, found: C 57.94, H 5.22, N 5.87.

4-((4-Amino-2,5-dimethoxyphenyl)(4-fluorophenyl)methyl)-2,5-dimethoxybenzene amine (3c): Following the general procedure described above, a solution of 2,5-dimethoxybenzenamine (50.2 mg, 0.33 mmol), 4-fluorobenzaldehyde (18.0 μL, 0.16 mol), and $Sc(OTf)_3$ (4.0 mg) in dry MeCN (2 mL) was irradiated for 10 min at 80 °C. After elimination of the solvent, the residue was purified by flash chromatography using hexane/EtOAc (1:1) to yield 59.7 mg (88%) of compound **3c** as an amorphous green solid: mp: 133–134 °C; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 3.60$ (s, 6H, 2′-MeO, 2″-MeO), 3.61 (s, 6H, 5′-MeO, 5″-MeO), 3.61 (bs, 4H, 4′-NH_2, 4″-NH_2), 5.96 (s, 1H, 1-H), 6.31 (s, 2H, 6′-H, 6″-H), 6.34 (s, 2H, 3′-H, 3″-H), 6.90 (t, $J = 8.5$ Hz, 2H), 6.99–7.03 ppm (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 42.0$ (CH, C-1), 56.5 (2 CH_3), 56.7 (2 CH_3), 100.9 (2 CH, C-3′, C-3″), 113.9 (2 CH, C-6′, C-6″), 114.6 (2 CH, d, $J^2_{C-F} = 20.9$ Hz, C-3‴, C-5‴), 122.7 (2 C, C-1′, C-1″), 130.4 (2 CH, d, $J^3_{C-F} = 7.6$ Hz, C-2‴, C-6‴), 135.1 (2 C, C-4′, C-4″), 141.0 (C, C-1‴), 141.2 (2 C, C-5′, C-5″), 152.0 (2 C, C-2′, C-2″), 161.1 ppm (C, d, $J^1_{C-F} = 241.4$ Hz, C-4‴); IR (CH_2Cl_2): $\tilde{\nu} = 3458$, 3368, 3197, 2997, 2937, 2834, 1622, 1600, 1511, 1464, 1415, 1326, 1246, 1209, 1042, 854, 831, 735 cm^{-1}; HRESIMS: 435.1705 (calcd for $C_{23}H_{25}N_2O_4NaF$ $[M]^+$ 435.1696).

4-((4-Amino-2,5-dimethoxyphenyl)(3-fluorophenyl)methyl)-2,5-dimethoxybenzen amine (3d): Following the general procedure described above, a solution of 2,5-dimethoxybenzenamine (50.2 mg, 0.33 mmol), 3-flurobenzaldehyde (18.0 μL, 0.17 mol), and $Sc(OTf)_3$ (4.0 mg) in dry MeCN (2 mL) was irradiated for 10 min at 80 °C. After elimination of the solvent, the residue was purified by flash chromatography using hexanes/EtOAc (1:1) to yield 66.8 mg (99%) of compound **3d** as an amorphous green solid: mp: 141–143 °C; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 3.60$ (s, 6H, 2′-MeO, 2″-MeO), 3.61 (s, 6H, 5′-MeO, 5″-MeO), 3.69 (bs, 4H, 4′-NH_2, 4″-NH_2), 5.99 (s, 1H, 1-H), 6.33 (s, 2H, 6′-H, 6″-H), 6.35 (s, 2H, 3′-H, 3″-H), 6.75 (d, $J_{oF} = 10.4$ Hz, 1H, 2‴-H), 6.80–8.84 (m, 1H, 4‴-H), 6.85 (d, $J = 7.6$ Hz, 1H, 6‴-H), 7.16 ppm (dd, $J = 7.6$ Hz, 1H, 5‴-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 42.5$ (CH, C-1), 56.5 (2 CH_3), 56.7 (2 CH_3), 100.9 (2 CH, C-3′, C-3″), 112.4 (CH, d, $J^2_{C-F} = 21.1$ Hz), 113.9 (2 CH, C-6′, C-6″), 115.9 (CH, d, $J^2_{C-F} = 21.3$ Hz), 122.2 (2 C, C-1′, C-1″), 124.9 (CH, C-6‴), 129.1 (CH, d, $J^3_{C-F} = 8.1$ Hz, C-5‴), 135.1 (2 C, C-4′, C-4″), 141.3 (2 C, C-5′, C-5″), 148.4 (C, d, $J^3_{C-F} = 6.6$ Hz, C-1‴), 152.0 (2 C, C-2′, C-2″), 162.9 ppm (C, d, $J^1_{C-F} = 242.6$ Hz, C-3‴); IR (CH_2Cl_2): $\tilde{\nu} = 3460$, 3370, 3201, 3052, 2997, 2937, 2869, 2834, 1620, 1590, 1515, 1464, 1416, 1326, 1251, 1209, 1042, 891, 829, 736 cm^{-1}; HRESIMS: 435.1697 (calcd for $C_{23}H_{25}N_2O_4FNa$ $[M]^+$ 435.1696); Anal. calcd for

$C_{23}H_{25}FN_2O_4$: C 66.98, H 6.11, N 6.79, found: C 66.74, H 6.34, N 6.23.

4-((4-Amino-2,5-dimethoxyphenyl)(4-chlorophenyl)methyl)-2,5-dimethoxy benzenamine (3e): Following the general procedure described above, a solution of 2,5-dimethoxybenzenamine (50.2 mg, 0.33 mmol), 4-chlorobenzaldehyde (23.8 mg, 0.16 mol), and $Sc(OTf)_3$ (4.0 mg) in dry MeCN (2 mL) was irradiated for 10 min at 80 °C. After elimination of the solvent, the residue was purified by flash chromatography using hexanes/EtOAc (3:2) to yield 69.0 mg (98%) of compound **3e** as a yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.53$ (bs, 4H, 4′-NH_2, 4″-NH_2), 3.59 (s, 6H, 2′-MeO, 2″-MeO), 3.61 (s, 6H, 5′-MeO, 5″-MeO), 5.95 (s, 1H, 1-H), 6.31 (s, 2H, 6′-H, 6″-H), 6.34 (s, 2H, 3′-H, 3″-H), 6.99 (d, $J = 8.1$ Hz, 2H, 2‴-H, 6‴-H), 7.17 ppm (d, $J = 8.1$ Hz, 2H, 3‴-H, 5‴-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 42.2$ (CH, C-1), 56.5 (2 CH_3), 56.7 (2 CH_3), 100.8 (2 CH, C-3′, C-3″), 113.9 (2 CH, C-6′, C-6″), 122.2 (2 C, C-1′, C-1″), 127.9 (2 CH), 130.5 (2 CH), 131.1 (C, C-4‴), 135.2 (2 C, C-4′, C-4″), 141.2 (2 C, C-5′, C-5″), 144.1 (C, C-1‴), 152.0 ppm (2 C, C-2′, C-2″); IR (CH_2Cl_2): $\tilde{\nu} = 3456$, 3368, 3199, 2996, 2936, 2833, 1622, 1514, 1463, 1415, 1325, 1246, 1209, 1042, 894, 847, 735 cm^{-1}; HRESIMS: 451.1394 (calcd for $C_{23}H_{25}N_2O_4Na^{35}Cl$ $[M]^+$ 451.1401); Anal. calcd for $C_{23}H_{25}ClN_2O_4$: C 64.41, H 5.88, N 6.53, found: C 63.98, H 5.74, N 6.28.

4-((4-Amino-2,5-dimethoxyphenyl)(phenyl)methyl)-2,5-dimethoxybenzenamine (3f): Following the general procedure described above, a solution of 2,5-dimethoxybenzenamine (50.2 mg, 0.33 mmol), benzaldehyde (17.0 μL, 0.16 mol), and $Sc(OTf)_3$ (4.0 mg) in dry MeCN (2 mL) was irradiated for 10 min at 80 °C. After elimination of the solvent, the residue was purified by flash chromatography using mixtures of hexane/EtOAc from 4:1 to 2:3 to yield 60.8 mg (94%) of compound **3f** as an amorphous white solid: mp: 148–150 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.59$ (s, 6H, 2′-MeO, 2″-MeO), 3.60 (s, 6H, 5′-MeO, 5″-MeO), 3.71 (bs, 4H, 4′-NH_2, 4″-NH_2), 6.00 (s, 1H, 1-H), 6.34 (s, 4H, 3′-H, 3″-H, 6′-H, 6″-H), 7.06 (d, $J = 7.2$ Hz, 2H, 2‴-H, 6‴-H), 7.13 (t, $J = 7.2$ Hz, 1H, 4‴-H), 7.21 ppm (t, $J = 7.2$ Hz, 2H, 3‴-H, 5‴-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 42.7$ (CH, C-1), 56.5 (2 CH_3), 56.9 (2 CH_3), 101.0 (2 CH, C-3′, C-3″), 114.1 (2 CH, C-6′, C-6″), 123.1 (2 C, C-1′, C-1″), 125.5 (CH, C-4‴), 127.9 (2 CH), 129.2 (2 CH), 134.9 (2 C, C-4′, C-4″), 141.3 (2 C, C-5′, C-5″), 145.3 (C, C-1‴), 152.1 ppm (2 C, C-2′, C-2″). IR (CH_2Cl_2): $\tilde{\nu} = 3456$, 3368, 3054, 2996, 2936, 2869, 2833, 1622, 1599, 1514, 1462, 1415, 1326, 1247, 1209, 1042, 839, 736, 703 cm^{-1}; HRESIMS 417.1791 (calcd for $C_{23}H_{26}N_2O_4Na$ $[M]^+$ 417.1790); Anal. calcd for $C_{23}H_{26}N_2O_4$: C 70.03, H 6.64, N 7.10, found: C 69.98, H 6.26, N 6.69.

4-((4-Amino-2,5-dimethoxyphenyl)(4-methoxyphenyl)methyl)-2,5-dimethoxybenzen amine (3g): Following the general procedure described above, a solution of 2,5-dimethoxybenzenamine (50.2 mg, 0.33 mmol), benzaldehyde (20.0 μL, 0.16 mol), and $Sc(OTf)_3$ (4.0 mg) in dry MeCN (2 mL) was irradiated for 30 min at 120 °C. After elimination of the solvent, the residue was purified by flash chromatography using a mixture of hexane/EtOAc (1:1) to yield 64.5 mg (93%) of compound **3g** as an amorphous brown solid: mp: 141–143 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.38$ (bs, 4H, 4′-NH_2, 4″-NH_2), 3.60 (s, 6H, 2′-MeO, 2″-MeO), 3.61 (s, 6H, 5′-MeO, 5″-MeO), 3.77 (s, 3H, 4‴-MeO), 5.95 (s, 1H, 1-H), 6.34 (s, 4H, 3′-H, 3″-H, 6′-H, 6″-H), 6.76 (d, $J = 8.4$ Hz, 2H, 2‴-H, 6‴-H), 6.97 ppm (d, $J = 8.4$ Hz, 2H, 3‴-H, 5‴-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 41.8$ (CH, C-1), 55.3 (CH_3, 4‴-MeO), 56.5 (2 CH_3), 56.9 (2 CH_3), 101.1 (2 CH, C-3′, C-3″), 113.3 (2 CH), 114.0 (2 CH), 123.4 (2 C, C-1′, C-1″), 130.0 (2 CH), 134.9 (2 C, C-4′,C-4″), 137.3 (C, C-1‴), 141.3 (2 C, C-5′, C-5″), 152.0 (2 C, C-2′,C-2″), 157.5 ppm (C, C-4‴); IR (CH_2Cl_2): $\tilde{\nu} = 3456$, 3367, 3199, 2996, 2936, 2834, 1621, 1512, 1463, 1415, 1326, 1299, 1246, 1209, 1177, 1041, 830, 735 cm^{-1}; HRESIMS: 447.1893 (calcd for $C_{24}H_{28}N_2O_5Na$ $[M]^+$ 447.1896); Anal. calcd for $C_{24}H_{28}N_2O_4$: C 67.91, H 6.65, N 6.60, found: C 67.56, H 6.43, N 6.38.

4-((4-Amino-2,5-dimethoxyphenyl)(3,4-methylenedioxyphenyl)-methyl)-2,5-dimethoxy benzenamine (3h): Following the general procedure described above, a solution of 2,5-dimethoxybenzenamine (50.2 mg, 0.33 mmol), piperonal (24.6 mg, 0.16 mol), and $Sc(OTf)_3$ (4.0 mg) in dry MeCN (2 mL) was irradiated for 30 min at 120 °C. After elimination of the solvent, the residue was purified by flash chromatography using mixtures of hexane/EtOAc (1:1) to yield 50.4 mg (70%) of compound **3h** as an amorphous brown solid: mp: 166–168 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.61$ (s, 6H, 2′-MeO, 2″-MeO), 3.62 (s, 6H, 5′-MeO, 5″-MeO), 3.72 (bs, 4H, 4′-NH_2, 4″-NH_2), 5.89 (s, 2H, 7‴-H), 5.92 (s, 1H, 1-H), 6.34 (s, 2H, 3′-H, 3″-H o 6′-H, 6″-H), 6.35 (s, 2H, 3′-H, 3″-H o 6′-H, 6″-H), 6.51 (d, $J = 8.0$ Hz, 1H, 5‴-H), 6.58 (s, 1H, 2‴-H), 6.67 ppm (d, $J = 8.0$ Hz, 1H, 6‴-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 42.2$ (CH, C-1), 56.6 (2 CH_3), 56.9 (2 CH_3), 100.8 (CH_2), 101.0 (2 CH, C-3′, C-3″), 107.7 (CH), 109.9 (CH), 114.0 (2 CH, C-6′, C-6″), 122.0 (CH), 123.1 (2 C, C-1′,C-1″), 135.0 (2 C, C-4′,C-4″), 139.4 (C), 141.3 (2 C, C-5′, C-5″), 145.4 (C), 147.3 (C), 152.0 ppm (2 C, C-2′,C-2″); IR (CH_2Cl_2): $\tilde{\nu} = 3462$, 3368, 3199, 2996, 2936, 2833, 1621, 1513, 1486, 1466, 1416, 1325, 1248, 1208, 1040, 930, 893, 825, 734 cm^{-1}; HRESIMS: 461.1685 (calcd for $C_{24}H_{26}N_2O_6Na$ $[M]^+$ 461.1689); Anal. calcd for $C_{24}H_{26}N_2O_6$: C 65.74, H 5.98, N 6.39, found: C 65.37, H 5.80, N 5.98.

4-((4-Amino-2,5-dimethoxyphenyl)(3-pyridyl)methyl)-2,5-dimethoxy benzenamine (3i): Following the general procedure described above, a solution of 2,5-dimethoxybenzenamine (50.2 mg, 0.33 mmol), 3-pyridylcarboxaldehyde (16.0 μL, 0.17 mol), and $Sc(OTf)_3$ (4.0 mg) in dry MeCN (2 mL) was irradiated for 30 min at 160 °C. After elimination of the solvent, the residue was purified by preparative TLC using EtOAc to yield 49.4 mg (76%) of compound **3i** as a green oil: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.60$ (s, 6H, 2′-MeO, 2″-MeO), 3.61 (s, 6H, 5′-MeO, 5″-MeO), 3.75 (bs, 4H, 4′-NH_2, 4″-NH_2), 5.97 (s, 1H, 1-H), 6.32 (s, 2H, 6′-H, 6″-H), 6.34 (s, 2H, 3′-H, 3″-H), 7.14 (dd, $J = 4.8$ Hz, 1H, 5‴-H), 7.34 (d, $J = 7.7$ Hz, 1H, 4‴-H), 8.34 (s, 1H, 2‴-H), 8.39 ppm (d, $J = 4.3$ Hz, 1H, 6‴-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 40.9$ (CH, C-1), 56.5 (4 CH_3, 2′-MeO, 2″-MeO, 5′-MeO, 5″-MeO), 100.6 (2 CH, C-3′, C-3″), 113.9 (2 CH, C-6′, C-6″), 121.1 (2 C, C-1′, C-1″), 122.9 (CH), 135.5 (2 C, C-4′, C-4″), 136.6 (CH), 141.0 (C, C-1‴), 141.2 (2 C, C-5′, C-5″), 146.6 (CH), 150.5 (CH), 151.9 ppm (2 C, C-2′, C-2″); IR (CH_2Cl_2): $\tilde{\nu} = 3451$, 3363, 3195, 2996, 2936, 2833, 1622, 1514, 1463, 1416, 1326, 1248, 1209, 1042, 893, 844, 732 cm^{-1}; HRESIMS: 418.1746 (calcd for $C_{22}H_{25}N_3O_4Na$ $[M]^+$ 418.1743); Anal. calcd for $C_{22}H_{25}N_3O_4$: C 66.82, H 6.37, N 10.63, found: C 66.42, H 6.74, N 10.28.

Biological methods

Mammalian cells: The human breast cancer cell lines T47D-kbluc (ATCC) and MDA-kb2 (ATCC) were maintained in 75 cm^2 culture flasks (Nunclon) in RPMI (Lonza) growth media, without phenol red, supplemented with 10% fetal bovine serum (FBS) (Lonza), 2 mM glutamine, 10 mM HEPES, and 1 mM sodium pyruvate. MCF-7 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 20 mM L-1 D-glucose. The medium was supplemented with 100 UI mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Cells were grown in a humidified incubator with 5% CO_2 at 37 °C.

Transcriptional activity studies: Compound stock solutions were prepared in 100% DMSO in glass amber vials and Teflon-lined caps and stored at −80 °C. Dosing solutions were prepared by diluting

compound stocks in fresh 5% dextran-coated charcoal-stripped FBS (DCC-FBS) (Hyclone) RPMI to desired concentrations. DMSO vehicle did not exceed 0.06%. Transcriptional activity was measured using a luciferase-based reporter gene assay in two breast cancer cell lines expressing different nuclear receptors: a) T47D-kbluc cells (ATCC), which naturally express both ERα and ERβ and are stably transfected with a triplet estrogen-responsive element (ERE) promoter–luciferase reporter plasmid (EREx3-Luc),[37] and b) MDA-kb2 cells (ATCC), which naturally express both glucocorticoid and androgen receptors and are stably transfected with an MMTV.neo promoter–luciferase reporter gene construct.[37] Cells were maintained in growth media, modified by replacement of 10% FBS with 10% DCC-FBS without antibiotic supplement four days prior to the assay to remove all steroids from the culture. After incubation in low-steroid media, T47D-kb cells or MDA-kb cells were seeded onto 96-well plates (Nunclon) at 6×10^4 cells per well or 3×10^4 cells per well, respectively, in 5% DCC-FBS RPMI and were allowed to attach overnight. Media was then replaced with 100 μL per well of dosing media and the test compound and incubated for 24 h. Then, cells were washed with cold phosphate-buffered saline, harvested in 80μL Passive Lysis Buffer (Promega) per well, and luciferase activity was determined with LAR (Promega) using a fluorescence Ascent FL multiplate reader (Thermo Fisher) and quantified as relative light units (RLU). Each compound was assayed independently at least three times with a minimum of three wells per each replicate. T47D-kb cells were screened with compounds using estrogen-positive (E2), -negative (vehicle only), antagonist (E2 plus ICI), and background (vehicle plus ICI) controls on every plate. Each compound was tested both alone and in the presence of an appropriate competitor, such as 0.1 nM E2 or ICI. MDA-kb cells were screened with compounds using agonist positive (testosterone or dexamethasone) or negative (vehicle only) controls on every plate. Agonist positive controls were monitored over time as an assessment of the stability of the cell lines. In those instances where cytotoxicity of a compound was suspected, duplicate plates were dosed in parallel.

Dynamic monitoring of chemical-induced changes in cell proliferation by IncuCyte ZOOM: Prior to beginning the assay, MCF-7 cells were seeded in a 96-well plate at 10000 cells per well and cultured overnight. Compounds were serially diluted with 0.5% DCC-FBS DMEM growth media containing YOYO-1 (Life Technologies) to a final concentration of 0.05 μM. Phase-contrast and fluorescent images were collected to detect morphological cell changes and plasma membrane permeability (by YOYO-1 DNA staining), both indicators of in vitro cytotoxicity. The YOYO-1 concentration did not affect proliferation or cell morphology of the cell types used in this study relative to identical cells placed in an IncuCyte with a 10× objective in a standard cell culture incubator at 37 °C and 5% CO_2. Two images per well were collected every 2 h in both phase-contrast and fluorescence. The assay was considered complete when a maximal response was achieved as determined by image analysis. The integrated object counting algorithm was used to isolate the fluorescent nuclear signal from background. Specifically, images were segmented in order to identify individual objects, counted, and reported on a per-area (mm^2) basis for each time point. For endpoint assays, in order to correct for differential proliferation of cells, the total number of DNA containing objects was counted at the final time point using Triton X-100 (0.0625%) to permeabilize the cells. Cells were incubated at 37 °C for 1 h to allow nuclear DNA staining by YOYO-1 prior to endpoint imaging. This number was used to calculate the "cytotoxic index", defined as the number of YOYO-1-positive objects divided by the total number of DNA-containing objects (fluorescent objects counted post Triton X-100 treatment).

Estrogen receptor competitive binding assay: Estrogen receptor was obtained from the uterine cytosol fraction from mature ovariectomized female Sprague-Dawley rats.[40] Aliquots of uterine cytosol (100 μL) were incubated with 5 nM [^{3}H]E2 and increasing concentrations of unlabeled competitors (0.1 nM–10 μM) for 18 h at 4 °C. Then, 200 μL of dextran-coated charcoal suspension (0.8% charcoal:0.08% dextran; DCC) in TRIS-EDTA-glycerol-Mg buffer was added to each tube and incubated for 10 min. The DCC was then removed by centrifugation at 3000 *g* for 10 min. The supernatant was measured for radioactivity in a 4 mL scintillation cocktail Optiphase Hisafe 2 (PerkinElmer) by LKB Wallac 1214-Rackbeta counter (LKB Instrument). Corrections were made for non-specific binding. The relative binding affinity (RBA) of agents was calculated as the ratio of agents and E2 or tamoxifen values as derived from dose–response curves.

Cytotoxicity assays: Cytotoxicity was evaluated by determining the mitochondrial function of the cells using the tetrazolium dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) following treatment with each compound. MTT is a yellow vital dye that is actively converted by mitochondrial oxidation–reduction reactions into blue formazan crystals. The formation of the blue MTT crystals within the cell decreases in direct proportion to the viability of cells.[47]

Data and statistical analysis: RLUs per mg protein for each run were normalized by converting values to fold induction by dividing the RLUs per mg protein for each well by the average RLUs per mg protein for the vehicle control. Fold induction values were then log transformed to control for heterogeneity of variance. Log_{10}-transformed fold induction values for each plate were further converted to a percentage of the maximal E2 response using the following procedure: The concurrent E2 curve for each run in log-fold (*y*-axis) was plotted in GraphPad 5.01 (GraphPad Software, San Diego, CA) versus compound concentration. Concentration values (*x*-axis) were log transformed, and then a nonlinear regression curve fit analysis (four-parameter logistic regression) were performed on the E2 data using a constrained bottom parameter held constant equal to 0.0. The top parameter (E_{max} = maximal response) of the nonlinear regression analysis for E2 was used to convert the log-fold data into a percent response for each test well. Specifically, the log-fold response was divided by the top parameter (in log fold) and then multiplied by 100. Once each run was converted to percent of the maximal E2 response, data from all runs for each compound were combined.

For ER competition assays, intra-assay data were normalized by reference to 100% binding of 5 nM [^{3}H]E2. Data were then expressed as a percentage of competition by increasing concentrations of unlabeled competitors (1 pM–10 μM). Dose–response curves were fitted to four-parameter logistic equations by nonlinear regression analyses.

Docking studies

Docking simulations were performed with Glide software (Glide, version 6.2; Schrödinger LLC, New York, NY (USA), 2014). The X-ray structures of ERα LBD to be used for docking were taken from the Protein Databank (www.rcsb.org).[48] The 3D coordinates of the corresponding ERα and ERβ complexes were extracted from PDB entries 1A52, 1ERR, 1X7J, 1X7R, and 3ERT. The PDB structures were prepared for docking using the Protein Preparation Workflow (Schrödinger) accessible from within the Maestro program (Maestro, version 9.7; Schrödinger). Shortly, the hydrogens were properly added to the complexes, water molecules more than 5 Å from

a heteroatom were deleted, and bond corrections were applied to the co-crystallized ligands. The receptor was optimized in Maestro 9.2 using the OPLS_2005 force field prior to the docking study. In the final stage, optimization and minimization of the ligand–protein complexes were carried out with the OPLS_2005 force field, and the default values for an RMSD of 0.30 Å for non-hydrogen atoms were used. The receptor grids were generated using the prepared proteins, with the docking grids centered on the center of the bound ligand for each receptor. A receptor grid was generated using a 1.00 van der Waals (vdW) radius scaling factor and a 0.25 partial charge cutoff. The binding sites were enclosed in a grid box of 20 $Å^3$ with default parameters and without constraints. The 3D structures of the ligands to be docked were generated and prepared for docking using the LigPrep as implemented in Maestro 9.7 (LigPrep, version 2.9; Schrödinger). In this stage, a series of treatments are applied to the structures. Finally the geometries are optimized using an OPLS_2005 force field. The ligands were docked using the extra precision mode (XP)[49] without using any constraints and a 0.80 vdW radius scaling factor and a 0.15 partial charge cutoff. The generated ligand poses were evaluated with an empirical scoring function, GlideScore a modified version of ChemScore,[50] was used to estimate binding affinity and rank ligands.[51] 3–5 poses per ligand were generated, and post-docking minimization was carried out. XP Pose Rank was used to select the best docked pose for each ligand.

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