Bioorganic & Medicinal Chemistry Letters 19 (2009) 2404–2408

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Biphenyl *C*-cyclopropylalkylamides: New scaffolds for targeting estrogen receptor β

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ARTICLE INFO

Article history:
Received 19 January 2009
Revised 14 March 2009
Accepted 20 March 2009
Available online 25 March 2009

Keywords:
Estrogen receptor
Chemical synthesis
Receptor binding
Antiproliferative

ABSTRACT

The *C*-cyclopropylalkylamide scaffold was previously identified as a new structural framework for antiestrogens. A second generation library provided three compounds that bind estrogen receptor (ER)α. Further screening of this library identified an ERβ hit and inspired another round of SAR. A new focused library was tested for binding to the ERs, and for effects on the growth of breast cancer cell lines and protein levels of common cell cycle regulators.

The estrogen receptors (ERs) are ligand activated transcription factors. ERα and ERβ demonstrate ~56% sequence homology in their ligand binding domains, but their ligand-binding pockets differ by only two amino acids: ERα Leu^{384} versus ERβ Met^{336}, and ERα Met^{421} versus ERβ ILe^{373}.[1] ERβ's pocket is also about 100 Å^3 smaller than that of ERα.[2] Additionally, the tissue distributions of these receptors are distinct.[3] There is much data demonstrating the benefits of selectively targeting the ERβ. ERβ expression inhibits estradiol (E2)-induced proliferation in T47D and MCF-7 breast cancer cells and leads to a reduction in xenograft volume.[4–6] ERβ has a growth inhibitory mechanism in medullary thyroid carcinoma, prostate cancer, and ovarian cancer.[7–10] Also, ERβ-selective agonists demonstrate potential as anti-inflammatory agents.[11] Several ERβ-selective agents have been identified (Fig. 1). The plant-derived ligand genistein (GEN (**1**)) has also been shown to induce DNA strand breaks and cause chromosomal aberrations.[3,12] The Katzenellenbogen group developed a diarylpropionitrile (DPN, **2**) that is a 100-fold selective ERβ agonist.[13] Recently, biphenyl derivatives have been investigated for ERβ-selectivity. Simple 4-OH-biphenyls demonstrated 20–70-fold selectivity for ERβ over ERα.[14] In order to attempt to mimic the C-ring of GEN, an oxime moiety was incorporated, and altering the substitutions on the biphenyl core provided various levels of selectivity: oximes **3** and **4** were 11-fold and 43-fold selective, respectively (Fig. 1).[15]

Previously, through screening of a library of homoallylic amides, allylic amides and *C*-cyclopropylalkylamides, we identified a new structural scaffold for antiestrogens.[16] The synthesis of a second generation library provided three additional agents that were found to bind tightly to ERα.[17] In the present analysis, we screened this second generation library with a fluorescent E2 derivative (ES2) for competitive binding to ERβ in a fluorescence polarization (FP) assay.[18] The percent displacement of the E2 derivative by the test compound was calculated from the difference in measured FP values upon incubation with and without the test sample. The efficiency of displacement demonstrated for ERβ by the initial screening library was significantly lower than that for

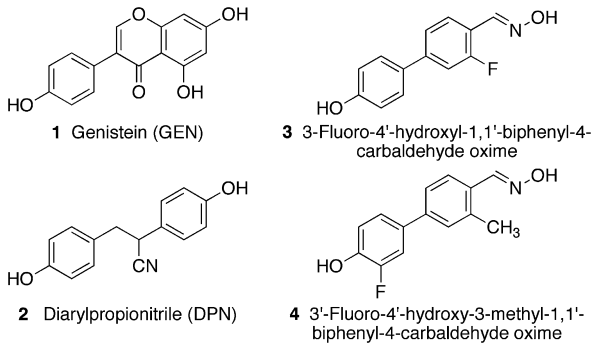

Figure 1. Structures of several known ERβ-selective ligands: GEN (**1**), DPN (**2**), and biphenyl carbaldehyde oximes **3** and **4**.

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doi:10.1016/j.bmcl.2009.03.075

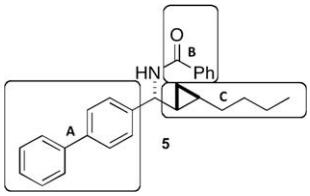
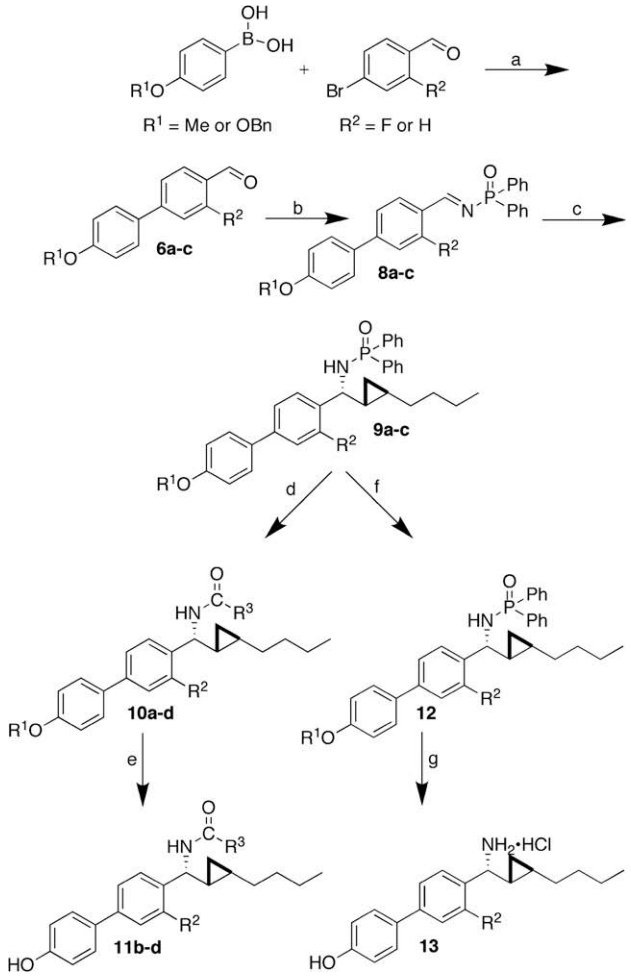


Figure 2. Lead compound **5**, and subunits A, B and C used in the SAR.

ERα. In fact, only a single compound (**5**; Fig. 2) demonstrated a concentration-dependent displacement over the concentration range tested and close to 50% displacement at the highest concentration. The displacement values were: 0.2 μM, 26%; 1 μM, 31%; and 5 μM, 46%. Compound **5** was chosen as the lead structure for efforts to improve ERβ-selectivity. Because **5** did not quite reach 50% displacement on ERβ, the selection criteria were modified to include structural features previously known to promote ERβ affinity.[15] Screening results on ERα were used as co-selection criteria, and **5** was not a hit on ERα.[17]

Figure 2 demonstrates the three regions in **5** that were further evaluated in the next round of our SAR. The biphenyl core A was shown to be beneficial for selective targeting of ERβ, in analogy to Yang et al.[15] who used GEN as a lead structure to search for new ERβ-selective ligands. The biphenyl moiety was synthetically readily accessible through diaryl Suzuki cross-couplings, and a large number of substituted phenyl precursors were commercially available. Biphenyl carbaldehyde oxime derivatives were synthesized with various substituents. Upon placement of a fluorine and/or chlorine substituent on the biphenyl core while maintaining the phenol group, agents selective for ERβ were obtained that demonstrated greater than 10-fold (**3**) and up to 40-fold (**4**) selectivity.[15] Among the second generation library compounds, only one compound containing the biphenyl system satisfied the criteria for hit selection in the screen on ERα; however, this compound failed to inhibit estradiol-stimulated growth of MCF-7 ER positive breast cancer cells.[17] Replacement of the biphenyl group with a smaller arene, that is, *p*-chlorobenzene, preserved the displacement activity on ERα and recovered the cell-based activity. This substituent effect indicates that the sterically more demanding biphenyl core is less tolerated by ERα if combined with larger substituents on the nitrogen atom in the benzylic position. When the biphenyl group in **5** was replaced with a phenyl ring while keeping the remaining structure constant, the activity at ERβ dropped to ~10% and activity at ERα was retained. Alterations to region B in **5** also led to detrimental consequences in the ability of the compound to displace ES2 from ERβ. Compound **5** demonstrated that the best-tolerated function in sector B was a phenylamide group, while other rings were better tolerated for ERα. When the phenylamide was replaced with a biphenylamide, activity at ERβ was abolished. Therefore, we concluded that only one biphenyl core was necessary and that increasing the size of the ligand would be detrimental to activity on ERβ. As far as the alkyl chain in region C was concerned, an increase in its size to a cyclohexyl group while keeping the remainder of the molecule constant abolished activity at both receptors. Therefore, region C was not altered in subsequent synthetic efforts. Previous results demonstrated that the cyclopropane ring was important for cellular activity.[16] When compounds containing the biphenyl core yet lacking the cyclopropane ring were tested in the FP assay, activity at both receptors was lost. Regions A and B were chosen for further elaboration and the structures were assembled with the biphenyl core, the cyclopropane ring and the amide linkage preserved. The biphenyl group was substituted with a key 4′-OH group expected to promote hydrogen bonding with ERβ Glu[305] and ERβ Arg[346]. A fluorine substituent was chosen based upon published SAR for biphenyl-containing agents.[15] The phenylamide was changed into a smaller acetylamide, or completely removed. Six new compounds, analogues of **5**, were synthesized, **10a**, **11b–d**, **12**, and **13** (Scheme 1). The palladium catalyst *trans*-$(Cy_2NH)_2Pd(OAc)_2$ (DAPCy) was used to perform Suzuki couplings in dioxane because it could be used at room temperature, thereby suggesting functional group tolerance, and due to its past use in similar couplings.[19,20] This catalyst was easily prepared in yellow crystalline form from palladium(II)acetate and dicyclohexylamine.[19] The cross-coupling of commercially available benzyl- or methyl-O-protected 4-hydroxybenzeneboronic acid to 4-bromobenzaldehyde using the DAPCy catalyst provided biphenyl aldehydes **6a–c** in good to excellent yields (65–88%). Imine formation using titanium(IV) chloride as the Lewis acid initially gave phosphinoylimine **8a** in poor to moderate yields (30–47%).[17] Changing the workup procedure to a fast filtration over a pad of silica gel and washing with ethyl acetate significantly improved the yields (80%) of the phosphinoylimines **8b and c**. One-pot hydrozirconation-transmetallation-aldimine addition in



Scheme 1. Overview of the synthesis of six new biphenyl substituted *C*-cyclopropylalkylamides. Reagents and conditions: (a) DAPCy (2 mol %), Cs_2CO_3, dioxane, rt; (b) $Ph_2P(O)NH_2$, $TiCl_4$, Et_3N, rt; (c) (i) $Cp_2Zr(H)Cl$, 1-hexyne, CH_2Cl_2, (ii) Me_2Zn, toluene, −78 to 0 °C, (iii) mW 100 °C, 300 W, 10 min, (iv) CH_2I_2, rt; (d) (1) HCl, MeOH, (2) R^3COCl, DMAP, $^iPr_2EtN$, rt; (e) H_2, $Pd(OH)_2/C$, THF, MeOH, rt; (f) H_2, $Pd(OH)_2/C$, THF, MeOH, rt; (g) HCl, MeOH. Percent yields: **6a**, 70; **6b**, 88; **6c**, 50; **8a**, 47; **8b**, 67; **8c**, 40; **9a**, 30; **9b**, 68; **9c**, 40; **10a**, 61; **10b**, 57; **10c**, 22; **10d**, 75; **11b**, 69; **11c**, 92; **11d**, 94; **12**, 80; **13**, 43. R^1 = Me, R^2 = F (**6a**, **8a**, **9a**); R^1 = Bn, R^2 = F (**6b**, **8b**, **9b**); R^1 = Bn, R^2 = H (**6c**, **8c**, **9c**); R^1 = Me, R^2 = F, R^3 = Ph (**10a**); R^1 = H, R^2 = F, R^3 = Ph (**10b**, **11b**); R^1 = H, R^2 = H, R^3 = Ph (**10c**, **11c**); R^1 = H, R^2 = F, R^3 = Me (**10d**, **11d**); R^2 = F (**12**, **13**).

dichloromethane using a microwave (100 °C, 300 W, 10 min) immediately followed by cyclopropanation in the presence of excess diiodomethane at room temperature (16–20 h) gave the phosphinoyl-protected biphenyl *C*-cyclopropylalkylamides **9a–c** in moderate overall yields (43–66%). Acidic deprotection followed by acylation gave the final product **10a** (61%) or benzyl-protected compounds **10b–d** (22–75%). Benzyl ethers were easily removed by hydrogenolysis at 1 atm in the presence of palladium hydroxide on carbon to give the final products **11b–d** and **12**. Deprotections and acylations were performed in parallel using a Bradley Greenhouse reactor, and final compounds were purified individually by column chromatography. The deacetylated primary amine **13** was purified as its HCl salt.

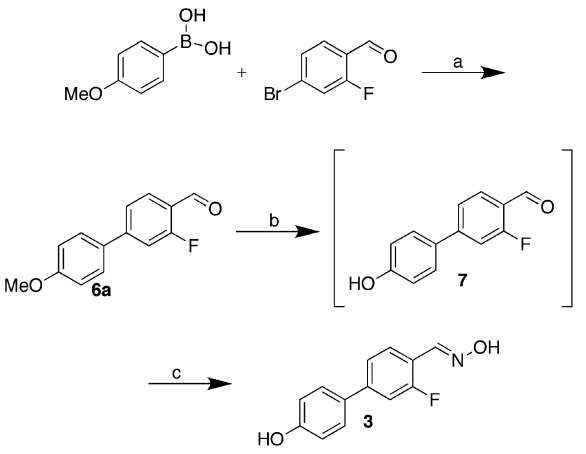
This synthetic scheme allowed for diversification in two ways. Substituents on the biphenyl core were varied by changing the substitution on the arylboronic acid and aryl bromide coupling components. The amide linkage also allowed for diversification by altering the acylating agents, analogously to the synthesis of the second generation library. After the acidic deprotection step, the amine salt could be coupled without further purification with diverse acyl chlorides, carbamoyl chlorides, and sulfonyl chlorides in the presence of DMAP.[17]

3-Fluoro-4′-hydroxy-1,1′-biphenyl-4-carbaldehyde oxime **3** served as a positive control. The past synthesis of **3** was modified as shown in Scheme 2.[15] Suzuki cross-coupling using DAPCy gave the desired biphenyl aldehyde **6a** in 70% yield. The aryl methyl ether was then removed with BBr_3 at low temperature, and, after simple basic aqueous work up, the resulting deprotected aldehyde was used without further purification. Biphenyl carbaldehyde oxime **3** was formed in two steps by treating the aldehyde **7** with hydroxyloxime hydrochloride in methanol at room temperature.

The six new compounds **10a**, **11b–d**, **12** and **13** and the control compound, oxime **3**, were first evaluated for their ability to compete with ES2 for binding to both ERs using the FP assay as described (Fig. 3).[18]

Only two compounds, **11b** and **13** (Fig. 4), significantly competed with ES2 as shown in Figure 3. The curves were constructed from one-site competition best-fit curves of polarization (mP) versus concentration using GraphPad Prism 4 software. The IC_{50} values determined from this fit are listed in Table 1. Biphenyl carbaldehyde oxime **3** and E2 were used as positive controls, the former giving an IC_{50} comparable to the previously determined value in a radioligand binding assay.[15]

The compounds were next evaluated for their ability to inhibit E2-induced proliferation of MCF-7 human breast cancer cells and



Scheme 2. Synthesis of positive control agent, 3-fluoro-4′-hydroxy-1,1′-biphenyl-4-carbaldehyde oxime **3**. Reagents and conditions: (a) DAPCy (2 mol %), Cs_2CO_3, dioxane, rt, 70%; (b) BBr_3; (c) $NH_2OH{\cdot}HCl$, 42%.

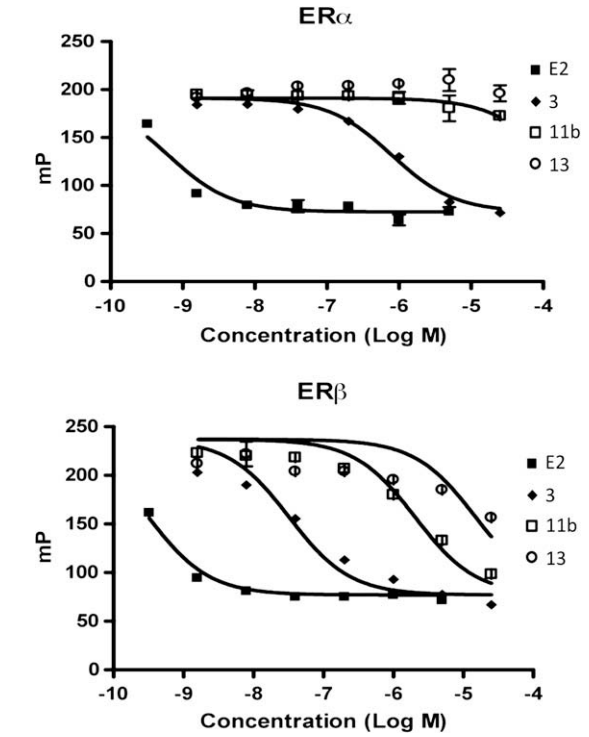


Figure 3. Competition FP assay for **11b** and **13** with ES2 for binding to ERα or ERβ. E2 and **3** were used as controls.

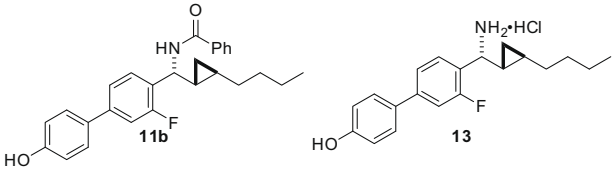


Figure 4. Lead compounds **11b** and **13**.

Table 1
IC_{50} values for competition at both ERs from the FP assay

Compounds	ERα IC_{50}[a] (nM)	ERβ IC_{50}[a] (nM)
E2	0.627	0.316
3	795	32.3
11b	>100,000	2048
13	na	15,030

[a] Values are means of three experiments, (na = not active).

demonstrate their potential as anti-estrogens. This was done using two variants of the MCF-7 cell line. The later passage ATCC variant (MCF7) that expresses only ERα, and an earlier passage (MCF-7) that expresses both ERα and ERβ. The western blot demonstrating this expression is in the Supplementary data. Figure 5 shows the growth inhibition curves generated for compounds determined to be selective for ERβ using the MTS assay.[21] Raloxifene (RAL), an antiestrogen known to inhibit the proliferation of MCF-7 cells, was included as a control.[22] GraphPad Prism 4 was used to estimate GI_{50} values using a non-linear best curve fit. In the ERα and ERβ expressing MCF-7 cells, compounds **11b** and **13** gave GI_{50} values of 4.1 and 1.2 μM, respectively.

In the ERα expressing ATCC MCF7 cells, compound **11b** did not demonstrate inhibition, while compound **13** gave a GI_{50} value of 51.7 μM. In these cells, all 3 compounds tested were toxic at a con-

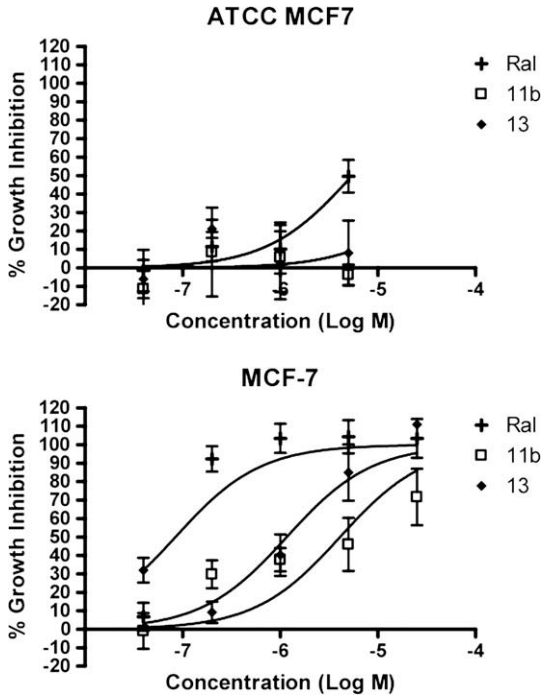


Figure 5. Inhibition of E2-stimulated growth of ATCC MCF7 (*top*) or MCF-7 (*bottom*) cells by biphenyl *C*-cyclopropylalkylamides **11b** and **13**. RAL was used as a positive control. Data represents % growth inhibition (mean ± SD, $N = 3$).

centration of 25 μM. Additionally, the growth inhibition of ER-negative MDA-MB231 cells was also assayed.[23,24] **11b**, **13**, and RAL did not demonstrate significant growth inhibitory activity against this cell line but the compounds again demonstrated toxicity at 25 μM. This can also be seen in the Supplementary data. Therefore, this provides support that the compounds are exerting their action through ERβ, because in breast cancer cells expressing both ERβ and ERα a significantly more potent anti-proliferative effect was demonstrated compared to breast cancer cells expressing only ERα or no ERs (Fig. 5).

c-Myc is known to play a major role in cell proliferation and malignant transformation in breast cancer.[25] E2 has been shown to cause a rapid increase in the level of c-Myc expressed by human breast cancer cells.[26] Induction of the expression of ERβ inhibits c-Myc at both the mRNA and protein level, while increasing the levels of its regulators, the cyclin-dependent kinase inhibitors, p21 (Cip1) and p27(Kip1).[5] Because of these known links to breast cancer and E2, we chose to investigate how our compounds affected

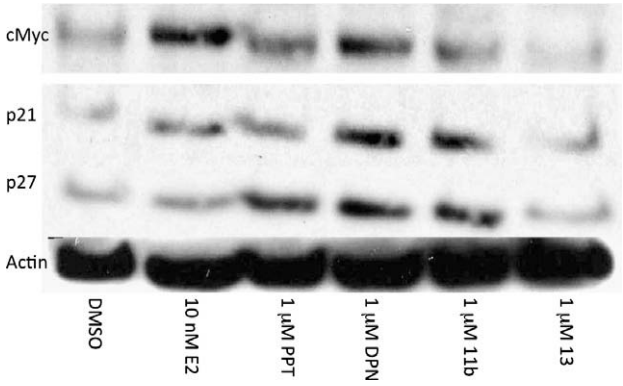


Figure 6. Western blot analysis of the levels of c-Myc, p21, p27, and actin in lysates of MCF-7 cells treated with 10 nM E2, 1 μM PPT, 1 μM **11b** and 1 μM **13** for 12 h.

Table 2
Relative fold changes of protein levels induced by the indicated treatment in MCF-7 cells compared to DMSO vehicle treatment

	10 nM E2	1 μM PPT	1 μM DPN	1 μM **11b**	1 μM **13**
cMyc[a]	3.84	2.17	2.67	0.77	0.59
p21[a]	1.15	4.05	3.47	3.64	1.10
p27[a]	1.76	2.47	3.32	3.90	1.51

[a] ImageJ was used to determine band intensity, and levels were standardized to actin.

the protein levels of c-Myc, p21, and p27. Western blot analyses (Fig. 6) from lysates of MCF-7 cells treated with the indicated compounds and densitometric analyses (Table 2) show compounds **11b** and **13** hold promise because of their ability to increase the levels of cyclin-dependent kinase inhibitors and decrease the levels of c-myc, likely inhibiting cell cycle progression and proliferation. PPT, a 410-fold ERα-selective agonist, and DPN, a 70-fold ERβ-selective agonist, were included as controls.[27,13]

In conclusion, compounds **11b** and **13** demonstrate selectivity for ERβ as well as promising antiproliferative effects in breast cancer cells. The compounds are likely acting as agonists for ERβ based on what has been shown about ERβ's activity in breast cancer,[4–6] and this encourages future investigation. Compared to the lead compounds **3** and **5**, **11b** and **13** show no affinity for ERα and moderate affinity for ERβ. This initial investigation into the biphenyl *C*-cyclopropylalkylamide pharmacophore demonstrates the potential of this scaffold to deliver ER-selective antiestrogens. Furthermore, the preliminary SAR provides clues for improving selectivity for ERβ. A subtype-selective agent will help to understand the complex biology of the ERs and their interplay. The arrival of new ER ligands with differential subtype selectivity ratios enables the tailoring of antiestrogenic or estrogenic therapy according to the condition and level of receptor isoforms present in patients.

Acknowledegments

Supported by grants from the Department of Defense (W81XWH-04-1-0413 and W81XWH-08-1-0290) and the National Institutes of Health (GM067082).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.075.

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18. The ER competitor assays were performed according to the manufacturer's (Invitrogen, Carlsbad, CA) recommendations with some modifications. Human recombinant ER was used at the recommended concentration of 15 nM and the Fluormone™ ES2 was used at a concentration of 1 nM. Aliquots (20 μL) of the mixture of ER and Fluormone™ ES2 were distributed in 384-well, black flat-bottom plates and serial dilutions of test compounds in DMSO were added. Each compound was tested in duplicate, and 1 μM E2 was used as a positive control. The DMSO concentration was kept at 1% (v/v) throughout the experiment. After 2 h, the fluorescence polarization (FP) was measured using an Analyst™ AD & HT Assay Detection Systems reader (Molecular Devices, Sunnyvale, CA) equipped with 485 nm excitation and 530 nm emission filters with the appropriate FL505 dichroic mirror. Data was then analyzed by linear regression using GraphPad Prism's one-site competition method. The fit was constrained by the high polarization control, which was the ER/ES2 complex with no competition, and the low polarization control, which was the ER/ES2 complex with 1 μM E2 for 100% competition.
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21. MCF-7 cells were from Dr. Marc E. Lippman MCF7 cells were from ATCC. Cells were seeded in 96-well plates at a density of 4×10^3 cells per well in phenol red-free RPMI-1640 medium containing 10% charcoal-dextran stripped FBS and allowed to adhere overnight. Test compounds were then added (concentrations ranging from 40 nM to 25 μM) along with 1 nM E2, and cells were incubated for 6 days. Effect of the test compounds on growth was determined using the CellTiter Aqueous One assay system (Promega, Madison, WI), which utilizes the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) dye reduction assay with phenazine methosulfate as the electron acceptor. Absorbance was measured at 490 nm using an M5 plate reader (Perkin Elmer, Boston, MA) and the background absorbance at 630 nm was subtracted after 2 h incubation with the reagents. A plate of control cells (16 wells) was measured at day 0 to establish 0% growth. E2-stimulated growth at day 6 was set to 100% growth. GraphPad Prism 4 (GraphPad Software Inc, La Jolla, CA) software was used for constructing dose-response curves and calculating GI_{50} values.
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