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Identification of a series of tetrahydroisoquinoline derivatives as potential therapeutic agents for breast cancer

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Abstract—A series of tetrahydroisoquinoline-*N*-phenylamide derivatives were designed, synthesized, and tested for their relative binding affinities, and antagonistic activities against estrogen receptor (ER). Compound **1f** (relative binding affinity, RBA = 5) showed higher binding affinity than tamoxifen (RBA=1), a potent ER antagonist and currently being used for breast cancer therapy. Compound 1f also exerted optimal antagonistic activity against ER in reporter and cell proliferation assays. Interestingly, compound **1j**, which only has a minor agonistic effect against ER, acted as a progesterone receptor (PR) antagonist and exerted agonistic activity against AP-1 through ER pathway. Our results show that these new compounds can be employed as leading pharmacophore for further development of potent selective ER and/or PR modulators or antagonists.

Breast cancer is the second leading cause of cancer-related deaths in women today and is the most common cancer among women, excluding non-melanoma skin cancers. The nuclear receptors, estrogen receptor (ER) and progesterone receptor (PR) and their associated steroid hormones, are known to play essential roles in the growth of breast tumors, and their status is also employed as diagnostic indicators for endocrine responsiveness and tumor recurrence.[1]

Physiologically, estrogen (E2, Fig. 1a) is involved in cellular proliferation and differentiation of organs such as breast, uterus, ovary, and bone.[2] Its action at the cellular level is exerted through ER. To date, two subtypes of human ERs have been cloned—hERα and hERβ.[3,4] Both proteins contain six functional domains, A to F.[5] The A and B domains, collectively referred to as the AF-1 domain, form the transactivation domain for ERs. The function of the AF-1 domain is regulated by promoter regions, as well as influenced or activated by E_2 and several antiestrogenic compounds upon binding to the ligand binding domain (LBD). The ER's C domain is the DNA binding domain (DBD). The D domain is a hinge domain, while the E and F domains are commonly referred to as the AF-2 domain and make up the second transactivation domain. The AF-2 domain contains the LBD and its function is highly regulated by compounds that bind to the LBD.[6] In addition to its ligand dependent properties, the AF-2 domain is also responsible for the interaction between ERs and coactivators/corepressors.[7] Traditionally, ERs exert their functions mainly via the genomic pathway where they serve as transcription factors. Initially, ERs are inactive and located in the cytoplasm.[8] Upon E_2 binding, they form an active complex that translocates to the nucleus, dimerizes, and binds to specific promoter or enhancer regions. Finally, this complex turns on transcription action as well as regulates the transcription actions of genes involved in several essential biological functions, including Bcl-2, cathepsin D, progesterone receptor, and VEGF.[9–12] Apart from the classic ligand dependent transactivation mechanism, there is a DNA binding independent transactivation mechanism for exerting estrogen's functions. The hER can mediate gene transcription through the AP-1 enhancer element that requires hER ligand and the AP-1 transcription factors, Fos and Jun, for the transcriptional activation.[13]

Progesterone (Fig. 1a) also plays a major role in the development, differentiation, and function of female reproductive tissues required for pregnancy, and recent

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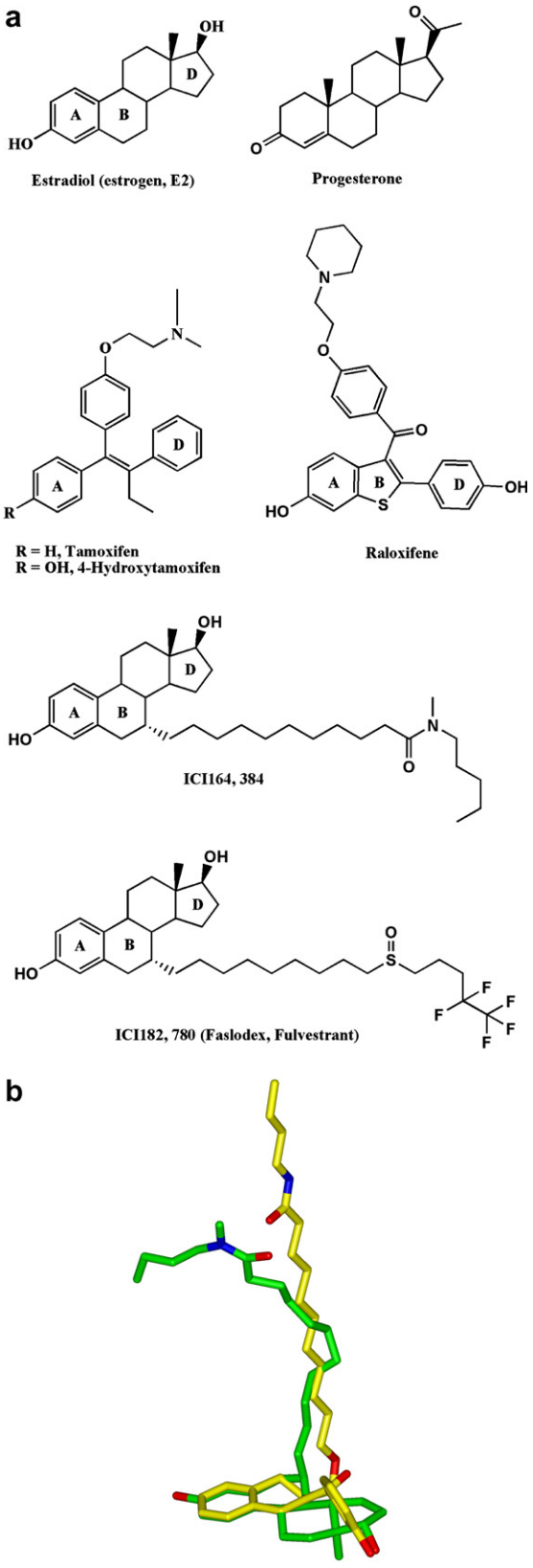


Figure 1. (a) Chemical structures of estrogen, progesterone and novel antiestrogens. (b): Superimposition of 3-D structures of 11-[2-(3-hydroxy-benzoyl)-1,2,3,4-tetrahydroisoquinolin-3-ylmethoxy]-undecanoic acid butylamide (yellow stick) and ICII64, 384 (green stick). These structures and the figures were generated with Sybyl 7.0 and InsightII.

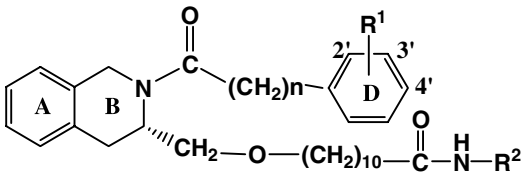
clinical data indicate that progesterone increases breast cancer risk and may also have a role in established breast cancer.[14] The biological effects of progesterone are mediated by PR. Like other nuclear receptors, PR also acts as a transcription factor and contains six functional domains (A–F), similar to those described for ER. The genes involved in cancer survival mechanism and regulated by progesterone include Bcl-$_X$L and transforming growth factors (TGF).[15,16]

Structurally, most non-steroid estrogen receptor modulators, including agonists and antagonists, contain three components: a core scaffold that mimics the A (phenolic moiety) and B rings of estrogen; a second aromatic ring that directly connects with the B ring of the core scaffold; and an antiestrogenic side chain that is substituted on the B ring of the core scaffold (such as found in these antiestrogen agents: ICI164, 384; ICI182, 782; and raloxifene; shown in Fig. 1a) and can interrupt the localization of helix 12 of hER/LBD. In this study, we employ a tetrahydroisoquinoline–*N*-phenylamide moiety (shown in Table 1) as a chemical core scaffold. It contains a carbonyl bridge between its core scaffold and a second aromatic ring to mimic the steroidal structure of estrogen. It also contains an antiestrogenic side chain, similar to that of ICI164, 384, the prototype of ICI182, 780 (Fig. 1a). Importantly, the 3-D structure of this new derivative can superimpose fairly well with that of the side chain of the pure antiestrogen, ICII64, 384 as shown in Figure 1b. The primary goal of this study is to determine whether this new pharmacophore is capable of serving as potential antiestrogen. The overall goal of our study is to generate new pure antiestrogens that can be more potent than ICI182, 780 and act as prodrugs but lack its complicated synthetic routes.

The synthesis of compound **1a**–**1 l** is generated by a simple amidation reaction to produce the core scaffold, tetrahydroisoquinoline-*N*-phenylamide, which is then followed by addition of hydrophobic antiestrogenic side chain as shown in Scheme 1.[17]

Table 1 summarizes the relative binding affinities for compounds **1a** to **1l** based on a fluorescence-based competitive binding assay.[18] This assay utilizes a synthetic estrogenic probe with high fluorescence polarization property following the binding of a compound to recombinant hERα. Individual binding affinity is further calculated as relative binding affinity by using tamoxifen as a standard. Our studies have identified compound **1f** as the most potent antiestrogen among the tested compounds with relative binding affinity (RBA) five times greater than that of tamoxifen. The structure–activity relationship from this study indicates that the hydroxyl group substituted on the D ring enhanced significant higher binding affinity compared to other substituents including halogens, methoxy, and hydrophobic groups. We note that a hydroxyl group at the 3′ position (**1f**, RBA = 5) of the second aromatic ring exerts better binding affinity than at the 4′ position (**1g**, RBA = 1.79). Also, it appears that a larger size connective bridge (**1l**, RBA = 0.267; compared to **1b**, RBA = 0.287) and different moieties at the side chain terminus (**1k**,

Table 1. Relative binding affinity of compounds **1a**–**1l** for hERα



Compound	N	R^1	R^2	Relative binding affinity
1a	0	2′-F	Butyl	0.225
1b	0	3′-F	Butyl	0.287
1c	0	4′-F	Butyl	0.264
1d	0	3′-OCH_3	Butyl	0.192
1e	0	4′-OCH_3	Butyl	0.14
1f	0	3′-OH	Butyl	5.0
1g	0	4′-OH	Butyl	1.79
1h	0	3′-CH_3	Butyl	0.156
1i	0	4′-CH_3	Butyl	0.132
1j	0	3′-Cl	Butyl	0.156
1k	0	3′-F	Octyl	0.177
1l	1	3′-F	Butyl	0.267
Tamoxifen				1

ER binding: fluorescence polarization competitive binding assay with recombinant ER protein. ER binding: the polarization values versus test compound concentration curves were analyzed by the graphfit software to generate IC_{50} value. The IC_{50} value was further converted to relative binding affinity (RBA) by using tamoxifen's IC_{50} as a standard. The RBA value of each test molecule was quantified as RBA = IC_{50} of tamoxifen/IC_{50} of test molecule.

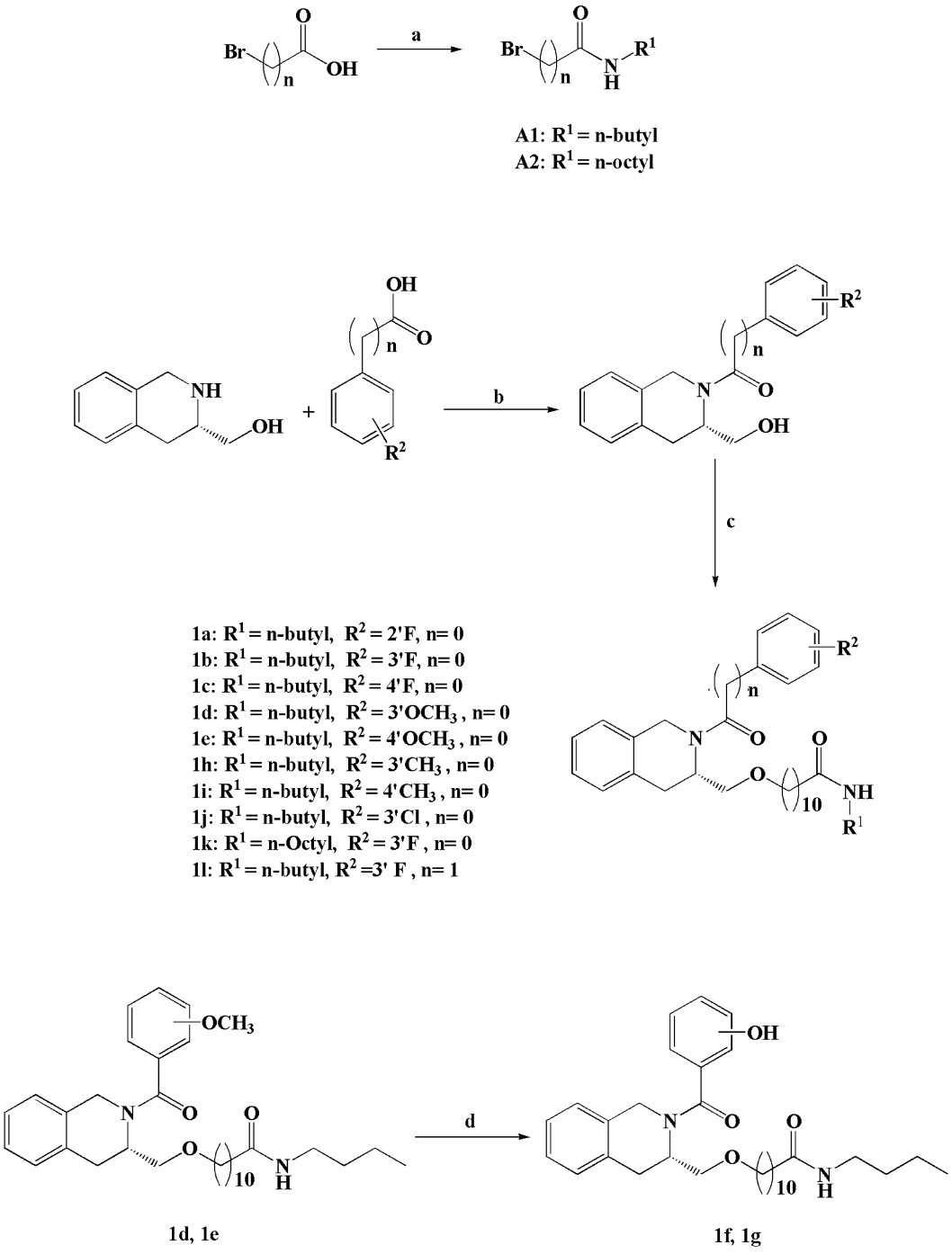
RBA = 0.177) do not appear to significantly influence the binding affinity.

Besides measuring the binding affinities, we tested the new analogs in transient transfection assays to determine their agonist/antagonist activity and selectivity for hERα.[19] Figures 2 and 3 outline the agonistic and antagonistic effects of these compounds, respectively, targeting hERα. At 2.5 μM, most of the tested compounds, except **1h**, **1i**, and **1l**, did not show any obvious estrogenic activity. **1f** (relative luciferase activity, RLA = 30% of 1 nM E2 effects) exerted the most significant antiestrogenic activity among the studied compounds, but slightly less than that of tamoxifen (RLA = 20% of 1 nM E2 effects). Compounds **1b**, **1d**, and **1h** also showed moderate antiestrogenic activity. Interestingly, **1l** with a larger core scaffold exerted minor estrogenic but no obvious antiestrogenic effects. Similar results were also observed for another core scaffold, benzofuran-phenylamide, with larger size than that of tetrahydroisoquinoline–*N*-phenylamide developed in our laboratory.

We also performed a cell proliferation assay using MCF-7 (ER positive) cells to evaluate the antiproliferation activities of the compounds, and the result is shown in Figure 4. At 5 μM, all tested compounds did not enhance the growth of MCF-7 cells. In line with the results observed with transient transfection assays, compound **1f** (relative cell viability = 0.48 of vehicle effects) exerted the most potent antiproliferation activity among the test compounds, but slightly less than that of tamoxifen (relative cell viability = 0.43 of vehicle effects).

In order to determine the specificity of pure antiestrogenic effects of compounds **1f**, **1b**, and **1d**, the compounds and tamoxifen were tested for their proliferation effects against MDA-MB-231 cells (ER negative breast cancer cells). As shown in Figure 5, unlike tamoxifen (relative cell viability = 0.65 of vehicle effects) that has been reported to exert significant antiproliferation activity against MDA-MB-231 cells through ER independent pathway to induce apoptosis, compound **1f** exhibited no obvious effects against the proliferation of MDA-MB-231 cells.[20] This result proved that compound **1f** can act as a pure antiestrogen and suggests that **1f** could potentially serve as a lead pharmacophore for the development of potent pure antiestrogens.

Based on the new core scaffold as well as the structure–activity relationship of the derivatives, there seem to be three essential moieties that could be suitable for designing potent antiestrogens. The first is the size of the core scaffold. It appears that a core scaffold larger than our new core scaffold might not serve as antiestrogens but rather weak estrogens. This is consistent with the fact that the other different core scaffolds (data not shown here) with a larger core size than **1f** but containing the same antiestrogenic side chain as compound **1f** showed weak estrogenic but no antiestrogenic effects. Second, the antiestrogenic side chain is essential for antiestrogenic effects. Crystallographic study of 4-hydroxytamoxifen bound to hER ligand binding domain shows 4-hydroxytamoxifen's antiestrogenic side chain bent toward helix 3 to make interaction with Asp351, and as a result the side chain does not directly interact with helix 12.[21] On the contrary, compound **1f** with a significantly longer side chain than that of 4-hydroxytamoxifen, as suggested by modeling studies, might protrude out of the ligand binding pocket and position in such a way as to directly interact with helix 12. In the modeling studies, we docked **1f** into the hERα LBD/4-hydroxytamoxifen



Scheme 1. Synthesis of compounds **1a**–**1l**. (a) i—isobutylchloroformate, CH_2Cl_2. ii—*N*-butylamine, (or *N*-octylamine), Et_3N; (b) DEC, HOBt, DMF, RT; (c) NaH, $Br(CH_2)_{10}CON(CH_2CH_2CH_2CH_3)H$ (or $Br(CH_2)_{10}CON$(n-octyl)H) DMF; (d) BBr_3, CH_2Cl_2.

structure (PDB code: 3ERT). After energy minimization, the result shown in Figure 6 suggests that the core structures of compound **1f** and 4-hydroxytamoxifen could superimpose closely, however as noted above the mode of binding of the side chain may be different. The suggested a side chain binding mode in **1f** would prevent helix 12 of the protein from moving toward the ligand binding pocket, resulting in the compound acting as a pure antiestrogen. We also point out that unlike the antiestrogenic side chain of ICI164, 384 which interacts with the side chain of Trp 290 of rERβ LBD, the longer antiestrogenic side chain of 1f may also prevent such an interaction.[22] The antagonistic mode of binding by GW5638, a tamoxfen derivative in hERα LBD, would support our hypothetical inference.[23] The main and only structural difference between tamoxifen and GW5638 is the terminus of their antiestrogenic side chains. Tamoxifen has a dimethylamino group at the antiestrogenic side chain terminus, while GW5638 has a terminus carboxylic acid, which does not interact with Asp 351, but rather with Leu 536 and Tyr 537 of helix 12 of hERα LBD. This interaction induces capping of helix

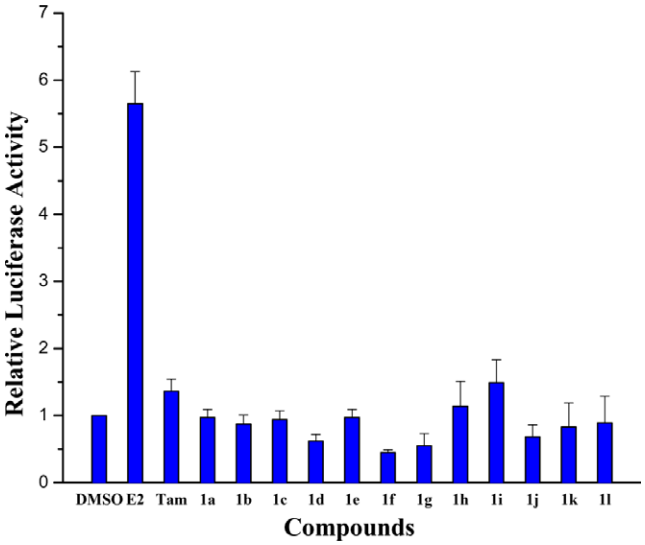


Figure 2. Transient transfection reporter assay in MCF-7 cells. ER agonistic effect was determined by the ERE-driven transactivation luciferase activity. The relative luciferase activity was quantified as RLA of 2.5 μM tested compound/RLA of DMSO (vehicle). The RLA data represent means ± SD for three determinations. Tam, tamoxifen.

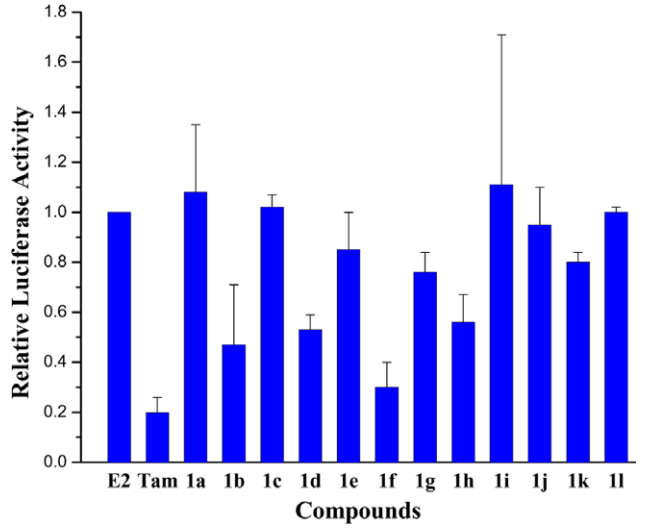


Figure 3. Transient transfection reporter assay in MCF-7 cells. ER antagonistic effect was determined by the ERE-driven transactivation luciferase activity in the presence of 1 nM estradiol. The relative luciferase activity was quantified as RLA of 2.5 μM tested compound/RLA of 1 nM estradiol. The RLA data represent means ± SD for three determinations. Tam, tamoxifen.

12 and causes it to shift to an antagonistic conformation. This antagonistic mode of binding by GW5638 is consistent with the hypothetic mechanism of the antiestrogenic side chain of **1f** observed in our modeling study. Further structure–activity relationship study in the modification of antiestrogenic side chain of **1f** is required to fully demonstrate this hypothesis. Finally, the last moiety that is essential in modulating antiestrogenic activity is the substituted group on the D ring of the core scaffold. Most novel antiestrogens (such as raloxifene, but not tamoxifen) contain a 4′-hydroxyl group on their D

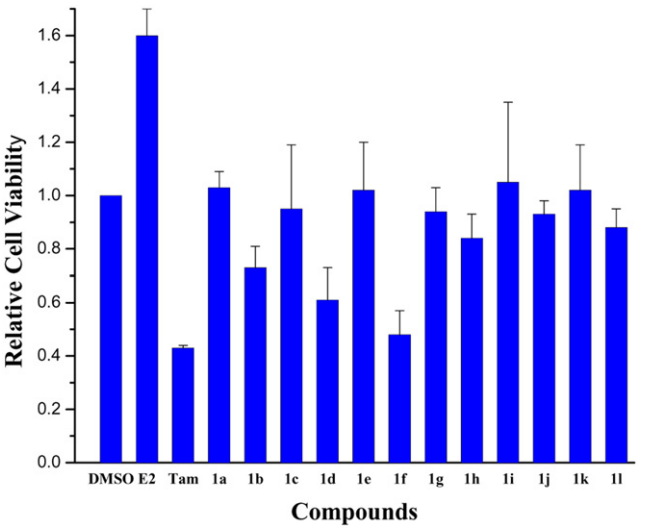


Figure 4. Cell viability assay in MCF-7 cells. After cells were inoculated into 12-well culture plate and attached to the bottom, 5 μM tested compound was added to the respective well and further incubated with cells for 48 h. The final relative cell viability activity was quantified as MTT assay value of 5 μM tested compound/MTT assay value of DMSO. The MTT assay data represent means ± SD for three determinations. Tam, tamoxifen.

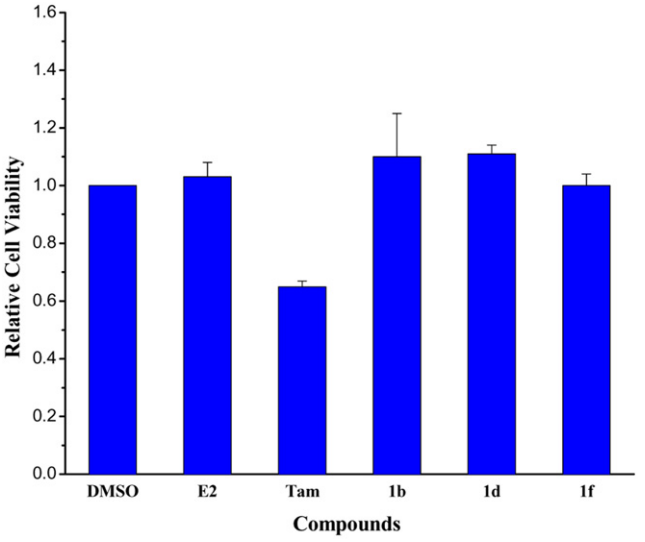


Figure 5. Cell viability assay in MDA-MB-231 cells. After cells were inoculated into 12-well culture plate and attached to the bottom, 5 μM tested compound was added to the respective well and further incubated with cells for 48 h. The final relative cell viability activity was quantified as MTT assay value of 5 μM tested compound/MTT assay value of DMSO. The MTT assay data represent means ± SD for three determinations. Tam: tamoxifen.

ring, which acts as an optimal substituent for activity. The structure–activity relationship study of raloxifene derivatives shows that the derivative with a 4′-hydroxyl group (raloxifene) exerted two-fold higher binding affinity than the derivative with 3′-hydroxyl group. On the other hand, both derivatives showed 10-fold higher binding affinity than the derivative with 2′-hydroxyl substituent.[24] In MCF-7 cell proliferation assays, raloxifene

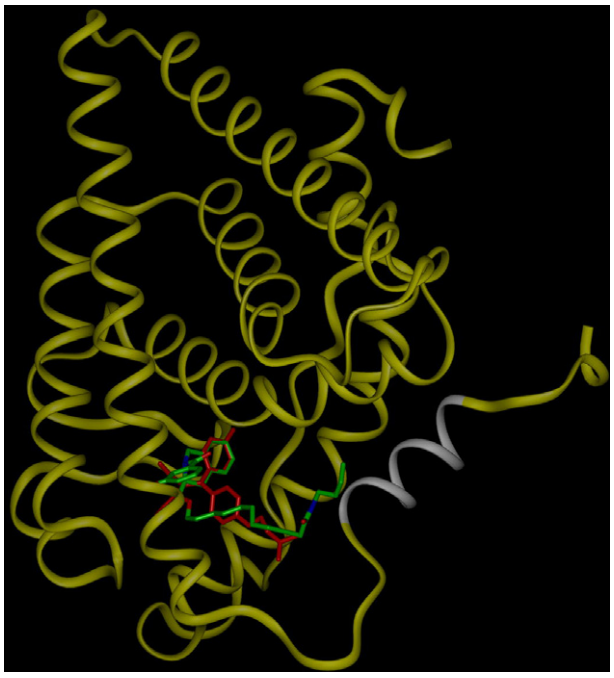


Figure 6. Hypothetical model of compound **1f** (in green color; 4-hydroxytamoxifen in red color; helix 12 of hERα LBD in white color) located in hERα LBD. The 3-D structure of compound **1f** was energy-minimized and generated using Sybyl 7.0 program. The minimized structure was further docked into hERα LBD crystal structure (3ERT, protein data bank) and superimposed with 4-hydroxytamoxifen using Insight II program to generate the model.

showed 16-fold better activity than its derivative with 3′-hydroxyl group. Addition of a 4′-hydroxyl group on the D ring of tamoxifen enhanced its higher binding affinity by six-fold.[25] On the other hand, the addition of a 3′-hydroxyl group decreased the binding affinity by one-third. Nevertheless, and unlike raloxifene derivatives, addition of a hydroxyl group to either the 3′ or 4′ position of tamoxifen's D ring did not significantly increase its in vivo antiestrogenic effects. For our new scaffold, a 3′ or 4′-hydroxyl substituent on the D ring also enhanced its binding affinity, with the 3′ hydroxyl substituent increasing binding affinity to five-fold that of tamoxifen. Interestingly, we observed an increase of antagonistic effects in the reporter assay. Wilhelm Stark et al. at Norvatis have reported a new class of antiestrogens with *N*-phenyl-tetrahydroisoquinoline as the core scaffold.[26,27] In their study, the 3′ and 4′-hydroxyl derivatives exerted similar binding affinity and cellular antiestrogenic effects. The above studies and results suggest substitution of an optimal substituent on the D ring varies with different core scaffold structures. In conclusion, the size of the core scaffold, the substitution on the D ring, and the antiestrogenic side chain play essential roles in the observed and future design of potent antiestrogens.

Although most antiestrogens can antagonize the actions of hER in many human organs, they are also found to exert estrogen-like actions in some tissues via hER. The AP-1 mechanism has been hypothesized to be the main pathway for estrogen-like actions of antiestrogens. Thus, we used AP-1 luciferase reporter in transient

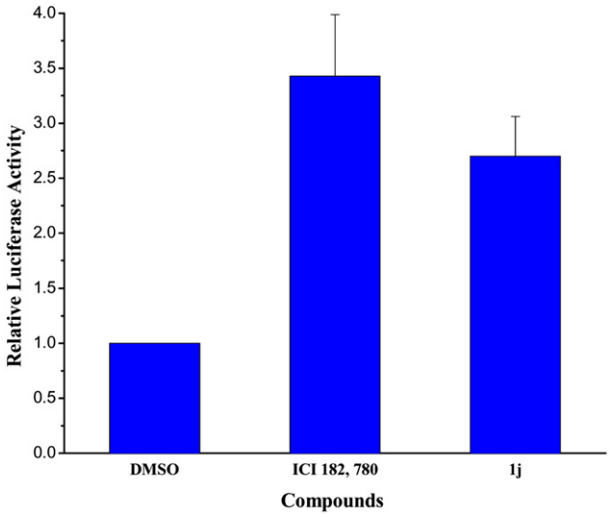


Figure 7. Transient transfection reporter assay in T47D cells. hERα/AP1 agonistic effect was determined by the AP1 promoter-driven transactivation luciferase activity. The relative luciferase activity was quantified as RLA of 5 μM tested compound/RLA of DMSO (vehicle). The RLA data represent means ± SD for three determinations.

transfection reporter assays to investigate whether any of our compounds can act as an estrogenic molecule to enhance AP-1 transcription actions through hERα. As shown in Figure 7, ICI 182, 780 at 5 μM significantly enhance AP-1 driven transcription effects as previously reported.[28] Compound **1j** also exerted a slightly less effect at the same concentration as ICI 182, 780. Importantly, although **1j** does not contain any hydroxyl group on its A ring like ICI 182, 780, it still can activate AP-1 dependent reporter action. This result suggests that the structural requirement of agonists in AP-1 dependent transcription in the presence of hERα/AP-1 pathway is different from the classical antiestrogenic structures. Especially, the position of hERα helix 12 in this mechanism could be different from that in the classical mechanism. It will be intriguing to study the structure–activity relationship of ER modulators against AP-1 actions.

In addition to ER, PR is also an essential diagnostic factor for breast cancer status, and its antagonists are known to show significant antiproliferation effects against ER+/PR+ mediated breast cancers. Even though PR antagonists have different structural requirement from ER antagonists, ICI182, 780 has been reported to contain antiprogestin activity. The antiprogestin activity of ICI182, 780 might be a contributing factor for its significant anti-breast cancer effects.[28] Since our compounds can structurally superimpose with ICI182, 780, we also evaluated compound **1j** against hPR by employing transient transfection reporter assays in T47D cells (ER positive/PR positive breast cancer cells). As shown in Figure 8, at 5 μM, **1j** (RLA = 54% of 1 nM progesterone effects) exerted similar antiprogestin activity as ICI182, 780 (RLA = 48% of 1 nM progesterone effects). Compound **1j** did exert moderate antiprogestin effects in PRB/MMTV dependent transcription actions although it has no obvious effects on ER. ER LBD and PR LBD have quite different structural require-

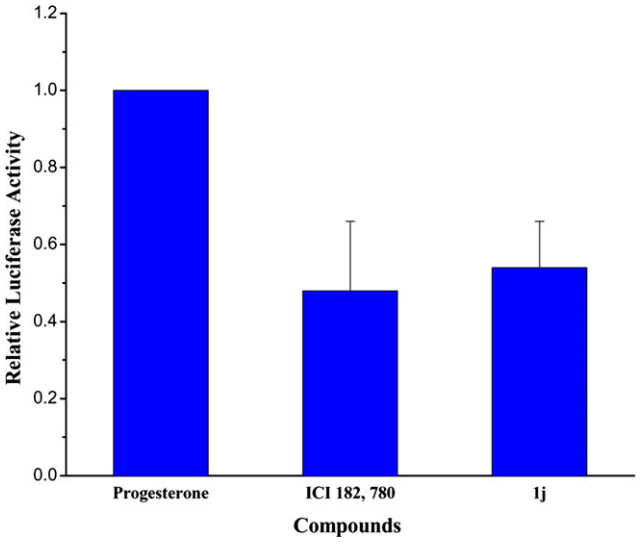


Figure 8. Transient transfection reporter assay in T47D cells. PR antagonistic effect was determined by the MMTV promoter-driven transactivation luciferase activity in the presence of 1 nM progesterone. The relative luciferase activity was quantified as RLA of 5 μM tested compound/RLA of 1 nM progesterone. The RLA data represent means ± SD for three determinations.

ments for their ligands due to structural differences in their ligand binding domains. In the crystal structure of PR LBD/progesterone complex, the carbonyl group on the 3 position of progesterone makes hydrogen-bond contact with Gln 725 and Arg 766.[29] Although, the carbonyl group on the 20 position of progesterone is in proximity to Thr 894 and Cys 891, it does not form hydrogen-bond interactions with any of the residues. Nevertheless, structure–activity relationship studies for progesterone derivatives suggest that the carbonyl group at the 20 position could make contact with PR LBD.[30] Like ICI182, 780, compound **1j** showed no agonistic but antagonistic effects against PR. Although both compounds do not contain a carbonyl group on the A ring, like progesterone, it is possible their long hydrophobic side chains could be responsible for their antiprogestin effects by directly interfering with helix 12. Unlike ICI 182, 780 that involves difficult synthetic routes, **1j** could easily be derivatized to generate potent antiprogestins.

In this study, we have identified a new type of pharmacophore and lead compound, **1f**, as a pure antiestrogen. Compound **1f** showed higher binding affinity than tamoxifen, and exerted no estrogenic but significant antiestrogenic effects in transient transfection reporter assays against hERα. It also selectively inhibited the proliferation of ER+ but not ER− breast cancer cells. Additionally, **1f** was also found to interfere with the dimerization of hERα in yeast two hybrid assays (data not shown). Apart from **1f**, **1j** exhibited selective action against PR and this discovery might lead to a new series of selective antiprogestins. Finally, we evaluated if these compounds can be employed as prodrugs by incubating compound **1k** with CYP2D6, a P450 enzyme that has been reported to transform tamoxifen to 4-hydroxytamoxifen.[31] HPLC analysis of the reaction products revealed a mixture of new products different from **1k**. However, the amount of mixture generated was not enough to run an NMR to identify these compounds. More study will be carried out in our laboratory to determine the possibility of our prodrug hypothesis. The results discussed above indicate that this new pharmacophore is capable of being further developed as potent pure antiestrogens or antiprogestins.

Acknowledgments

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17. *Synthesis of the hydrophobic antiestrogenic side chain A1 and A2*
*Synthesis of N-butyl-11-bromoundecanamide (A1) and N-octyl-11-bromoundecanamide (***A2***).* 11-Bromoundecanoic acid (12 g, 45 mmol) or 11-bromoundecanoic acid (12 g, 45 mmol) was dissolved in dry CH_2Cl_2 (200 ml) and tributylamine (10 g, 54 mmol). After cooling the mixture to −10 °C, isobutylchloroformate (8.2 g, 60 mmol) was added and stirred for 2 h. Excess *N*-butylamine (4.43 g,

60.8 mmol) was then added, and later the cooling bath was removed. After 3 h, CH_2Cl_2 was added and the organic phase was washed with 1 N HCl, saturated $NaHCO_3$, and water. After drying with $MgSO_4$, the solvent was removed and the crude product purified by flash column chromatography to generate compounds **A1** and **A2**.

Synthesis of compounds **1a**–**1l**. Benzoic acid (7 mmol) and (s)-(−)-1,2,3,4-tetrahydro-3-isoquinoline methanol (1 g, 6.7 mmol) were dissolved in DMF (30 mL) under nitrogen gas. 1-Hydroxybenzotriazole hydrate (HOBT) (1 g, 7.4 mmol) and 1-(3-(dimethylamino) propyl)-3-ethyl-carbodiimide) hydrochloride (DEC) (1.5 g, 7.8 mmol) were added to the solution and the reaction mixture stirred overnight. EtOAc was added to the reaction mixture and subsequently washed with 10% $KHSO_4$ (30 mL), saturated $NaHCO_3$(30 mL), and brine (30 mL). After drying with $MgSO_4$, the solvent was removed and the crude product was purified by flash column chromatography. The purified compound was dissolved in DMF and mixed with sodium hydride (0.17 g, 7 mmol) and *N*-butyl-11-bromoundecanamide (**A1**, 3.2 g, 10 mmol) or *N*-octyl-11-bromoundecanamide (**A2**, 3.76 g, 10 mmol). The reaction mixture was refluxed under nitrogen overnight. EtOAc (100 mL) was added to the reaction mixture and the solution was subsequently washed with water (30 mL) and brine (30 mL). After drying with $MgSO_4$, the solvent was removed and the crude product was further purified by flash column chromatography to generate the final pure product.

Synthesis of compounds **1f** *and* **1g**. Compound **1f**. A solution of compound **1d** (0.54 g, 1 mmol) in dry CH_2Cl_2 (50 mL) was cooled to −60 °C under a nitrogen atmosphere. Following, BBr_3(1 mL, 10 mmol) was added. After 30 min, the cooling bath was removed and the mixture stirred overnight. After cooling, the mixture was poured into an aqueous solution of $NaHCO_3$.The organic layer was separated, and the aqueous layer was extracted with EtOAc (50×3 ml). The combined organic layers were washed with water and dried with $MgSO_4$. After evaporation of the solvent, the remaining residue was purified by flash column chromatography (SiO_2, hexane/EtOAc) to generate compound **1f**.

Compound **1 g**. Using compound **1e** (0.54 g, 1 mmol) in dry CH_2Cl_2 (50 mL) and then BBr_3(1 mL, 10 mmol), compound **1g** was synthesized following the above procedure for compound **1f**.

18. Fluorescence polarization competitive binding assay: the estrogen receptor competitor assay kit (Panvera, Madison, WI) was used to determine the ability of test molecules to displace the synthetic estrogenic probe, ES2, with high fluorescence polarization property when it binds to hERα, from hERα-ES2 complex. Serial dilutions of each test molecule were prepared in DMSO. The recombinant hERα (7 nM) was preincubated with ES2 (1 nM) in screening buffer. After preincubation, the test molecules and ER/ES2 complex solution were added into a 96-well microplate to produce a final volume of 100 μl per well. The reaction mixture was incubated at room temperature for 1 hr and the polarization values were measured by using fluorescence micro plate reader, Polarion (Tecan, Research Triangle Park, NC) with excitation wavelength 495 nm and emission wavelength 535 nm. The polarization values versus test compound concentration curves were analyzed by the graphfit software to generate IC_{50} value. The IC_{50} value was further converted to relative binding affinity (RBA) by using tamoxifen's IC_{50} as a standard that was set to 1. The RBA value of each test molecule was calculated by using the equation, RBA $=IC_{50}$ of tamoxifen/IC_{50} of test compound.
19. Cell culture, transient transfection reporter, and cell viability assay: Human breast cancer cells, MCF-7, MDA-MB-231, and T47D, were purchased from ATCC (Manassas, VA). The cells routinely were cultured as monolayer in Dulbecco's modified minimal essential medium for MCF-7 and MDA-MB-231 cells, and RPMI 1640 (Gibco/BRL, Grand Island, NY) for T47D cells supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), Penicillin (100 U/ml)/streptomycin (100 μg/ml) (GIBCO/BRL, Grand Island, NY), and incubated at 37 °C in a humidified atmosphere of 5% CO_2/air. For the transient transfection reporter assays, the MCF-7 or T47D cells were plated in triplicate in 12-well plates at a density of 3×10^5 cells/well in the phenol red-free DMEM or RPMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone, Logan, UT), Penicillin (100 U/ml)/streptomycin (100 μg/ml). Twenty-four hours later, the cells were transfected with three plasmids by using the Superfect transfection kit (Qiagen, Valencia, CA). For the detection of wild type hERα activity, cells were transfected with 2 μg wild type hERα expression plasmid (pCMV-hERα), 6 μg luciferase reporter plasmid containing estrogen receptor response element (3x-ERE-TATA-Luc) or luciferase reporter plasmid containing AP-1 response element (AP1-Luc, Clontech, Palo Alto, CA) , and 1 μg normalization control, β-galactosidase reporter plasmid (pCMV-β, Clontech, Palo Alto, CA). For the activity against hPR, cells were transfected with 2 μg wild type hPR expression plasmid (pCMV-hPR), 6 μg luciferase reporter plasmid containing progesterone receptor response element (pMMTV-Luc), and 1 μg normalization control, β-galactosidase reporter plasmid (pCMV-β). After transfection, the cells were treated with test compounds and one positive control (vehicle, DMSO) in phenol red-free culture medium. After incubation for further 24 h, the cells were washed with PBS and lysed with lysis buffer (Pierce, Rockford, IL). The lysate was used to determine the luciferase activity for ER's activity and the β-galactosidase activity for the normalization of transfection efficiency. For the luciferase activity assay, 20 μl of lysate and 100 μl luciferase assay buffer (Promega, Madison, WI) were added into a well of 96-well plate. The luminescence was detected by using luminescence microplate reader, LumiCount (Packard, Boston, MA). For the β-galactosidase activity assay, 20 μl of lysate and 180 μl β-galactosidase assay buffer (Clontech, Palo Alto, CA) were added into a well of 96-well plate. The β-galactosidase activity was measured as luminescence strength by using LumiCount (Packard, Boston, MA). The normalized luciferase activity was calculated by the equation, normalized luciferase activity =luciferase activity/β-galactosidase activity. For the estrogenic, antiestrogenic or antiprogestin effects of test molecules, the normalized luciferase activity value was further converted to relative normalized luciferase activity by using vehicle (DMSO) for estrogenic effects, 1 nM E_2's value for antiestrogenic effects or 1 nM progesterone's value for antiprogestin effects as a standard that was set to 1. In cell viability assays, MCF-7 or MDA-MB-231 cells were inoculated into 12-well culture plates at 2×10^5 cells in 2 ml maintained medium per well. Cells were allowed to attach to the bottom for 24 h incubation, then the seeding medium was removed and replaced by the experimental medium (phenol red-free DMEM supplemented with 5% charcoal-stripped fetal bovine serum and penicillin (100 U/ml)/Streptomycin (100 μg/ml). After further 24 h incubation, the test molecules dissolved in DMSO were added in the wells. The final concentration of DMSO in the culture medium did not exceed 0.1%. The culture was continued

for 2 days. The final relative cell viability was estimated with Cell Titer proliferation assay kit (Promega, Madison, WI) by measuring the absorbance at 570 nm, which is directly proportional to the number of living cells in the culture. The testing was performed by following the manufacturer's protocol. Experiments were triplicated for each compound.

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