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Discovery of Potent Ligands for Estrogen Receptor β by Structure-Based Virtual Screening

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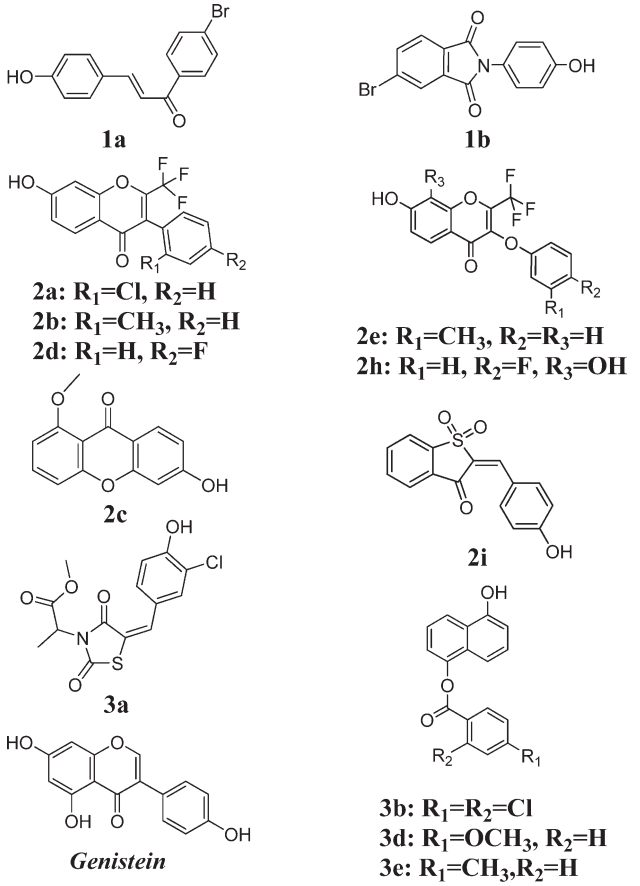
With virtual screening based on a structure optimized through molecular dynamics (MD) and bioassays, 18 potent ligands of estrogen receptor (ER) β were discovered from 70 purchased compounds here. Among them, dual profile was observed in two ligands (**1a** and **1b**), as agonists for ERβ and antagonists for ERα, and they might serve as lead compounds for selective ER modulators. The results also suggest that structures optimized through MD are applicable to lead discovery.

Introduction

Estrogen receptors (ERs[a]), including two subtypes, ERα and ERβ, belong to the nuclear receptor superfamily. Both subtypes play curial roles in the human body, but their distributions and functions are different. ERα is predominantly expressed in breast, uterus, and vagina, while ERβ is widely expressed in several tissues including the central nervous system, cardiovascular system, gastrointestinal system, and immune system.[1,2] Therefore, ERs are regarded as important drug targets for many diseases. For example, ER ligand tamoxifen has been widely used for the treatment of breast cancer.[3] Tamoxifen is the first selective ER modulator (SERM) acting as antagonist in breast tissue (ERα) but as agonist in bone (ERβ). Since tissue- or subtype-selective activity of SERM shows great advantage in clinic due to less adverse side effects,[4,5] it is important to seek novel subtype selective ligands or SERMs. Extensive studies have suggested that selective ERβ ligands would shed light on new avenues for clinical exploration.[6] For example, ERβ might be related to inflammatory diseases such as chronic intestinal and joint inflammations.[7,8] In addition, selective ERβ ligands could serve as probes to investigate the biological functions of ER subtypes.[8,9] Hence, discovering novel selective ERβ ligands has attracted more and more attentions.[4,10−17]

To date, besides steroidal estrogens, few scaffolds were developed as ERβ ligands. Most of nonsteroidal ligands contain a dicyclic part and a benzene ring connected with a single bond, such as benzonfuran/benzoxazole analogues[10−12] and genistein (see Chart 1) analogues.[13−16] Recently, the bibenzyl core and its analogues were also identified to have specific affinities for ERβ.[17] Nevertheless, there are growing needs to discover novel ERβ ligands, especially those with novel scaffolds. Virtual screening (VS) is an efficacious strategy for rapid lead discovery, which has been successfully applied in discovering ER ligands.[18,19] In most cases of previous studies, the crystal structures of ERs were adopted as the targets for VS, ignoring the dynamic nature of proteins.

Chart 1. Representative Structures of ER Ligands Discovered in This Study



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[a]Abbreviations: ER, estrogen receptor; LBD, ligand binding domain; SERM, selective estrogen receptor modulator; VS, virtual screening; SBDD, structure-based drug design; MD, molecular dynamics; Y2H, yeast two-hybrid system; LBP, ligand binding pocket; rmsd, root-mean-square deviation; MM-GBSA, molecular mechanics−generalized Born surface area; SAR, structure−activity relationship; E2, 17β-estradiol; SRC 1, steroid receptor coactivator-1.

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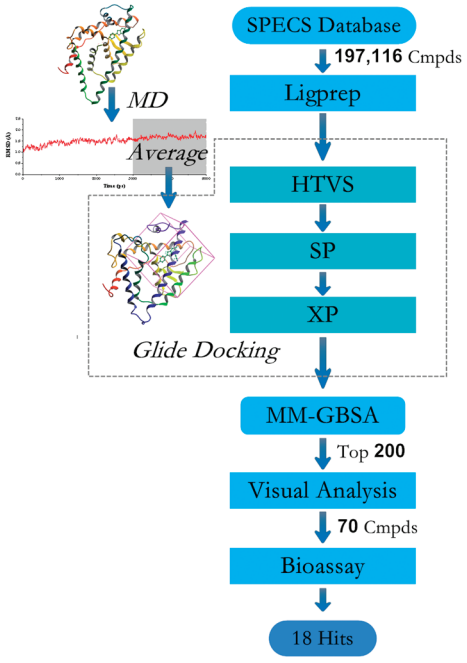

Figure 1. Schematic representation of hit discovery strategy in this study.

In the present study, we employed a strategy that combines molecular dynamics (MD) simulation with VS to discover novel ERβ ligands from a commercial database (Figure 1 and Experimental Section). The structure of the target was prepared by averaging a series of snapshots extracted from the trajectory of the MD simulation, and three rounds of VS were subsequently performed. Among 70 hits selected by our strategy, 18 compounds were identified as ERβ ligands with the yeast two-hybrid (Y2H) bioassays, in which not only the active value but also the behavior (agonistic or antagonistic) could be obtained for each compound simultaneously.[20] Meanwhile, the bioassays were also performed on ERα to evaluate the selectivity of those compounds. In addition, the antiproliferative activities of the compounds with antagonistic activities on ERs were also evaluated via MTT assays.

Results and Discussion

As illustrated in Figure 1, 197 116 compounds in the SPECS database were virtually screened against the dynamically averaged structure of ERβ LBD, which is obtained from the equilibrated stage of MD simulation (Figure S1 of Supporting Information). After three rounds of screening, then molecular mechanics−generalized Born surface area (MM-GBSA) calculation, and finally visual analysis, 70 compounds were selected and purchased from SPECS for bioassay.

The MM-GBSA score and ranking of each active compound and results of bioassays are presented in Table 1. Eighteen active compounds were discovered with our VS strategy. Among them, two compounds showed "dual-profile" activities, i.e., as agonists for ERβ and antagonists for ERα (Chart 1, **1a** and **1b**). Both compounds have potent activities for both subtypes with EC_{50}/REC_{10} or IC_{50} values below 10 μM. The docking pose of **1a** revealed that the phenolic part of the compound fitted well in the hydrophobic pocket (S2 subset), composed of residues Leu301, Ala302, Glu305, Met336, Leu339, Met340, Leu343, Arg346, Phe356, and Leu380 (Figure 2a). The hydroxyl group formed hydrogen bonding interactions with Glu305 and a water molecule, which made an important contribution to the binding affinity. In addition, an "edge to face" $\pi-\pi$ interaction was formed between Phe356 and the phenolic part of the ligand. Such $\pi-\pi$ interaction was also found in the docking poses of other ligands (Figure 2 and Figure S2). The bromobenzene part of the ligand was located in the S1 subset of the LBP, and the carbonyl group was toward the S1′ subset. Besides **1a** and **1b**, we also discovered 11 agonists (**2a**−**k**) and 5 antagonists (**3a**−**e**) (Chart 1 and Chart S1) in the study. Some active compounds discovered in this study were similar to known ER ligands. For example, among the 11 agonists, **2a**, **2b**, **2d**, **2e**, and **2h** were similar to genistein, which is a plant-derived nonsteroidal estrogen (Chart 1). Such results also proved that the model used for VS was reliable. There were five potent agonists (**2a**−**e**) with EC_{50} less than 100 nM. The docking pose of **2a** demonstrated that the benzopyran part adopted in the same manner as **1a**, where the hydrogen bonding network was formed with Glu305 and the water in the LBP (Figure 2b). The chlorobenzene part of **2a** was oriented into the S1′ subset of the LBP, and the CF_3 group was oriented toward the S1 subset. These halogen atoms contributed to the electrical interactions with the ligand. Similar binding modes were also found for **2b**, **2e**, **2h**, and **2i** (Figure S2), where the benzopyran part fitted the S2 subset and formed hydrogen bonds with residues in LBP. The aromatic ring of the ligand was extended into the S1′ subset of the receptor. To determine the agonistic effectiveness of these compounds, we also evaluated the 10% relative effective concentration (REC_{10}), which is the concentration of the tested compound showing 10% agonistic activity of 17β-estradiol (E2). The REC_{10} values were interrelated with EC_{50} for most compounds. Although the EC_{50} of compound **2i** on ERα was 18.7 μM, this compound could not activate ERα effectively (REC_{10} could not be determined). This made **2i** to have specific agonistic activity on ERβ. Besides, most compounds (**2c**−**f**) also exhibited certain selectivity for ERβ according to their REC_{10} values of the two subtypes.

According to the bioassay results, fundamental structure−activity relationships (SARs) were analyzed among the analogues. The benzopyran analogue **2a** behaved as a potent agonist for both ERβ and ERα. Once the ortho-substituent (R_1) was replaced by a neutral methyl (**2b**), the activity was reduced for ERβ but increased for ERα. It is difficult to explain from the docking results, since the ortho-substituent positioned into the conserved part of LBP where the environment was almost the same for both subtypes. However, this was in good agreement with the conclusion from our previous study that the polar functional group on the phenol part could improve the ERβ selectivity.[21,22] When a substituent was introduced into the para-position (R_2, **2d**), the activities for both ERβ and ERα were reduced significantly. In this case, the steric clash in the para-position forced the ligand to adjust its binding pose. The ligand pose of **2d** was reversed in the LBP compared to **2a** and **2b** (Figure S2), where the hydroxyl group formed a hydrogen bond with the backbone of Leu339 and the halobenzene part fitted into the S1 subset. Interestingly, **2e** and **2h** exerted significantly different activities only because of minor differences. When the *m*-methyl (R_1) was changed to the para position (R_2) with a fluorine and a hydroxyl group was added to the benzopyran part, the EC_{50} values were increased 75.4- and 140.7-fold for ERβ and ERα, respectively. The docking poses of **2e** and **2h** were almost the same (Figure S2), and two hydroxyl groups in **2h** could also serve as strong

Table 1. Agonistic or Antagonistic Activities of the Active Compounds and Standard Compounds on Both Subtypes[a] and MM-GBSA scores and Rankings of Active Compounds[b]

			agonistic activity (EC_{50} and REC_{10}[c], μM) or antagonistic activity (IC_{50}, μM)							
	MM-GBSA (kcal·mol^{-1})		ERβ			ERα				
compd	DG bind	rank	IC_{50}	EC_{50}	REC_{10}	IC_{50}	EC_{50}	REC_{10}	selectivity α/β[d]	MCF-7 inhibition (%)[e]
Dual-Profile Ligands										
1a	−55.15	3		3.91 ± 0.37	1.11	4.66 ± 1.29				71.44
1b	−41.36	68		4.07 ± 0.48	1.55	7.98 ± 1.07				35.12
Agonists										
2a	−45.13	36		0.0338 ± 0.0048	0.0081		0.0591 ± 0.00054	0.025	3.13	
2b	−41.76	59		0.0463 ± 0.0045	0.0074		0.0438 ± 0.00285	0.018	2.47	
2c	−51.02	10		0.0498 ± 0.0018	0.021		0.415 ± 0.024	0.61	29.9	
2d	−38.84	98		0.0672 ± 0.0144	0.036		0.331 ± 0.01	0.24	6.65	
2e	−41.26	71		0.071 ± 0.008	0.021		0.231 ± 0.01	0.16	7.60	
2f	−49.38	16		0.894 ± 0.14	0.21		4.13 ± 0.39	3.0	14.4	
2g	−39.15	93		4.93 ± 0.25	1.5		27.2 ± 7.4	8.6	5.65	
2h	−51.99	9		5.36 ± 0.73	4.1		35.8 ± 8.7	25	6.06	
2i	−48.31	22		5.77 ± 0.29	2.1		18.7 ± 3.2	>100	∞	
2j	−52.73	7		7.06 ± 0.59	1.3		3.08 ± 0.28	1.4	1.10	
2k	−42.72	54		34.9 ± 0.76	18		12.8 ± 0.77	7.0	0.40	
E2				0.00108 ± 0.00007	0.00018		0.00105 ± 0.00006	0.00010	0.55	
Antagonists										
3a	−43.83	44	0.0497 ± 0.002			2.32 ± 0.20			46.7	47.44
3b	−58.90	5	1.52 ± 0.55			1.20 ± 0.12			0.79	57.25
3c	−38.66	103	2.09 ± 0.39			6.13 ± 0.60			2.93	35.12
3d	−44.27	40	2.67 ± 0.33			0.795 ± 0.14			0.30	56.19
3e	−45.90	30	3.91 ± 1.24			31.6 ± 3.1			8.09	29.69
tamoxifen			1.66 ± 0.003			2.54 ± 0.12			1.53	93.33

[a] Data shown are the mean ± SD for at least triplicate measurements determined by Y2H assays. This system employs the interaction between hER LBD and the coactivator SRC 1 (see Experiment Section). [b] MM-GBSA scores are represented by free energies of binding (DG bind) of active compounds with ERβ, and their rankings (rank) in the top 200 compounds are also given. [c] 10% relatively effective concentration, which is the concentration of the tested chemical showing 10% of agonistic activity of the maximum activity of E2. REC_{10} provides the estrogenic activity relative to that of E2. [d] Determined by REC_{10} on ERα and ERβ for agonists and by IC_{50} of ERα and ERβ for antagonists. [e] Relative inhibition rate of antiproliferative potencies determined by MTT assays. The final concentration of the compounds is 100 μM, and cells treated with DMSO as control were set to 100% viability.

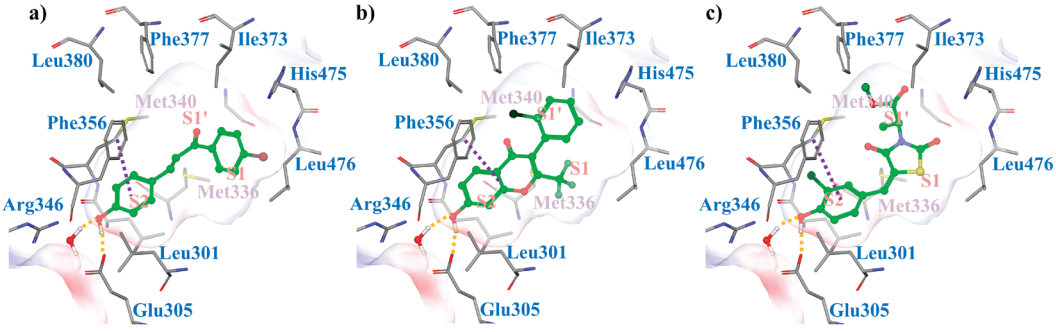

Figure 2. LBP of ERβ and docking poses of **1a** (a), **2a** (b), and **3a** (c). The LBP consists of three hydrophobic subsets (S1, S1′, and S2). Dash lines represent the noncovalent interactions between the ligand and receptor: yellow, H-bonding; violet, $\pi-\pi$ interaction; magenta, halogen bonding.

hydrogen bond donors, which should contribute the binding affinity of **2h**. However, the activity of **2h** is much less than **2e**. This could also be explained by our previous conclusion that the polar ligands would dissociate from the LBP easily and this would affect more on ERα than ERβ because of the "transmitter".[21]

To evaluate the transcriptional activities of these agonists, **2a**, **2c**, and **1a**, which have better activities in Y2H assay, were selected for transient co-transfection assay. The results are presented in Figure 3. In these assays, CHO-K1 cells were transfected with ER expression vectors for ERα or ERβ and an ERE-driven reporter plasmid and a Renilla luciferase transfection control plasmid pRL-SV40. Cells were treated with compounds at the concentrations indicated. Compounds **2a** and **2c** were proved to be ER agonists, and their maximum activities were even better than that of E2 (Figure 3). Compound **2a** efficiently stimulated ERα and ERβ, with EC_{50} of 0.97 and 0.75 μM, respectively. Compound **2c**, exhibiting a higher agonistic activity in stimulating ERβ in the Y2H assay, also showed its potency and selectivity to ERβ on cell-base transcription, with an EC_{50} of 0.88 μM. While the activation EC_{50} is 4.16 μM on ERα. It was consistent with the results of Y2H that the "dual-profile" **1a** could activate reporter gene transcription through ERβ but not through ERα (Figure S3).

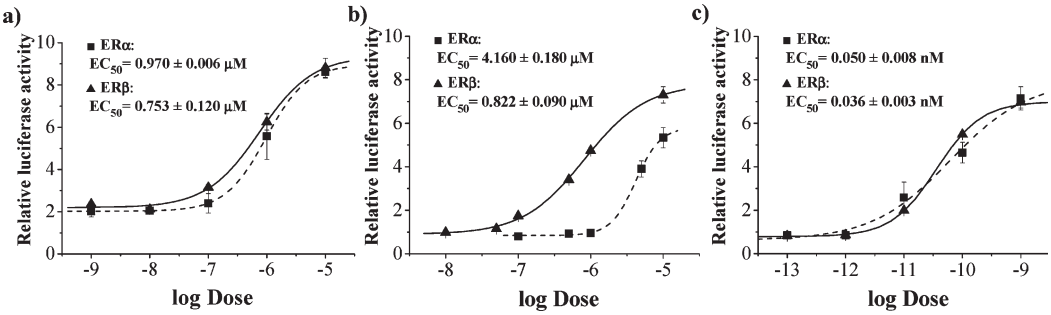

Figure 3. Dose-response curves for transcriptional activation by **2a** (a), **2c** (b), and E2 (c) through ERβ (solid line) and ERα (dashed line). CHO-K1 cells were transfected with expression vectors for ERα and ERβ, the estrogen responsive gene pGL2-ERE3-luc, and internal control pRL-SV40. Luciferase activity was performed as the ratio of the firefly luciferase activity to the Renilla luciferase activity, and the luciferase activity of cells treated with DMSO was set as 1.0. Values are presented as the mean ± SD of duplicate determinations.

Among the five antagonists, **3a** not only performed potent inhibitory activity (49.7 nM) but also showed significant selectivity (46.7-fold) for ERβ. Docking pose of **3a** showed that the phenolic part of the compound was positioned in the S2 subset and hydrogen-bonded to Glu305 and a water molecule. The thiazole part of the compound was located in the S1 subset, and the methyl ester part was extended into the S1′ subset (Figure 2c). For antagonists **3b**, **3d**, and **3e**, their binding modes revealed that the naphthalene part of the compounds was adopted in a vertical position (Figure S2), which was different from other diphenyl compounds, such as **2a** (Figure 2b). The hydrogen bonding network was also formed by the hydroxyl group together with Glu305 and the water molecule. The dichlorobenzene part connected with the naphthalene ring by an ester linkage was oriented to the S1 subset. SAR analysis demonstrated that when the polar chlorine atoms in ortho- and para-positions (R_2 and R_1, **3b**) were substituted by a *p*-methoxyl group (**3d**), the antagonistic activity was decreased to ERβ but increased to ERα. Furthermore, the activities for ERβ and ERα were reduced by substitution of the *p*-methoxyl group with a nonpolar one (**3e**).

The compounds that showed antagonistic activities on ERβ or ERα were selected for the assessment of antiproliferative potencies on human MCF-7 breast cancer cell line (Table 1), and all of them displayed certain antiproliferative activities. The most potent compound in suppressing the proliferation of MCF-7 cell was **1a** with an inhibitory ratio of 71.44%, which was close to that of tamoxifen. Furthermore, **1a** was a "dual-profile" compound, which showed agonistic activity on ERβ. Among those compounds, **3a** and **3c** showed relatively low MCF-7 inhibition percentages, while their inhibitory capacities determined in the Y2H assays were high. This might be caused by the big size and slight acidity of the two compounds, which might hamper cell penetration and therefore decrease the antiproliferative activity on the MCF-7 cell line. In addition, subtype selectivity of a compound might also affect the antiproliferative activity, since ERα was dominantly expressed in the human MCF-7 breast cancer cell line. Actually, the antagonistic activities of **3a** and **3e** on ERα were not very high. This might also affect the overall antiproliferative activities. Nevertheless, since these active compounds exhibited certain antiproliferative activities on human MCF-7 breast cancer cell, they could be used as starting points to find novel anti-breast cancer agents.

Conclusions

By use of a combined strategy of VS and MD, 18 compounds were discovered as potent ligands of ERβ. Among them, two "dual-profile" ligands (**1a** and **1b**) could be potential leads for novel SERMs, which could benefit the therapy of ER sensitive breast cancer. Eleven agonists for ERβ and ERα were identified (**2a**−**k**), where six compounds had potency at the submicromolar level (**2a**−**f**) and three compounds showed certain ERβ selectivity (**2c**, **2f**, and **2i**). Five antagonists for both subtypes were also discovered, which showed inhibitory activities on the proliferation of MCF-7 cell line, too. These compounds could be used as lead compounds for the development of new anti-breast cancer agents. By virtue of the different or opposed actions of the compounds on ERβ and ERα, including **1a**, **1b**, **2c**, **2f**, **2i**, and **3a**, they could also be used as potential molecular probes to differentiate the biological roles of the two subtypes.

Some analogues identified in the study also presented good SARs and provided us useful information to optimize the ligands. These active compounds not only provided good starting points for finding and optimizing potent ligands of ERs but also suggested that the target structures optimized through MD are applicable in VS and lead discovery.

Experimental Section

Molecular Modeling and Virtual Screening. The crystal structure of ERβ LBD in complex with Way-244 (PDB entry 1X78)[10] was used as a starting point. The average structure taken from the equilibrated stage (3−5 ns) of a 5-ns MD simulation was then prepared with the "Protein Preparation Wizard" workflow in Maestro, version 8.5.[23] The database of the SPECS (197116 compounds, March 2008, http://www.spces.net, accessed on May 20, 2009) was used to be the source for screening and prepared using Ligprep 2.1.[23] Afterward, three rounds of VS, including high throughput virtual screening (HTVS), standard precision (SP) and extra precision (XP) dockings, were adopted.

The top 200 compounds ranked by MM-GBSA were stored for visual analysis to check the docking poses and interactions between ligands and receptor. Finally, 70 compounds were selected for purchase from SPECS vendor for bioassay. The computational details are presented in the Supporting Information.

Chemistry. The purity of 18 hit compounds discussed in the text was determined by HPLC equipped with a ZORBAX RX-C18 column (250 mm × 4.6 mm, 5 μm particle size) and a UV/vis detector setting of λ = 254 nm. All compounds were eluted with the two solvent systems (CH_3OH as organic phase in method I and CH_3CN as organic phase in method II) listed in Table S1 at a flow rate of 1 mL/min. HPLC analysis confirmed ≥95% purity for the tested compounds.

In Vitro Agonistic and Antagonistic Activities Assay. To evaluate the activity and selectivity of the compounds, Y2H assays of ERα and ERβ with the steroid receptor coactivator-1 (SRC 1) were carried out as described previously.[20,24] Yeast

strain AH109 was transformed with pGADT7-SRC 1 (amino acid residues 613−773) and pGBKT7-ER LBD (amino acid residues 301−553 of ERα and 248−510 of ERβ). Yeast transformants were incubated with compounds for 24 h, and in antagonist assays 1 nM E2 was added. The quantitative α-galactosidase activity assays were determined according to the Clontech manual.

Cell Transfection and Reporter Gene Assays. In this study, CHO-K1 cells were maintained in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) with 10% charcoal stripped FBS without phenol red at 37 °C in 5% CO_2. Cells were co-transfected with pGL2-ERE3-luc and ER expression plasmid pRST7-ERα or pCMV5-ERβ. A Renilla luciferse gene pRL-SV40 was also co-transfected as internal control. Cells were incubated with different concentrations of compounds, and luciferase activity was measured with dual-luciferase assay system according to the Promega manual.

MTT Assays. Cell proliferation was quantified by MTT assay as previously described.[18] Human MCF-7 breast cancer cells were cultured using the same conditions as CHO-K1 cells. Cells were seeded at a density of 1.5×10^4 in a 96-well plate in the presence of compound solution at a final concentration of 100 μM. After incubation for 24 h, 20 μL of 5 mg/mL MTT was added and the plate was incubated for a further 4 h. Then the converted dye was dissolved in 100 μL of DMSO and the absorbance was measured at 570 nm.

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Supporting Information Available: Computational details, HPLC data, structures, MD simulation results, docking poses, and dose response results. This material is available free of charge via the Internet at http://pubs.acs.org.

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