

Letters

Structure-Based Virtual Screening for Plant-Based ER β -Selective Ligands as Potential Preventative Therapy against Age-Related Neurodegenerative Diseases

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Abstract: ER β has been associated with estrogen-induced promotion of memory function and neuronal survival. Based on the optimized complex structure of human ER β LBD bound with genistein, computer-aided structure-based virtual screening against a natural source chemical database was conducted to determine the occurrence of plant-based ER β -selective ligands. Twelve representative hits derived from database screening were assessed for their binding profiles to both ERs, three of which displayed over 100-fold binding selectivity to ER β over ER α .

Two nuclear receptors for estrogen (ERs), ER α and ER β , have been identified. In the central nervous system, both ER α and ER β are expressed in the hippocampus and cortex of rodent and human brains.^{1,2} Our previous studies have demonstrated that both ER α and ER β can equivalently promote neuronal survival by activating estrogen mechanisms of action in rat hippocampal neurons.³ In addition, increasing evidence indicates that ER β is a key requirement for activation of mechanisms that underlie estrogen-inducible neuronal morphological plasticity, brain development, and cognition.^{4–6} ER α , on the other hand, is more predominant in mediating the sexual characteristics of estrogen effects in the reproductive organs such as breast and uterus.^{1,7} Taken together, these data establish a potential therapeutic application for ER β as a pharmacological target to promote memory function and neuronal defense mechanisms against age-related neurodegeneration such as Alzheimer's disease (AD), while avoiding activating untoward estrogenic proliferative effects in the breast and uterus, although this might be at the cost of lower efficacy due to the lack of activation of ER α in the brain. Other potential therapeutic advantages associated with ER β include regulation of estrogen vasculoprotective action⁸ and development of interventions targeting diseases such as depression, colon cancer, prostate cancer, obesity, leukemia, and infertility.⁹ However, a potential disadvantage of an ER β -selective ligand is the lack of activation of ER α in bone, as ER α has been demonstrated to mediate estrogen regulation of bone density.⁷

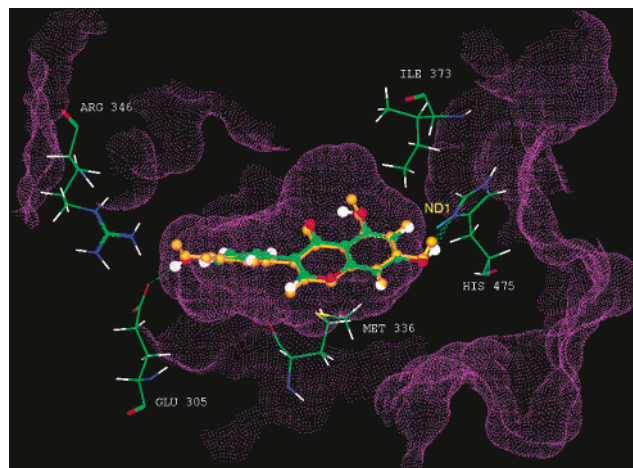


Figure 1. Comparison of experimentally observed binding mode (colored in atom types) and GOLD solution (colored in orange) of genistein in the ER β binding site. ER β is depicted as solvent accessible surface, and only a few important residues are highlighted. Hydrogen bonds are labeled in dashed green lines.

The ligand binding domains of the human ER α and ER β are approximately 60% homologous.¹⁰ Structural modeling and mutational analyses indicate that two variant amino acid residues along the ligand binding pocket, Leu 384 and Met 421 in ER α , which are replaced with Met 336 and Ile 373, respectively, in ER β (see Figure 1), are the key molecular constituents underlying discriminative binding of selective ligands to either receptor subtypes.^{11,12} This slight structural variance serves as the foundation for both design and discovery of ER specific ligands. Meanwhile, the similarities in the chemical features of both pairs of residues presents a substantial challenge to discover a selective ligand based on this difference. Of the known natural source ER β -selective ligands, genistein remains the most selective. However, an increasing number of synthetic compounds are emerging showing greater selectivity than genistein for ER β , as evidenced by the compound DPN developed in Katzellenebogen's laboratory.¹³ Computer-aided structure-based virtual database screening provides an efficient approach to rationally highlight a small group of lead candidates from a large number of compounds for investigation at the bench.

In our preliminary work, we have pursued both direct receptor-based molecular docking and indirect ligand-based pharmacophore mining approaches focusing on the search for the plant-based ER β -selective ligands. Here, briefly summarized, is one portion of our efforts in conducting the ER β 3D structure-based virtual screening of a natural source chemical collection.

All computational work was performed on a SGI Octane workstation equipped with the IRIX 6.5 operating system (Silicon Graphic Inc.). First, the 3D crystallographic structure of human ER β LBD complexed with genistein was downloaded from the Protein Data Bank

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(PDB ID: 1QKM).¹⁴ The complex structure was fixed and energy minimized with the Accelrys molecular modeling software package InsightII 2000 (Accelrys Inc.). An in-house 2D natural source chemical collection containing approximately 25 000 plant-based natural molecules or derivatives was converted to a 3D multi-conformational database with the Accelrys modeling software package Catalyst 9.8 (Accelrys Inc.).

The receptor-docking site was defined based on the binding position of genistein in the receptor and specified as all atoms within 10 Å of the center carbon of genistein. GOLD 2.0 (Genetic Optimization for Ligand Docking),¹⁵ an automated ligand docking program distributed by CCDC (Cambridge Crystallographic Data Center), was applied to calculate and rank the molecules based on their complementarities with the receptor binding site, on both geometrical and chemical features.

Prior to the database screening, initial validation using genistein as the test ligand was conducted. The aim of the validation test was to evaluate the effectiveness of the algorithm of the docking program in identifying the experimentally observed binding mode of the ligand in the receptor, to determine whether the program is applicable to the specific target system in this study. In addition, the validation test was used to determine the optimal parameter settings for the later database screening. Twenty docking runs were carried out on the test complex, using the fastest default generic algorithm parameters optimized for virtual library screening, and the GoldScore fitness function was applied. The validation test demonstrated that, based on the specified parameter settings, GOLD was effective in capturing the contributive hydrogen bond donor (ND1 in His 475) crucial to the binding and reproducing the nearly coincident solution in terms of both the binding orientation and conformation of genistein as observed in the experimental measurement (see Figure 1). The root-mean-square (RMS) deviations were computed between the observed experimental position and the GOLD solutions, with RMSD 0.3299 and 0.4483 compared to top-ranked and worst solutions, respectively. The average RMSD of all solutions was 0.3566, which is regarded as a *good* prediction based on the subjective classifications defined by the program developer (refer to the program manual), suggesting that this program is reliable and applicable to the database screening toward ER β .

Using the parameter settings determined in the validation test, the 3D natural source chemical database was input and docked into the prepared ER β binding site in a flexible docking manner (full ligand and partial protein) and scored based on the GoldScore fitness function. Five hundred resultant top-scoring molecules were filtered via visual screening in the context of the receptor in InsightII. Based on visual analysis, 100 molecules underwent further analysis by Affinity, a more complex and predictive ligand docking program to refine the binding modes predicted by GOLD. The criteria used for the selection of candidate molecules for investigation included the following (a) formation of hydrogen bond with donor atom ND1 in His 475; (b) hydrophobic and hydrophilic balance appearing in the structure (e.g., the molecule should potentially have two relatively hydrophilic sides and a hydrophobic center

to enhance both the steric and electrostatic complementarity with the receptor); (c) bound pose of the molecule in the receptor; and d) structural diversity. Finally, molecules that met the above criteria were computationally predicted for their drug-likeness (Lipinski's Rule of Five) and blood-brain barrier (BBB) penetration properties.

As a result, 31 molecules that can form a hydrogen bond with ND1 in His 475 were selected and grouped into three categories based upon the chemical features that favored both the van der Waals (VDW) contact (the number of the rings in the structure) and electrostatic interactions (the number of the hydrogen bonds) with the receptor. In addition, 10 molecules that have strong VDW interactions with the receptor, but without contributive hydrogen bonding, were grouped in Category IV. These molecules contain three or four five- or six-membered rings in their structures that could promote the hydrophobic interactions with the center of the receptor binding site as observed in endogenous estrogen 17 β -estradiol that consists of four rings in its structure and binds to the estrogen receptor with a high affinity.

The binding affinity and selectivity of candidate molecules yielded from database screening were determined by a fluorescent polarization competitive binding assay using purified baculovirus-expressed human ER α or ER β and a fluorescent estrogen ligand EL Red (PanVera Corp.). Test molecules were serially diluted to a 2 \times concentration in assay buffer (200 μ M to 200 pM). Fifty microliters of preincubated 2 \times complex of ER α (30 nM) or ER β (60 nM) and EL Red (2 nM) was added to each well in a 96-well Non-binding Surface black microplate (Corning Life Sciences) for a final volume of 100 μ L. Negative controls containing ER and EL Red (equivalent to 0% inhibition) and positive controls containing only free EL Red (equivalent to 100% inhibition) were included. After a 2-h incubation period at room temperature, the polarization values were measured using a Tecan GENios Pro reader at 535 nm/590 nm excitation/emission and plotted against the logarithm of the test molecule concentration. IC₅₀ values (concentration of test molecule that displaces half of the EL Red from ER) were determined from the plot using a nonlinear least-squares analysis. Figure 2 presents the competition binding curves of four known ER ligands for both ER α and ER β . The IC₅₀ determined for these ligands from the binding curves are consistent with the previously reported values using alternative methods such as radioligand assay, demonstrating the reliability of this assay in determining the binding profiles of small molecules to both ERs. Table 1 summarizes the IC₅₀ binding results of test molecules to both ER α and ER β as well as the binding selectivity of representative molecules selected from four categories.

As expected, the negative control steroid, progesterone, does not bind to either ER. As a positive natural source estrogen control, genistein was found to bind to ER β with a 47.2-fold greater binding selectivity over ER α , but at an affinity one-fourth of 17 β -estradiol. Among 12 molecules tested, five molecules, **1**, **2**, **5**, **7**, and **8**, showed binding selectivity to ER β over ER α , 3 of which, **2**, **5**, and **8**, displayed the selectivity over 100-fold. Preliminary structure and binding activity rela-

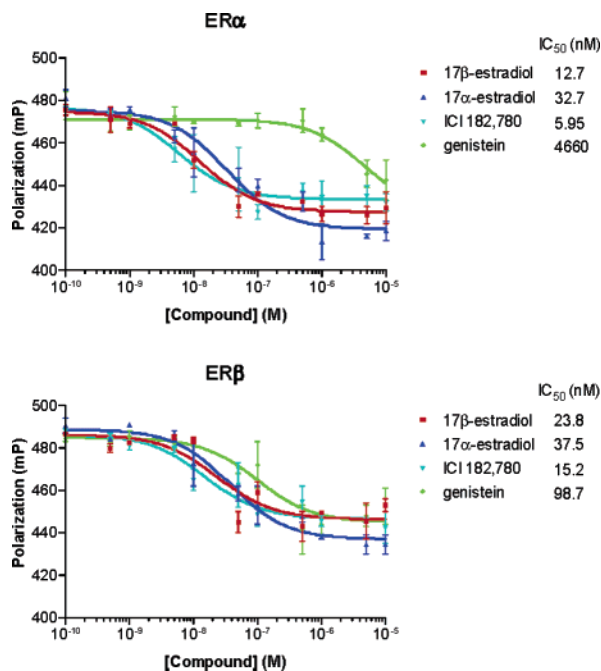


Figure 2. Competition binding curves of ER ligands for both ER α and ER β . The concentration of a test ligand resulting in a half-maximum shift in polarization value equals its IC₅₀. Curve fitting was performed using Prism 4.0 from GraphPad Software, Inc.

tionship analyses revealed that both the central hydrophobic skeletal structure and the connected two polar ‘arms’ contribute to the binding affinity of ligands to both ERs. The enhanced VDW contact derives mainly from the central hydrophobic feature of the molecule. For example, the number of rings increases the binding affinity of molecules to the receptor, as indicated by the VDW value of 17 β -estradiol (−67.98) versus that of genistein (−60.75) and molecule **9** (−58.04), which are well correlated with their order-different binding affinities. Meanwhile, the hydrogen bonds derived from the two polar ‘arms’ of the molecule are essential for the binding as well. The lack of one ‘arm’ of the hydrogen bond, as represented by molecule **4** and **6**, or two ‘arms’, as represented by **10** and **12**, even though the latter two molecules can elicit strong VDW interactions with the receptor, with the VDW value of −72.58 and −69.19, respectively, leads to either very weak or no binding. With respect to the binding selectivity, as demonstrated in the modeling complex structures of a synthetic ER α -selective agonist, PPT,¹⁶ and a synthetic ER β -selective agonist, DPN,¹³ with both ERs,¹¹ relatively larger molecular size favors the binding selectivity for ER α over ER β , as represented by molecule **3** and **11**. These analyses shed light on the future search and design of more active and selective ER subtype-selective ligands. Further, 3 out of 12 representative molecules yielded from database searching displayed over 100-fold selectivity toward ER β over ER α , indicating the effectiveness of this computer-aided virtual screening approach applied in the present study in the discovery of potential molecules that preferentially interact with ER β . However, it should be noted that the ER β agonist versus antagonist feature of the identified selective molecules requires further neurobiological evaluation.

Table 1. Binding Affinity (IC₅₀) and Selectivity of Representative Molecules for Estrogen Receptor α and β

Compd	Structure	IC ₅₀		Selectivity (ER α /ER β)
		ER α	ER β	
Progesterone		NC*	NC	
genistein		4.66 μ M	98.7 nM	47.2
1		75.7 nM	18.6 nM	4.07
2		NC	0.68 μ M	> 100
3		120 nM	250 nM	0.48
4		NC	NC	
5		NC	2.80 μ M	> 100
6		NC	NC	
7		85.7 μ M	43.0 μ M	1.99
8		NC	4.48 μ M	> 100
9		NC	NC	
10		NC	NC	
11		2.32 μ M	NC	<0.01
12		NC	NC	

*NC: Nonconvergence within the dose range, predicting that either the molecule does not bind to the receptor or that the binding affinity is very low, with an IC₅₀ greater than 1 mM.

Estrogen/hormone therapy (ET/HT) has been associated with the reduced risk of developing AD when treated at the menopausal transition in women.¹⁷ For example, results of the Cache County Study indicate that women who receive ET/HT at the time of menopause and continue for 10 years have a 3-fold lower risk of developing AD,¹⁸ whereas the recent data from the Women’s Health Initiative Memory Study indicate that women who begin the therapy late in menopause have a greater risk of developing AD.^{19,20} These clinical observations are consistent with our basic science analyses of estrogen-inducible molecular mechanisms in the brain, indicating a healthy cell bias of estrogen action.¹⁷

Consistently, a recent clinical trial of phytoestrogens reported that a soy protein supplement containing a complex formulation of isoflavones did not improve cognitive function in postmenopausal women when treated at the age of 60 years or older,²¹ further

indicating that when started 10 or more years following menopause in postmenopausal women when age-related neuronal reorganization has taken place, ET/HT has no benefit on neural function.^{18–20} Another issue that can substantially impact the efficacy of a mixture of phytoestrogens action in the brain is the formulation of phytoestrogens, since we have found that when administered alone, a number of phytoestrogens were protective to neurons from neurodegenerative insults.²² Gustafsson et al. indicate that ER α and ER β have a yin/yang relationship in many contexts where one receptor may antagonize the actions of the other.²³ Our studies further confirmed this observation, showing that coadministration of ER α -selective agonist PPT and ER β -selective agonist DPN was less efficacious than either PPT or DPN alone in protecting hippocampal neurons against excitotoxic insults.³ Based on this analysis, a presumption can be made that the ineffectiveness of administering a mixture of phytoestrogens (i.e. a soy protein supplement) may partly come from the antagonizing actions among different phytoestrogens, which may be ER α selective or ER β selective. Therefore, the efficacy of a single ER specific phytoestrogen, in particular the ER β specific phytoestrogen in the brain, remains to be determined. The plant-based molecules identified in this study serve as important probing molecules for this purpose.

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