**Regular Article**

**Discovery of Novel Selective ERα/ERβ Ligands by Multi-pharmacophore Modeling and Virtual Screening**

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Estrogen receptor α (ERα) and estrogen receptor β (ERβ) regulate different sets of gene expression, and have different ligand responses, which make the estrogen tissue-specific. Thus, the estrogen receptor (ER) subtype-selective ligands can improve the target-site selectivity and decrease the off-target effect. In order to discover the selective ER subtype ligands with novel scaffolds, in this work three-dimensional (3D) pharmacophore models of the ERα ligands (Hypo 1) and the ERβ ligands (Hypo 2) were established (correlation coefficients were 0.959 and 0.966) and validated (R²=0.936 and 0.879; enrichment factors (EFs) at 2% were 16.2 and 8.4; areas under the concentration–time curve (AUC) of the receiver operating curve (ROC) were 0.88 and 0.91) using the Discovery Studio 4.0 software package. Hypo 1 and Hypo 2 were then employed for virtual screening and ten hits were found as potential candidate leads. Based on their ERα/ERβ binding affinity results by fluorescence polarization technology, two of these leads, AH-262/34334025 (AH) and AG-670/08803023 (AG) with novel scaffolds were identified as selective ERα ligands. A molecular docking study was also performed, which provided the explanation for the ER subtype preferences for AH and AG.

**Key words** estrogen receptor ligand; pharmacophore model; virtual screening

Estrogen exerts its cascade of biochemical events through estrogen receptors (ERs). Mainly, there are two ER subtypes, estrogen receptor α (ERα) and estrogen receptor β (ERβ). ERα predominates in the uterus and the mammary gland, thus being mostly responsible for the female reproductive functions, whereas ERβ plays roles in many other body compartments, such as the central nervous, urogenital and immune systems. ERα and ERβ regulate different sets of gene expression, and have different ligand responses, which make the estrogen tissue-specific. For instance, since estrogen can promote growth in breast cancers via the activation of ERα, ERα antagonists can be used for treating breast cancer. An ERβ-selective ligand may promote ER activity in the bone without promoting tumorigenesis in the breast. Furthermore, ER subtype ligands could present opposite effect in ovarian. ERα antagonist could suppress the cell growth in ovarian cancer cell lines SKOV3 and OV2008, whereas ERβ antagonist could significantly enhance cell growth. Therefore, subtype-selective ligands can improve the target-site selectivity and decrease the off-target effect.

Until now, many ER ligands have been reported. Most of them (such as 1, 3, 5, 8, 48 in Fig. 1) have the similar scaffold of diphenylethane or diphenylpropylene. In this work, we focused on discovering the lead compound with new scaffold. Pharmacophore model-based virtual screening is a common fast way to find the lead compounds. Therefore, pharmacophore models of the ERα and ERβ ligands were built and employed for discovering selective ER subtype ligands (Fig. 2).

**Experimental**

**Data Preparation** Sixty-nine positives with binding affinities (range from 0.3 to 7980 nmol) of ERα and seventy-one positives with binding affinities (range from 0.5 to 27500 nmol) of ERβ for the pharmacophore model study were taken from the literatures, and one thousand two hundred negatives were retrieved from the Available Chemical Directory database using Random Percent Filter protocol by Pipeline Pilot software. The training set including 16–25 compounds with 3–4 different scaffolds and more than 1000-fold activity range would properly generate the suitable pharmacophore model by Discovery Studio 4.0 software package (Accelrys Software Inc., DS, U.S.A.). Therefore, all the compounds were divided into training set and test set. The training set α for the ERα model consisted of nineteen positives (compounds 1–19, Fig. 3), while the training set β for the ERβ model consisted of twenty-one positives (compounds 10, 11, 20–38, Fig. 4). The test set α consisted of fifty positives (compounds 39–88, Figs. 5, 6) and the test set β consisted of fifty positives (compounds 89–128, Figs. 7, 8). Both were used for the test set prediction method validation of the ERα and ERβ models. The test set α and β, both of which were used for the enrichment factor (EF) method and the receiver operating curve (ROC) method validation of the models respectively, consisted of fifteen positives (compounds 39–52, 85 for ERα and compounds 85–99 for ERβ) and one thousand two hundred negatives. All compounds were optimized by Discovery Studio 4.0 software package.

**The Validation of the Pharmacophore Model** Three validation procedures were followed: test set prediction method, EF method and ROC method. Both positives and negatives in test set were predicted by the pharmacophore models. We employed the DS/Ligand Pharmacophore Mapping protocol to estimate the test set with the default values except the flexible fitting method and the best conformation generation.

Test set prediction method used the relationship between the actual activities and the predicted activities, which generated pharmacophore hypotheses to predict positives of test set to evaluate the validation of the models.

EF at a given percentage, the simplest and most commonly used method, is defined as:

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where TP is the number of true positives, FP is the number of false positives, N is the number of the total compounds and n is the number of the total positive compounds.

Moreover, the ROC method, allowing quick calculation of sensitivity and specificity from a comparison between in vitro and in silico, was also applied to evaluate the validation of the models. Area under concentration–time curve (AUC) values of ROC would be obtained by the following three steps:

a) Sensitivity (Se): the likelihood that an event will be detected if that event is present. Se is defined as:

$$\text{Se} = \frac{TP}{TP + FN}$$

where FN is the number of false negatives.

b) Specificity (Sp): the likelihood that the absence of an event will be detected. Sp is defined as:

$$\text{Sp} = \frac{TN}{TP + FP}$$

where TN is the number of true negatives.

c) The ROC is a function of (1−Sp) versus the Se, and the
area under the ROC \((AUC)\) is the important way of measuring the performance of the test.

\[
AUC = \sum_{x=2}^{N} Se(x)[(1 - Sp)(x) - (1 - Sp)(x-1)]
\]

Here, \(Se(x)\) is the percent of the true positives versus the total positives at rank position \(x\), \(1 - Sp(x)\) is the percent of the false positives versus the total negatives at rank position \(x\).

**Database Screening** The pharmacophore Hypo 1 and Hypo 2 were used as 3D queries to screen the Specs database and the Drug Bank-approved database which consisted of 206615 and 1491 small molecules, respectively. The databases were converted into the DS database, which consisted of a maximum of 255 conformations of each compound. Then DS/ Ligand Pharmacophore Mapping protocol was used to estimate compounds with flexible fitting method.

**Estrogen Receptor Binding Affinity Assay** Tamoxifen was purchased from International Laboratory. The binding affinities were assessed by the fluorescence polarization technology using a Synergy 2 SLFPA MODEL Multi-Detection Microplate Reader (Bio Tek Instruments). The \(IC_{50}\) values of \(17\beta\)-estradiol and test compounds were determined by inhibiting the binding of the fluorescent estrogen ES2 (Invitrogen) to the isolated recombinant human ER\(\alpha\) or ER\(\beta\) (Invitrogen) by 50%. These values were used to normalize as relative binding affinity (RBA). Curve fitting was performed by using GraphPad Prism\textsuperscript{®} software from GraphPadTM Software Inc.
Results and Discussion

Building of the Pharmacophore Models  One hundred twenty-eight compounds with ER affinities and HypoGen algorithm in DS/3D pharmacophore generation protocol were employed to generate pharmacophore models of the ERα and ERβ ligands. Both of the Conformation Generation and Fitting parameters were set as the “BEST” method. Then the top 10 scoring hypotheses for the ERα and ERβ ligand models were output respectively. On the basis of analysis, Hypo 1 for ERα and Hypo 2 for ERβ, both of which consisted of two hydrogen bond acceptors and two hydrophobic chemical features, were the best pharmacophore models (Hypo 1: Δcost = 42.505, correl = 0.959, Config = 16.773; Hypo 2: Δcost = 41.952, correl = 0.966, Config = 15.137). The statistical relevance of a generated hypothesis was estimated by comparing its Δcost values, a value of which between 40 and 60 would suggest a 75–90% probability for the experimental and predicted activity correlation. In other words, the Δcost values of Hypo 1 and Hypo 2 suggested that both of them would be the reliable pharmacophore models. Another important parameter for pharmacophore models is correl, the maximum value of which is 1, and the correl values of Hypo 1 and Hypo 2 also gave us the confidence of these models (Figs. 9, 10).

The Validation of the Pharmacophore Models  Every model should be ascertained, and three validation procedures (test set prediction method, EF method and ROC method) were employed to validate Hypo 1 and Hypo 2. For Hypo 1, the result of the test set prediction method validation by using the test set α1 is shown in Table 1. As shown in Fig. 11, the R value of the plot of the linear regression between the experimental and predicted activities for the test set α1 is 0.936, which means the predicted activities by Hypo 1 is close with the experimental activities respectively. EF at a given percentage of the database is the most commonly used and simplest validation method. The EFs of the test set α2 consisting of one thousand two hundred and twenty compounds screened by Hypo 1 at 2, 5 and 10% are 16.2, 14.6 and 8.1, respectively. Furthermore, the ROC of the test set α2 screened by Hypo 1 is shown in Fig. 12, and the AUC is 0.88 which means that a randomly selected active compound has a higher score than
a randomly selected inactive compound 8.8 times out of 10. The pharmacophore model Hypo 2 was also validated by three validation procedures (test set prediction method validation results are shown in Table 2 and Fig. 13, and $R$ value is 0.879, EF screened by Hypo 2 at 2% is 8.4, ROC is shown in Fig. 14 and $AUC$ is 0.91). The results of these three validation procedures suggested that the Hypo 1 and Hypo 2 would be valuable and reliable in identifying the compounds for the ER$\alpha$ and ER$\beta$ binding affinities.

**Virtual Screening** Virtual screening of small molecule
libraries is an important and rapid approach to discover the lead compounds. Database search using the DS/3D query represented by the Hypo 1 retrieved 31430 hits from the Specs database and the Drug Bank-approved database. According to the combination of Lipinski’s rule of five, these hits were then filtered by the Hypo 2. Finally, ten hits with high predicted values were selected for further biological test (Fig. 15). As shown in Table 3, the estimated IC$_{50}$ values for ER$\alpha$ range from 0.146 to 37.591 nM, whereas for ER$\beta$ range from 13.929 to 11832.9 nM. The fact that the RBA (relative binding affinity) ER$\beta$/ER$\alpha$ values of these ten hits are more than 20 indicated that they would be the potential selective ER$\alpha$ ligands.

**Biological Activities** The binding affinities of these ten hits on ER were determined by the fluorescence polarization technology using estrogen as the positive compound. As shown in Table 3, three hits exhibit potent ER$\alpha$ binding affinity whereas only one of them can bind to ER$\beta$. These results indicated that hits AH and AG showed the potent activity to
ERα and high selectivity over ERβ which could be used as leading compounds for further optimization.

**Molecular Docking** In an attempt to understand the molecular interactions between ligands and ERα/ERβ, a molecular docking study was performed using the Discovery Studio 4.0/CDOCKER and Dynamics protocols. The crystal structures of estrogen/ERα complex (PDB ID: 1A52) and estrogen/ERβ complex (PDB ID: 3OLS) were used as the templates. Based on methods within CHARMM to sample side-chains and ligand conformations, the side-chains of amino acids in the ligand-binding domain were allowed to move during the docking in an induced fitting model. The docking model was first validated by the redock method which used estrogen to dock into ERα (PDB ID: 1A52). During the docking and subsequent scoring, Best Conformation Method was used for the Generate Ligand Conformation parameter. As shown in Fig. 16a, the amide group of AH forms a hydrogen bond interaction with ERα/His524 (the distance of the hydrogen bond is 2.43 Å) which is one of the most important amino acids in the binding mode between estrogen and ERα. An additional hydrogen bond is also observed between AH and Thr347 with a distance of 2.50 Å. Similarly, AG also makes the hydrogen bond network with His524 and a water molecule. (Fig. 16b) In contrast, AH and AG cannot form any hydrogen bond with ERβ residues. (Figs. 16c, d) These results could provide partial explanation of the ER subtype preferences for AH and AG.

Furthermore, the result of the docking energy could also give us some hints for ER subtype preferences. AH and AG exhibited similar docking energy to ERα (ΔG_{AH} = −34.21 kcal/mol, ΔG_{AG} = −27.4 kcal/mol) compared to the estrogen (ΔG_{Estrogen} = −88.53 kcal/mol). When docking to ERβ, however, a significant increase of the docking energy could be observed (ΔG_{AH} = 11.49 kcal/mol, ΔG_{AG} = 43.89 kcal/mol). As we reported before,7 the binding cavity size of ERβ is slightly smaller than that of ERα. Since AH is a small molecule with 19 heavy atoms (molecule weight = 299), the increase of the docking energy might be due to the loss of the hydrogen bonds.

**Fig. 7. The Structure of the Test Set β1 (Part 1)**
bonds compared to ERα. For AG, which has 32 heavy atoms (molecule weight=492), only the distorted molecule can dock to the ERβ binding cavity, increasing the docking energy to 43.89 kcal/mol, which was greater than 0. It meant that AG could not dock to the ERβ binding cavity actually.
Table 1. Experimental and Predicted Activities of the Test Set $\alpha_1$ Based on the Pharmacophore Model Hypo 1

| Compound | Experimental $IC_{50}$ (nM)* | Predicted $IC_{50}$ (nM) | Compound | Experimental $IC_{50}$ (nM)* | Predicted $IC_{50}$ (nM) |
|----------|-------------------------------|--------------------------|----------|-------------------------------|--------------------------|
| 39       | 0.3                           | 0.122                    | 64       | 3                             | 10.075                   |
| 40       | 0.51                          | 0.244                    | 65       | 14.4                          | 11.152                   |
| 41       | 1.2                           | 0.378                    | 66       | 17.3                          | 24.491                   |
| 42       | 1.2                           | 0.645                    | 67       | 16.2                          | 13.597                   |
| 43       | 1.1                           | 0.819                    | 68       | 928                           | 519.813                  |
| 44       | 3.1                           | 1.169                    | 69       | 2655                          | 853                      |
| 45       | 4.1                           | 1.211                    | 70       | 25.5                          | 16.096                   |
| 46       | 6.4                           | 4.494                    | 71       | 171                           | 535.317                  |
| 47       | 1                             | 2.13                     | 72       | 61.9                          | 63.723                   |
| 48       | 4.7                           | 2.364                    | 73       | 1083                          | 305.452                  |
| 49       | 8.8                           | 2.59                     | 74       | 35                            | 14.717                   |
| 50       | 5                             | 2.782                    | 75       | 30                            | 14.43                    |
| 51       | 8.4                           | 3.311                    | 76       | 1300                          | 813.931                  |
| 52       | 5.8                           | 3.757                    | 77       | 1300                          | 818.763                  |
| 53       | 7.7                           | 9.335                    | 78       | 16                            | 15.038                   |
| 54       | 1.2                           | 4.76                     | 79       | 8.3                           | 14.057                   |
| 55       | 9.9                           | 4.928                    | 80       | 11                            | 9.53                     |
| 56       | 3.3                           | 5.837                    | 81       | 14.9                          | 9.69                     |
| 57       | 3.3                           | 6.024                    | 82       | 10                            | 12.372                   |
| 58       | 6.5                           | 9.954                    | 83       | 6.2                           | 13.492                   |
| 59       | 9.3                           | 8.662                    | 84       | 50                            | 83.686                   |
| 60       | 11.3                          | 8.734                    | 85       | 6.7                           | 1.71                     |
| 61       | 12.3                          | 8.877                    | 86       | 3                             | 6.538                    |
| 62       | 7.6                           | 9.197                    | 87       | 16                            | 15.674                   |
| 63       | 9.3                           | 10.931                   | 88       | 8.77                          | 13.268                   |

* All the data come from the literatures. 7–32)

Fig. 11. Plot of Linear Regression between Experimental and Predicted Activities for the Test Set $\alpha_1$

Table 2. Experimental and Predicted Activities of the Test Set $\beta_1$ Based on the Pharmacophore Model Hypo 2

| Compound | Experimental $IC_{50}$ (nM)* | Predicted $IC_{50}$ (nM) | Compound | Experimental $IC_{50}$ (nM)* | Predicted $IC_{50}$ (nM) |
|----------|-------------------------------|--------------------------|----------|-------------------------------|--------------------------|
| 79       | 1100.0                        | 738.0                    | 104      | 611.0                         | 145.7                    |
| 80       | 43.0                          | 185.4                    | 105      | 143.0                         | 149.8                    |
| 81       | 234.0                         | 165.0                    | 106      | 433.3                         | 157.4                    |
| 82       | 50.5                          | 70.3                     | 107      | 137.0                         | 170.7                    |
| 83       | 183.0                         | 191.1                    | 108      | 5500.0                        | 32951.5                  |
| 84       | 180.0                         | 286.5                    | 109      | 1833.0                        | 1126.0                   |
| 85       | 4.6                           | 5.0                      | 110      | 3910.0                        | 2419.3                   |
| 86       | 34.0                          | 84.3                     | 111      | 125.0                         | 199.6                    |
| 87       | 105.0                         | 90.9                     | 112      | 262.0                         | 202.9                    |
| 88       | 46.0                          | 75.5                     | 113      | 309.0                         | 215.0                    |
| 89       | 0.5                           | 1.4                      | 114      | 168.8                         | 235.4                    |
| 90       | 232.1                         | 126.0                    | 115      | 161.8                         | 239.3                    |
| 91       | 5.0                           | 10.1                     | 116      | 374.6                         | 273.1                    |
| 92       | 15.0                          | 15.3                     | 117      | 113.0                         | 295.5                    |
| 93       | 86.0                          | 42.5                     | 118      | 1428.0                        | 301.0                    |
| 94       | 16.0                          | 46.1                     | 119      | 80.3                          | 333.0                    |
| 95       | 35.5                          | 111.4                    | 120      | 1100.0                        | 342.5                    |
| 96       | 73.0                          | 65.9                     | 121      | 1494.3                        | 353.1                    |
| 97       | 70.0                          | 134.8                    | 122      | 1000.0                        | 379.8                    |
| 98       | 54.2                          | 58.7                     | 123      | 137.0                         | 460.1                    |
| 99       | 60.0                          | 80.9                     | 124      | 303.7                         | 496.0                    |
| 100      | 100.0                         | 105.7                    | 125      | 90.3                          | 629.4                    |
| 101      | 57.9                          | 131.0                    | 126      | 2750.0                        | 2572.7                   |
| 102      | 275.0                         | 119.6                    | 127      | 343.0                         | 890.3                    |
| 103      | 79.0                          | 140.7                    | 128      | 5500.0                        | 1050.0                   |

* All the data come from the literatures. 7–32)

Fig. 13. Plot of Linear Regression between Experimental and Predicted Activities for the Test Set $\beta_1$

Fig. 12. The ROC Curve of Hypo 1
The curve with square dots is the ROC of the test set $\alpha_2$. The curve with triangle dots is the ROC of the random classification of the compounds.

Fig. 14. The ROC Curve of Hypo 2
The curve with square dots is the ROC of the test set $\beta_2$. The curve with triangle dots is the ROC of the random classification of the compounds.
Conclusion
In conclusion, a virtual screening platform, which consisted of two pharmacophore models of the ERα ligands and the ERβ ligands, were established to discover novel selective ER subtype ligands. After being ascertained by three validation methods, these two pharmacophore models were used for virtual screening, and ten hits were found and their ER subtype affinities were further evaluated. The fact that two hits with novel scaffolds had the ERα preferment also certified the reliability of these two pharmacophore models. This work may be contributed to the identification and design of novel selective ERα/ERβ ligands, and compound AH can be further optimized as a selective ERα lead.

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Conflict of Interest The authors declare no conflict of interest.

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