Rational questing for inhibitors of endothelin converting enzyme-1 from \textit{Salvia miltiorrhiza} by combining ligand- and structure-based virtual screening

Xing Wang, Yuhong Xiang, Zhenzhen Ren, Yanling Zhang, and Yanjiang Qiao

Abstract: In this study, a virtual screening approach based on pharmacophore and molecular docking was proposed to identify endothelin converting enzyme-1 (ECE-1) (EC 3.4.24.71) inhibitors from \textit{Salvia miltiorrhiza}. First, the pharmacophore models were generated to recognize the common features of the ECE-1 inhibitors. The models were validated by a test database composed by a set of compounds known as ECE-1 inhibitors and nonactive compounds and proven to be successful in discriminating active and inactive inhibitors. Then, the best pharmacophore model was used to screen the compounds from \textit{S. miltiorrhiza}. Furthermore, the Surflex-Dock procedure was used for molecular docking. All compounds from \textit{S. miltiorrhiza} were docked into the active site of the target protein. An empirical scoring function was used to evaluate the affinity of the compounds and the target protein. Comparing the virtual screening results based on pharmacophore and molecular docking, respectively, 11 communal compounds with higher QFIT and docking score were hit, and the activity of some compounds was validated in the literature. The binding modes between these compounds and the ECE-1 binding site were predicted and used to identify the key interactions that contribute to the inhibitory activity of ECE-1 activity. The results show that the two methods have good consistency and can be validated and supplemented with each other.

Key words: ECE-1 inhibitors, \textit{Salvia miltiorrhiza}, virtual screening, pharmacophore, molecular docking.

Introduction

Endothelin (ET), first described in 1988 by Yanagisawa as a vasconstrictor, is an important regulator of cardiovascular function.\textsuperscript{1} It is synthesized in vascular endothelial cells and acts on vascular smooth muscle cells in the form of paracrine. Hyperactivation of the endothelin system has been implicated in the pathogenesis of various cardiovascular disorders including myocardial infarction, restenosis, hypertension, heart failure, and Chagas cardiopathy.\textsuperscript{2} ET mediates its effects through two distinct G-protein-coupled receptor subtypes, ETA and ETB.\textsuperscript{3–5} Three isoforms of ET (ET-1, ET-2, and ET-3) have been identified, each with one encoded by a different gene. ET-1 has the highest proportion forms of ET (ET-1, ET-2, and ET-3) have been identified, with each G-protein-coupled receptor subtypes, ETA and ETB.\textsuperscript{3–5} Three isoforms of ET (ET-1, ET-2, and ET-3) have been identified, each with one encoded by a different gene. ET-1 has the highest proportion forms of ET (ET-1, ET-2, and ET-3) have been identified, with each G-protein-coupled receptor subtypes, ETA and ETB.

ET is produced by the cleavage of its precursor big ET (BigET), which is a 38 amino acid peptide with unknown biological functions. BigET is cleaved by the highly specific metalloprotease endothelin converting enzyme-1 (ECE-1) (EC 3.4.24.71) between Trp21 and Val22 to produce the 21 amino acid bioactive ET.\textsuperscript{9} Cloning studies have shown ECE-1 to be a type II membrane-bound metalloprotease that exists as a dimer on the cell surface. The amino acid sequence of ECE-1 includes a zinc co-ordinating motif (HEXXH) and shows significant homology (40%) with human neutral endopeptidase.\textsuperscript{9} ECE-1 catalyzes the final step in the biosynthesis of ETs in a rate-limiting fashion through post-translational conversion of the biologically inactive BigETs,\textsuperscript{10} so inhibiting ECE-1 is a pivotal and effective step to reduce ET. Various attempts have been made to study the structure of ECE-1 and its inhibitor.\textsuperscript{11–13} In a recent work,
Fig. 1. Chemical structures and activities of ECE-1 inhibitors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC₅₀</th>
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<tbody>
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<td>1</td>
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<td>410 nmol/L</td>
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<tr>
<td>2</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>16</td>
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</table>
P. Ajay Babu et al. retrieved the reported inhibitors of ECE-1 as well as their IC50 values from the literature and docked their structures with ECE-1 protein using the Molegro virtual docker. The MolDock scores of each compound were calculated and subjected to graphical analysis in conjunction with their respective IC50 values to characterize potent inhibitors.

Danshen, the dry root and rhizome of *Salvia miltiorrhiza*, is one of the popular and important traditional Chinese herbs used in China and neighboring countries. This herb can improve blood microcirculation, dilate coronary arteries, and protect blood vessels from thrombosis and atherogenesis, so it is widely used for the treatment of cardiovascular diseases, inflammatory diseases, blood stasis, and neurodegeneration diseases. It has been reported that *S. miltiorrhiza* and its prescription can decrease the content of plasma ET-1 and endothelin gene expression in circulating endothelial cells for the treatment of coronary heart disease and congenital heart disease. However, the active ingredients and mechanism of *S. miltiorrhiza* are not entirely clear. In this paper, a virtual screening approach based on ligand and structure was proposed to quest for potential inhibitors of ECE-1 from *S. miltiorrhiza*. The pharmacophore model was generated to identify the critical pharmacophoric features of ECE-1 inhibitors. Meanwhile, the chemical components were subjected to molecular docking studies using Surflex-Dock, and an empirical scoring function was used to evaluate the binding capacity of each compound.

**Materials and methods**

Each step of the studies was performed with the SYBYL X-1.2 package (Tripos Inc., St. Louis, Missouri) running on a Red Hat Linux workstation. The genetic algorithm with linear assignment of hypermolecular alignment of datasets (GALAHAD) module was used to generate the pharmacophore model of ECE-1 inhibitors, the UNITY module was used to perform a flex search for the potential inhibitors based on the pharmacophore query, and the Surflex-Dock (SFXC) module was used to perform molecular docking. Traditional Chinese Medicine Database (TCMD) version 2009 was used to establish the chemical structure database of *S. miltiorrhiza*.

The studies were implemented on a series of ECE-1 inhibitors reported in the literature. The structures of ECE-1 inhibitors are listed in Fig. 1. The chemical structures were drawn and saved in SYBYL mol2 format and all of the 2D structures were converted to 3D structures with SYBYL X-1.2 software. Considering both the distribution of biological data and structural diversity, 10 compounds were selected to generate the pharmacophore model.

**Pharmacophore studies**

GALAHAD uses Tripos’ proprietary technology to generate pharmacophore hypotheses and alignments from sets of ligand
molecules that bind at a common target site. Two special algorithms named genetic algorithm (GA) and linear assignment for molecular dataset alignments (LAMDA) are included in GALAHAD. A set of ligand conformations that have an optimal combination of low strain energy (SE), steric overlap (SO), and pharmacophoric similarity (PhS) can be identified by GALAHAD. The multi-objective (MO) functions in each term (SE, SO, and PhS) are considered independently and constitute a multi-objective triage (MOTriage) approach. They are adopted to assess reproductive fitness and select which candidates should survive to the next generation by making use of the Pareto rank for each individual model. The GALAHAD models derived from the training set were compared according to Pareto ranking and a small value of SE and high values of SO and PhS were considered to be a good model.

Before establishing the model, all of the compounds were prepared as follows: the structures were checked for bond orders, hydrogen atoms were added, and a minimization procedure was implemented using the MMFF94 force-field. Ten compounds were selected as training set using the Kennard−Stone method, which could ensure the compounds of training set to be good representative and evenly distributed in a space distance. GALAHAD was run for 130 generations with a population size of 100. The rest of the parameters were set as default values. The generated models were evaluated by a test database that consisted of 101 experimentally known ECE-1 inhibitors and 310 nonactive compounds selected from the MDL Drug Data Report (MDDR) version 200712 database.

**Molecular docking**

**Preparation of the ligands**

A total of 80 compounds from *S. miltiorrhiza* were chosen from TCMD and saved as the ligands in mol2 format. The 2D structures

<table>
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<tr>
<th>Model</th>
<th>SE</th>
<th>SO</th>
<th>PhS</th>
<th>Ht</th>
<th>Ha</th>
<th>A%</th>
<th>Y%</th>
<th>N</th>
<th>CAI</th>
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<td>3668.6</td>
<td>167.2</td>
<td>118</td>
<td>58</td>
<td>0.57</td>
<td>0.49</td>
<td>2.00</td>
<td>1.15</td>
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<tr>
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<td>45.83</td>
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<td>80</td>
<td>61</td>
<td>0.60</td>
<td>0.76</td>
<td>3.10</td>
<td>1.87</td>
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<tr>
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<td>218</td>
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<td>0.75</td>
<td>0.35</td>
<td>1.42</td>
<td>1.07</td>
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<td>3104.8</td>
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<td>61</td>
<td>0.60</td>
<td>0.76</td>
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<tr>
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<td>0.60</td>
<td>2.46</td>
<td>1.85</td>
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<td>0.80</td>
<td>0.33</td>
<td>1.35</td>
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**Table 1.** Parameter values for each pharmacophore model.

<table>
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<th>Compounds hit by pharmacophore Model_015.</th>
<th>ID</th>
<th>QFIT</th>
<th>Name</th>
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<tr>
<td>14927</td>
<td>23.69</td>
<td>Monomethyl lithospermate</td>
<td></td>
</tr>
<tr>
<td>19202</td>
<td>21.12</td>
<td>Salvinolic acid B</td>
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</tr>
<tr>
<td>12926</td>
<td>17.53</td>
<td>Lithospermic acid B</td>
<td></td>
</tr>
<tr>
<td>13370</td>
<td>17.35</td>
<td>Magnesium lithospermate B</td>
<td></td>
</tr>
<tr>
<td>14580</td>
<td>15.9</td>
<td>Methyl melitrate A</td>
<td></td>
</tr>
<tr>
<td>19123</td>
<td>9.72</td>
<td>Sagecoumarin</td>
<td></td>
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<tr>
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<td>8.49</td>
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<td></td>
</tr>
<tr>
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<td>8.33</td>
<td>Salvinolic acid D</td>
<td></td>
</tr>
<tr>
<td>12420</td>
<td>7.67</td>
<td>Labiatic acid</td>
<td></td>
</tr>
<tr>
<td>13709</td>
<td>7.18</td>
<td>Melitric acid A</td>
<td></td>
</tr>
<tr>
<td>19205</td>
<td>5.52</td>
<td>Salvinolic acid E</td>
<td></td>
</tr>
<tr>
<td>19203</td>
<td>5.48</td>
<td>Salvinolic acid C</td>
<td></td>
</tr>
<tr>
<td>13709</td>
<td>5.13</td>
<td>Melitric acid A</td>
<td></td>
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</tbody>
</table>

**Fig. 3.** Pharmacophore Model_015 and molecular alignment of the compounds used to generate the model.
were converted to 3D structures in SYBYL X-1.2 software. For each ligand, the structure was checked and the hydrogen atoms were added. Then, a minimization procedure was implemented using the Tripos force-field for 1000 iterations.

Preparation of the target protein
The crystal structure of ECE-1 (2.38 Å, 3DWB.pdb) was selected as the docking template. The ligand phosphoramidon was extracted, the crystallographic water molecules in the structure were removed, and the hydrogen atoms of the modeled structure were added to define the correct configuration and tautomeric states. With the standard parameters, the modeled structure was energy-minimized using the AMBER7 F99 force-field with the Powell energy minimization algorithm, distance-dependent dielectric function, and current charges.

Docking strategy
Surflex-Dock is a well-recognized method in the field and plays a crucial role in calculating the ligand–receptor interaction. After extracting the binding ligand phosphoramidon, the structure of ECE-1 was used for redocking with phosphoramidon and the score was calculated to check the accuracy of the Surflex-Dock program. The default parameters, as implemented in the SYBYL X-1.2 software, were used. All of the compounds from S. miltiorrhiza were prepared to dock into the active site of the ECE-1. The highest scored conformation based on the Surflex-Dock scoring functions was selected as the final bioactive conformation. Then, a virtual screening tool was used that combines Hammerhead’s empirical scoring function with morphological similarity to generate putative poses of ligand fragments.

Virtual screening
To quest for the potential inhibitors of ECE-1, the pharmacophore model generated by GALAHAD was used as the query to perform a flex-UNITY search of all of the known compounds from S. miltiorrhiza. To evaluate the performance of the models, four parameters (i.e., $A\%$, $Y\%$, $N$, and CAI) were introduced and calculated as follows:

\[
A\% = \frac{Ha}{A} \times 100%
\]

\[
Y\% = \frac{Ha}{Ht} \times 100%
\]

\[
N = \frac{Ha \times D}{Ht \times A}
\]

\[
CAI = N \times A\%
\]

$D$ is the total number of compounds in the test database and $A$ is the number of active compounds. $Ht$ is the total number of hit compounds from the test database and $Ha$ is the number of active hit compounds from the test database. The relationship of four parameters is revealed in Fig. 2. $A\%$ represents the ability to identify active compounds from the test database and $Y\%$ represents...
the proportion of active compounds in hit compounds. N, the index of effective identification, is used to evaluate the activity of the models to identify active compounds from nonactive compounds. CAI, a comprehensive evaluation index, is used to identify the best pharmacophore model. The model with the highest value of CAI is considered to be the best.

Meanwhile, the compounds were automatically docked into the binding site of ECE-1 successively. A protomol-based method and an empirically derived scoring function were used to evaluate the interaction between the ligands and ECE-1. The scoring function includes hydrophobic, polar, repulsive, entropic, solvation, and crash terms, and a higher total score implies a better binding capacity. The crash value represents the degree of inappropriate penetration by the ligand into the protein and of interpenetration (self-clash) between ligand atoms that are separated by rotatable bonds. Crash scores close to 0 are favorable. Negative numbers indicate penetration. A smaller crash score indicates a better ability to exclude the false positives screened. Polar represents the contribution of the polar interactions to the total score. The polar score is useful for excluding docking results that make no hydrogen bonds.

Compared with the biological activity data reported, the results of virtual screening were analyzed comprehensively. The binding mode between the compounds and ECE-1 was identified and discussed.

**Results and discussion**

**Pharmacophore model**

**GALAHAD**

Twenty pharmacophore models were generated by using 10 ECE-1 inhibitors as a training set (compounds 3–5, 9, 11–13, and 15–17), which have good activity and drug-likeness ability according to Lipinski’s “rule of five”.

Each model represented a different trade-off among the conflicting demands of maximizing steric consensus (measured by SO), maximizing pharmacophore consensus (measured by PhS), and minimizing energy (measured by SE). All of the obtained models were derived from more than eight ligands and had Pareto rank 0, which means that no one model is superior to any other. Models 17, 16, 11, 18, and 6 had high energy (SE = 6.3 × 10^-10, 3.4 × 10^-9, 3.1 × 10^-9, 5.5 × 10^-5, and 2.2 × 10^-10, respectively), which could be due to steric clashes, leading to their exclusion from our analysis. The other 15 models were evaluated successively by the test database constructed previously. The SE, SO, and PhS values and predictive results for each model are listed in Table 1. In this study, Model_015, with the highest value of CAI, high values of SO and PhS and a low value of SE, was considered to be the best model.

Model_015 is displayed in Fig. 3 where cyan, green, magenta, and blue spheres (color in inline version only) indicate hydrophobes, HB acceptors, HB donors, and negatively charged group, respectively. Model_015 consists of eight pharmacophore features: three hydrophobes (HY2, HY4, and HY7), two HB acceptors (AA5 and AA8), two HB donors (DA1 and DA3), and one negatively charged group (NC6).

**Virtual screening**

Model_015 was used as a 3D query to screen the compound database of *S. miltiorrhiza*. A query fit (QFIT) value was computed for each screened compound hit to rank the matching rate of its required structural features on the pharmacophoric query. A high QFIT score corresponds to a good alignment between the pharmacophore model and compound conformer. According to the QFIT values, the top 13 compounds are listed in Table 2 and the best compounds mapping on Model_015 are shown in Fig. 4.

**Molecular docking**

To determine the probable binding conformations of these compounds, the Surflex-Dock program was used to dock all of the compounds from *S. miltiorrhiza* into the active site of ECE-1. The docking reliability was validated using the known X-ray structure of ECE-1 complexed with phosphoramidon. As shown in Fig. 5, the low root mean-square deviation of 1.91 Å between the docked and the crystal conformation of phosphoramidon indicated the high reliability of Surflex-Dock in reproducing the experimentally observed binding mode for ECE-1 inhibitor.

The Surflex-Dock program was used to search the binding conformations and 27 compounds from *S. miltiorrhiza* with a docking score ≥ 6.0 were hit. The calculated hydrophobicity (ClogP), docking scores, crash, and polar are summarized in Table 3. Here, the ClogP descriptor is important in determining whether ECE-1 inhibitors can reach the site of action. The hit compounds, ID 16072, 4680, 19983, and 16073, with high ClogP (>5) were excluded. The
The total score is calculated by an empirically derived scoring function that is based on the binding affinities of ligand–protein complexes.

**Binding mode**

The correct mode of ligand–protein binding is extremely important not only for molecular recognition but also for the identification of active compounds from traditional Chinese medicine. In this study, the binding mode of ECE-1 with phosphoramidon, ECE-1 inhibitors, and compounds from *S. miltiorrhiza* were investigated.

From Fig. 5, it can be seen that phosphoramidon could bind to ECE-1 via six H bond interactions: the tetrahydropyran –OH with the side chain –OH of GLU667, the phosphate –OH with the backbone C=O of ASN567 and the side chain –OH of GLU608, the –NH adjacent to phosphate with the backbone C=O of ASN566 and ASN567, the amide –NH with the backbone =O of ASN566, the amide C=O with two –NH of ARG738, the carboxyl –OH with –NH of ASN566, and the indole –NH with the backbone =O of ASN565. Our docking results were similar to most of the evidence described in the literature and were confirmed to be accurate and reliable.

Fourteen ECE-1 inhibitors from the literature with a docking score ≥8.0 were chosen to study the binding mode with ECE-1. From Table 4, it can be seen that ARG738 (11 times), ASN566 (10 times), GLU608 (8 times), ALA567 (7 times), and TYR569 (7 times) are the main amino acid residues binding to ECE-1 inhibitors.

Fifteen compounds from *S. miltiorrhiza* with a docking score ≥8.0 were chosen to study the binding mode with ECE-1. From Table 5, it can be seen that GLU608 (13 times), TYR569 (10 times), and ARG738 (9 times) are the main amino acid residues binding to the compounds from *S. miltiorrhiza*. The interactions between ECE-1 and two compounds, phosphoramidon and salvianolic acid B, are shown in Fig. 6.

Notably, the docking analysis revealed that most of the ECE-1 inhibitors and compounds from *S. miltiorrhiza* have their own similar binding patterns in the active pocket of ECE-1. However, most of the ligands can be combined with GLU608 and ARG738, which

<table>
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**Fig. 6.** Interactions between ECE-1 and (a) phosphoramidon and (b) salvianolic acid B.

**Fig. 7.** Interactions between Compound 3 and the target protein.
implies that GLU608 and ARG738 may be critical amino acid residues binding to inhibitors in the active sites of ECE-1.

The pharmacophore can describe the common characteristics of the active ingredients, while molecular docking can reflect the detailed binding mode of the ligand and target protein. The combination of pharmacophore and molecular docking is capable of finding the communal active groups in the structure of ingredients. As shown in Fig. 7, Compound 3 can interact with the target protein through eight H-bond interactions. The –NH group close to the benzene ring can match HB donor site 3 (DA3) and bind with the backbone C=O of both ASN566 and ALA567. The other –NH group can match HB donor site 1 (DA1) and bind with the backbone C=O of ASN566. The C=O group, mapped with HB acceptor site 1 (AA8), can bind with two –NH2 groups of ARG738. It implies that pharmacodynamic features of DA1, DA3, and AA8 play important roles in the activity of ECE-1 inhibitors.

Top scoring compounds
Comparing the virtual screening results based on pharmacophore and molecular docking, respectively, 11 communal compounds from *S. miltiorrhiza* with high QFIT (>5.0) and docking scores (>8.0) were hit, which are salvianolic acid B, melitric acid A, lithospermic acid B, salvianolic acid E, methyl melitrate A, sagecoumarin, salvianolic acid D, salvianolic acid A, salvianolic acid C, monomethyl lithospermate, and labiatenic acid. The molecular structures of these hits are shown in Fig. 8. Among them, salvianolic acid B, E, D, A, and C, lithospermic acid B, and labiatenic acid, with the same mother nucleus structure of cinnamic acid, belong to the water-soluble compounds from salvianolic acid. Melitric acid A, sagecoumarin, monomethyl lithospermate, and methyl melitrate A are esterified derivatives of cinnamic acid.

Several related studies have proven that salvianolic acid compounds have activities in decreasing the content and activity of ET-1. Zhang\(^43\) found that three doses of salvianolic acid B can significantly inhibit the contraction effects of ET-1 on human hepatic stellate cells (all \(P < 0.01\)) through collagen gel contraction experiments. Xu\(^44\) found that salvianolic acid B can decrease the contraction by ET-1-activated human hepatic stellate...
cells by 66.5% in vitro and lower the portal pressure in rats with dimethylnitrosamine-induced cirrhosis in vivo.

Conclusions

In this study, a systematic computational method based on pharmacophore and molecular docking was established to screen ECE-1 inhibitors from S. miltiorrhiza. The pharmacophore model generated can characterize the common features of the ECE-1 inhibitors. Molecular docking studies can describe the detailed binding mode of the ligand and ECE-1. The virtual screening approach based on pharmacophore and molecular docking can be used to quest for ECE-1 inhibitors from S. miltiorrhiza rationally. To the best of our knowledge, this is the first in silico study of ECE-1 inhibitors from S. miltiorrhiza. The generated can characterize the common features of the ECE-1 activity. In conclusion, ligand- and structure-based screenings have good consistency and can be validated and supplemented with each other. The main mechanism of S. miltiorrhiza for dilatation of blood vessels may be related to inhibition of ECE-1 activity, which is helpful for further in-depth study.

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References


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