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（收稿日期：2012年11月27日）

·Treatis·

Identification of thrombin inhibitors from *Salvia Miltiorrhiza* by pharmacophore based virtual screening and molecular docking

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Abstract: Objective: To quest for anticoagulation active constituents from *Salvia Miltiorrhiza* based on thrombin target. Methods: The chemical compounds of *Salvia Miltiorrhiza* were collected from Traditional Chinese Medicine Database (TCMD). Virtual screening based on pharmacophore and molecular docking were used to identify the thrombin inhibitors from *Salvia Miltiorrhiza*. Results: The pharmacophore model of thrombin inhibitors established includes seven features: four hydrophobic groups, two hydrogen-bond donors and one positive nitrogen group. Then, virtual screening using the pharmacophore model was performed resulting in a hit list of 14 compounds with QFIT≥9.0. Molecular docking studies hit 17 constituents with the docking score≥6.0. There were 8 mutual compounds both hit by pharmacophore-based and docking-based screening. Parts of the hit have been reported to have the activity of thrombin inhibition by literatures. Conclusion: Virtual screening based on pharmacophore and molecular docking can be used to identify thrombin inhibitors from *Salvia Miltiorrhiza*. The result indicates that the mechanism of *Salvia Miltiorrhiza* for anticoagulation mechanism maybe related to the thrombin inhibitory activity, and virtual screening based on pharmacophore and molecular docking approach can provide a helpful tool to identify thrombin inhibitors from *Salvia Miltiorrhiza*.

Key words: Thrombin inhibitor; *Salvia Miltiorrhiza*; Virtual screening; Pharmacophore; Molecular docking; Identification of active ingredients

Fund assistance: National Key Technology R&D Program (No.2008BAI51B01)

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基于药效团与分子对接筛选丹参药材中凝血酶抑制活性成分

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摘要：目的：辨识丹参药材中抑制凝血酶的活性成分。方法：从中药化学成分数据库（TCMD）收集丹参药材已知的化学成分群。分别采用基于药效团与基于分子对接的方法，筛选丹参药材的化学成分群以辨识凝血酶抑制剂。结果：所建凝血酶抑制剂药效团包含7个药效特征：4个疏水基团，2个氢键给体和1个正氮中心。将所建药效团筛选丹参化学成分数据库，命中14个匹配度（QFIT）在9.0以上的化合物。分子对接结果显示，17个化合物对接得分（docking score）在6.0及以上。2种方法筛选结果相比，有8个共有化合物，部分命中化合物的凝血酶抑制活性已被相关文献所佐证。结论：基于药效团与分子对接的虚拟筛选方法可用来鉴别丹参药材中凝血酶抑制活性成分，丹参的抗凝血机制与其凝血酶抑制活性有关，该结果为进一步研究丹参作用分子机制提供参考和依据。

关键词：凝血酶抑制剂；丹参；虚拟筛选；药效团；分子对接；有效成分辨识

基金资助：国家科技支撑计划项目（No.2008BAI51B01）

Thrombin, an important factor of clotting system, takes part in a variety of physiological actions, such as blood clotting, anticoagulation, thrombosis fibrinolysis, angiogenesis, invasion and metastasis, growth of cancer[1-6]. Platelet activation by thrombin is a key factor leading to stasis syndrome. So thrombin may be an important target to promote blood circulation for removing blood stasis.

Danshen, the dry root and rhizome of *Salvia Miltiorrhiza*, is one of the popular and important traditional Chinese herbs. It can improve blood microcirculation, dilate coronary arteries, protect blood vessels from thrombosis and atherogenesis, it 's widely used for the treatment of cardiovascular diseases, inflammatory diseases, blood stasis and neurodegeneration diseases[7-9]. Virtual screening is a computational technique used in drug discovery research. It can deal with the quick search of large molecular libraries to identify the structures which are most likely to bind to the drug target, typically a protein receptor or enzyme. In this paper, virtual screening based on pharmacohpore and molecular docking was proposed to quest for potential inhibitors of thrombin from *Salvia Miltiorrhiza*, the association between the potential activity of chemical compositions and the efficacy of *Salvia Miltiorrhiza* was also discussed.

Materials and Methods

1. Materials and instruments All the studies were performed with SYBYL 7.0 package running on Xeon Intel(R) X5460 CPU 3.16 GHz, RAM Memory 12 GB under the Windows XP system. GALAHAD module was used to generate pharmacophore models of thrombin inhibitors. UNITY module was used to search for the potential inhibitors and Surflex-Dock module was used to perform molecular docking. TCMD (version 2009), was used to establish the chemical structure database of *Salvia Miltiorrhiza*.

Compounds 1-30, which can inhibit thrombin, were collected from the literatures[10-12] and served as the training set for the pharmacophore modeling. The structures and inhibitory activities are listed in color interpolation 1 Figure1.

2. Pharmacophore studies GALAHAD (Genetic Algorithm with Linear Assignment of Hypermolecular Alignment of Datasets) uses Tripos' proprietary technology to generate pharmacophore hypotheses and alignments from sets of ligand molecules that bind at a common target site. Before establishing the model, all the structures of ligands were checked, hydrogen atoms were added and a minimization procedure was implemented using the MMFF94 force-field. 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 using UNITY module. The test database composed of 112 experimentally known thrombin inhibitors[13-18] and 323 non-active compounds selected from the MDL Drug Data Report (MDDR) database (MDDR is a database

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covering the patent literatures, journals, meetings and congresses. Produced by MDL and Prous Science, the database contains over 100 000 biologically relevant compounds and well-defined derivatives).

3. Molecular Docking

3.1 Preparation of the ligands and target protein Eighty-eight compounds from *Salvia Miltiorrhiza* were picked out from TCMD. The structure was checked and hydrogen atoms were added, then a minimization procedure was implemented using the Tripos force-field for 1 000 iterations.

The 3D coordinates of the crystal structure of thrombin in complex with 1TS inhibitor was collected from PDB under code 3UTU identified by X-ray diffraction (Resolution: 1.55Å). After extracting the ligand 1TS, all 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 AMBER7 F99 force field with the Powell energy minimization algorithm, distance-dependent dielectric function and current charges.

3.2 Docking strategy The factors of the scoring function in Surflex-Dock contain hydrophobic, polar, repulsive, entropic and solvation terms. It is a well-recognized method in the field and plays a crucial role in calculating the ligand-receptor interaction[19-20]. The default parameters, as implemented in the SYBYL 7.0 software, were used. All the compounds from *Salvia Miltiorrhiza* were prepared to dock into the active site of the thrombin. The highest-scored conformation based on the Surflex-Dock scoring functions, was selected as the final bioactive conformation.

4. Virtual Screening The pharmacophore model generated by GALAHAD was used as a query to screen the compounds from *Salvia Miltiorrhiza*. A test database was used to evaluate the performance of the models. The schematic diagram was listed in color interpolation 1 Figure2. D is for the total number of compounds in test database and A represents the number of active compounds. Ht is the total number of hit compounds from test database and Ha represents the number of active hit compounds from test database, A% represents the ability to identify active compounds from test database, Y% represents the proportion of active compounds in the hit compounds. N, the index of effective identification, is used to evaluate the ability of the models to identify active compounds from the non-active compounds. CAI, a comprehensive evaluation index, is used to identify the best pharmacophore model. A higher CAI value suggests that the model is better.

Meanwhile, the compounds were docked into the binding site of thrombin successively, an empirically derived scoring function was used to evaluate the interaction of the ligands and thrombin.

Results and Discussion

1. Generation of pharmacophore model GALAHAD models were derived by using ten active ligands as a training set (compounds 1-4, 7, 8, 14, 22, 23, 25), 20 pharmacophore models were derived by using the training set after GALAHAD run. All the 20 models were evaluated successively by the test database constructed previously. Table 1 shows the predictable results of the test database for models.

Table 1 The parameter values for each pharmacophore model

1	Ht	Ha	A%	Y%	N	CAI
MODEL_01	232	82	0.73	0.35	1.37	1.01
MODEL_02	2	0	0.00	0.00	0.00	0.00
MODEL_03	394	112	1.00	0.28	1.10	1.10
MODEL_04	95	35	0.31	0.37	1.43	0.45
MODEL_05	410	106	0.95	0.26	1.00	0.95
MODEL_06	145	46	0.41	0.32	1.23	0.51
MODEL_07	222	90	0.80	0.41	1.57	1.27
MODEL_08	261	86	0.77	0.33	1.28	0.98
MODEL_09	365	93	0.83	0.25	0.99	0.82
MODEL_10	311	94	0.84	0.30	1.17	0.99
MODEL_11	126	78	0.70	0.62	2.40	1.67
MODEL_12	76	42	0.38	0.55	2.15	0.80
MODEL_13	43	26	0.23	0.60	2.35	0.55
MODEL_14	136	92	0.82	0.68	2.63	2.16
MODEL_15	394	108	0.96	0.27	1.06	1.03
MODEL_16	40	20	0.18	0.50	1.94	0.35
MODEL_17	91	55	0.49	0.60	2.35	1.15
MODEL_18	127	79	0.71	0.62	2.42	1.70
MODEL_19	262	94	0.84	0.36	1.39	1.17
MODEL_20	5	0	0.00	0.00	0.00	0.00

MODEL_14 is displayed in color interpolation 1 Figure3, it includes seven pharmacophore features: four hydrophobes (HY), two hydrogen-bond donors (DA) and one positive nitrogen (NP).

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2. Virtual Screening MODEL_14 was used to screen the compounds of *Salvia Miltiorrhiza*. A query fit (QFIT) value was computed for each 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 pharmacophore model and compound conformer. According to QFIT values, the top 12 compounds with QFIT>9.0 were listed in Table 2.

Table 2 The hit compounds screened by pharmacophore MODEL_14

ID	QFIT	Name
15374	31.04	Neocryptotanshinone
12924	23.23	Lithospermate B
19203	23.20	Salvianolic acid C
12926	21.39	Lithospermic acid B
13370	20.16	Magnesium lithospermate B
4631	17.27	Danshensuan B
19202	16.09	Salvianolic Acid B
19983	15.66	β-Sitosterol
4680	14.61	Daucosterol
14927	13.46	Monomethyl lithospermate
16073	9.46	Oleoyl neocryptotanshinone
18011	9.03	Przewaquinone A

3. Molecular Docking All the compounds from *Salvia Miltiorrhiza* were docked into the active site of thrombin. Docking reliability was validated using the known X-ray structure of thrombin complexed with 1TS. As shown in color interpolation 1 Figure4, the low root mean-square deviation (RMSD) of 0.94 Å between the docked and the crystal conformation of 1TS indicated the high reliability of Surflex-dock in reproducing the experimentally observed binding mode for thrombin inhibitor, key residues are displayed and hydrogen bonds are displayed in dotted lines.

Surflex-Dock program was used to search the binding conformations and seventeen compounds from *Salvia Miltiorrhiza* with docking score ≥6.0 were hit, the docking scores and calculated hydrophobicity (ClogP) were summarized in Table 3. The hit compound, ID 16073, with high ClogP (>5) was excluded according to lipinsk's rule of five.

Considering the calculated scores and inhibitory activity in the training set, the hits with QFIT values ≥9 and docking score ≥6.0 show good activity. Therefore,

Table 3 Hydrophobicity of the compounds from Salvia Miltiorrhiza and the docking results to thrombin

ID	Total_Score	ClogP	Name
12926	8.95	1.84	Lithospermic acid B
6452	7.64	2.75	Diosmetin
16073	7.17	12.97	Oleoyl neocryptotanshinone
19203	7.13	3.09	Salvianolic acid C
14085	7.08	3.08	7-Methoxyrosmanol
19205	6.84	2.45	Salvianolic acid E
4631	6.78	1.84	Danshensuan B
12420	6.72	1.10	Labiatenic acid
19202	6.62	2.09	Salvianolic Acid B
13370	6.48	-4.05	Magnesium lithospermate B
14927	6.44	1.77	Monomethyl lithospermate
18925	6.40	1.39	Rosmarinic acid methyl ester
12924	6.25	1.63	Lithospermate B
19201	6.24	2.53	Salvianolic acid A
13709	6.13	2.34	Melitric acid A
19204	6.07	1.37	Salvianolic acid D
3745	6.00	2.86	Cirsimaritin

9 and 6.0 were set to be the threshold in identifying thrombin inhibitors by pharmacophore-based screening and molecular docking respectively.

As shown in Table 2 and Table 3, there are 8 mutual molecules, ID 12924, 19203, 12926, 13370, 4631, 19202, 14927 and 16073, were both hit by pharmacophore-based and docking-based screening respectivel. All the compounds belong to the water soluble Salvianolic acids, and exist in a variety of *Salvia Miltiorrhiza* preparations. They are the main active ingredients of *Salvia Miltiorrhiza*. As far as we know, the literature has reported that Magnesium lithospermate B[21] can inhibit thrombin-induced platelets aggregation and Salvianolic Acids[22-24] have the active of inhibiting thrombin. To a certain extent, this can prove the accuracy and reliability of the virtual screening results and explain the anticoagulation mechanism of *Salvia Miltiorrhiza*.

Conclusion

Virtual screening based on pharmacophore and molecular docking can be verified mutually in questing for thrombin inhibitors from *Salvia Miltiorrhiza*. The computational approaches are effective and have the advantage in saving time and cost. Several active compounds were successfully identified from the

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structurally diverse mixture in *Salvia Miltiorrhiza* and by corroborated the relevant literatures. Thus, virtual screening revealed as a powerful tool to identify thrombin inhibitors, it can provide a way for the identification of active ingredients in other Chinese herbal medicine and the association of active ingredient groups with specific efficacy.

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(收稿日期：2012年11月26日)

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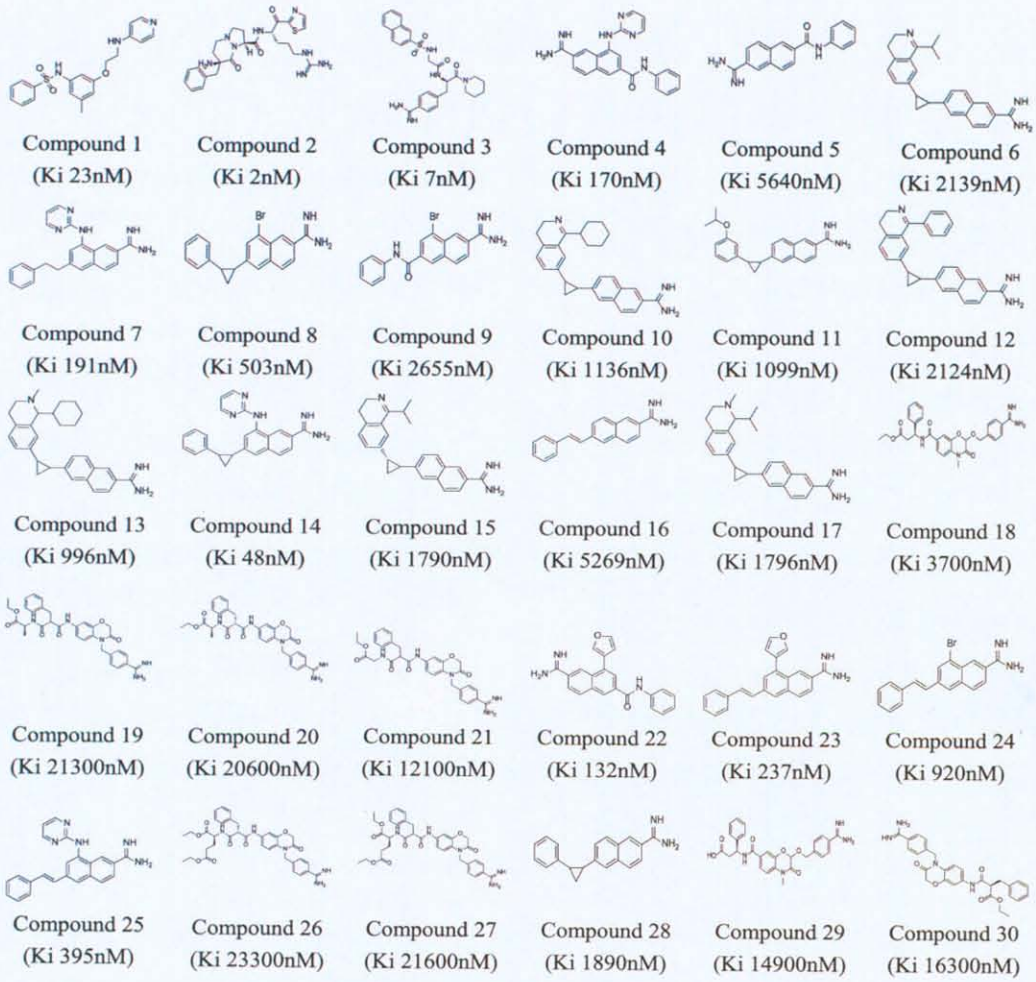

Figure1 Chemical structure of thrombin inhibitors

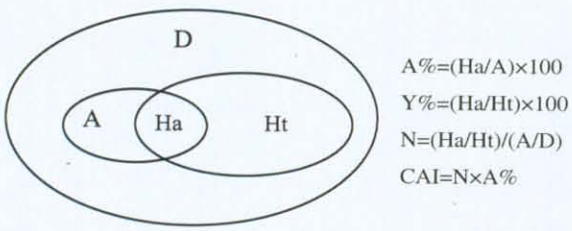

Figure2 The schematic diagram of indicators used to evaluate the pharmacophore model

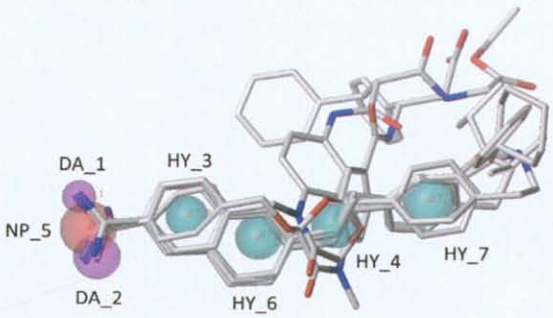

Figure3 Pharmacophore MODEL_14 and molecular alignment of the compounds

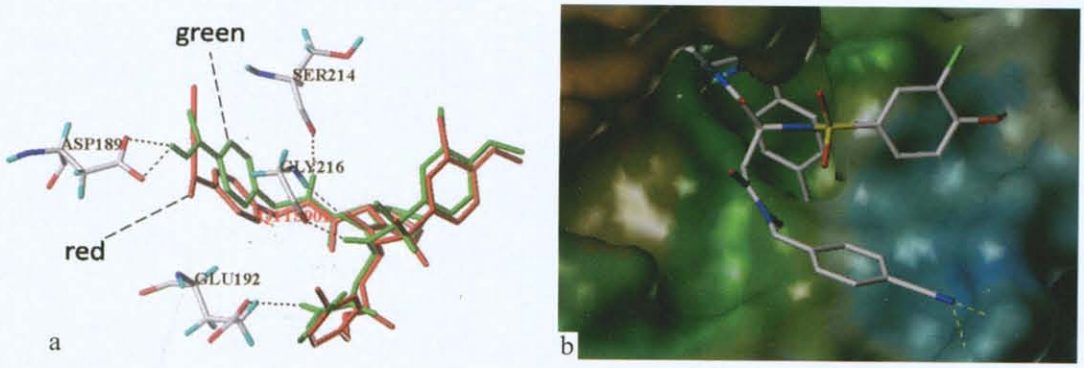

Figure4 Binding conformation of ligands at the active site of thrombin

Note: (a) Co-crystallized (red) and re-docked (green) 1TS. (b) MOLCAD lipophilic potential surface of the binding pockets with the docked compound 1TS.

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