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FULL-LENGTH PAPER

System-level multi-target drug discovery from natural products with applications to cardiovascular diseases

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Abstract The term systems pharmacology describes a field of study that uses computational and experimental approaches to broaden the view of drug actions rooted in molecular interactions and advance the process of drug discovery. The aim of this work is to stick out the role that the systems pharmacology plays across the multi-target drug discovery from natural products for cardiovascular diseases (CVDs). Firstly, based on network pharmacology methods, we reconstructed the drug–target and target–target networks to determine the putative protein target set of multi-target drugs for CVDs treatment. Secondly, we reintegrated a compound dataset of natural products and then obtained a multi-target compounds subset by virtual-screening process. Thirdly, a drug-likeness evaluation was applied to find the ADME-favorable compounds in this subset. Finally, we conducted in vitro experiments to evaluate the reliability of the selected chemicals and targets. We found that four of the five randomly selected natural molecules can effectively act on the target set for CVDs, indicating the reasonability of our systems-based method. This strategy may serve as a new model for multi-target drug discovery of complex diseases.

Keywords Systems pharmacology · Multi-target drugs · Cardiovascular diseases · Natural products · Polypharmacology

Introduction

Although most new drugs often fail in Phase II and Phase III trials, they are usually promising in cell-based assay. Nowadays, developing drugs through classical empirical methods is not proven to be productive. There is an urgent need of new guidelines to decrease the high drug attrition rates in changing the current unsatisfactory drug discovery model. Polypharmacology describes an innovative area in drug discovery that integrates the concept of systems biology and pharmacology, which expedites drug discovery by systems modeling among human diseases, biology, and chemistry. Therapeutic polypharmacology derives from two aspects: (1) an effective drug modulates multiple targets, and (2) several drugs bind to one protein molecule, leading to activating multiple pathways, such as signaling and functional pathways [1].

Multi-target drugs (MTDs) are believed to be promiscuous and might affect the entire cellular networks equilibrium more than single-targeted drugs, resulting in fully correcting complex disease conditions such as cancer [2]. There are many marketed drugs labeled as MTDs by FDA. Although some of them were purposefully synthesized, the majority of them were randomly discovered. Presently, the development of polypharmacology faces two challenges: identifying a target or combination of targets in a biological system whose perturbation results in an expected curative effect, and discovering multi-target agents with the desired polypharmacology profile to perturb those targets.

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How should we find the relevant target-sets of MTDs? Systems biology is likely to expand the druggable genome to benefit polypharmacology through a comprehensive perspective on disease and drug-action mechanisms. Systems analysis methods provide computational assistance for pharmacologists in identifying the efficacy rooted in the comprehensive drugs-targets interactions exposing to the whole cellular network [3–6]. How could we find the clinically relevant target-combinations? Markedly, platforms are moving right into generating reliable data to accelerate drug development. However, the problem is limited due the difficulty of integrating data and performing dynamic simulation between two states [7]. Hence, many promising tools might be useful to estimate the matching probability between our present knowledge and the cell complexity in pathological statuses such as systems pharmacology. Systems pharmacology aims at decoding the mechanisms of drug actions by integrating systems biology, pharmacokinetics, and pharmacodynamics methods [8–14]. As a major constituent part of systems pharmacology, network provides the framework to explore possible knock-on and knock-out effects interfering with the target and decides whether a target is worth pursuing or not.

As we know, herbal medicine, featured as multiple components and multiple targets, has shown a tremendous promise both for clinical practices and drug discovery. Contemporarily, accompany with the development of combinatorial chemistry and high-throughput screening (HTS) technologies, large natural products databases [15] have come to exist, such as the National Institutes of Health (NIH), Molecular Library Initiative [16], and ZINC [17]. These databases play a vital role in the drug discovery processes and have been analyzed utilizing physicochemical properties, molecular fingerprints and many other methods [18].

Presently, how to identify effective targets or target networks for a particular disease is still a debating problem. And it is still a big challenge to identify MTDs from the huge pool of natural products. In this work, we propose a systems biology-based approach termed reverse Network Targeting and Screening (rNTS) to discover MTDs from natural products for a specific disease, i.e., cardiovascular diseases (CVDs). The rNTS is a target-based drug discovery approach, which consists of network analysis, drug targeting, in silico ADME screening, and experimental validations. Unlike the traditional pharmaceutical development approach, network targeting is first made to obtain a set of therapeutic targets which are then applied to identify compounds that bind with high affinity to them. The main protocols are as follows:

(1) Reconstructing drug–target and target–target networks for a disease of interest and then selecting several putative protein targets based on network pharmacology techniques.
(2) Reintegrating the compound dataset of natural products involved in specific disease therapy, and then a virtual screening was performed for the selected targets.
(3) Refining the subset by a drug-likeness filtering to obtain the multi-target agents.
(4) Conducting in vitro experiments to evaluate the reliability of the proposed strategy.

Materials and methods

In this section, we consider to provide a simple, efficient as well as dependable way to explore MTDs at a system level. An example utilizing this rNTS approach is introduced to discover MTDs in nature products for CVDs treatment. The detailed descriptions of the rNTS method are shown as following (Fig. 1).

Potential multi-target identification

To capture the targets for MTDs, firstly, the molecular networks were constructed, including drug–target (D–T) network, in which a drug and a target are linked if a drug targets a known protein; and target–target (T–T) network, whose nodes are targets that are linked to each other if they simultaneously focused by the same set Gene Ontology (GO) [19] terms. GO (http://www.geneontology.org) was constructed by Ashburner et al. in 2000 and published in the UK journal Nature. Subsequently, the network topology analysis was performed to obtain the most potential drug targets. The bipartite graphs are generated by Cytoscape version 2.8 [20]. Cytoscape is a desktop Java application released by Smoot *et al.* in 2010 under the Library Gnu Public License (LGPL). Binary install bundles and source code for Cytoscape 2.8 are available for download from http://cytoscape.org.

D–T network

According to the current research status of CVDs, a hierarchical approach integrating text mining, database searching, network construction, and analysis was applied to identify the key CVDs therapeutic targets. Firstly, a large-scale text mining of PubMed and the manual extraction in the English literatures (from 1995 to 2012) were performed to acquire the CVDs therapeutic targets with the keywords “CVDs” and “target”; secondly, mapping these targets to the DrugBank Database (http://www.drugbank.ca/) to achieve their corresponding approved and experimental drugs; then the D–T network was constructed by linking the drugs with their targets; finally, the key topological parameter degree was ana-

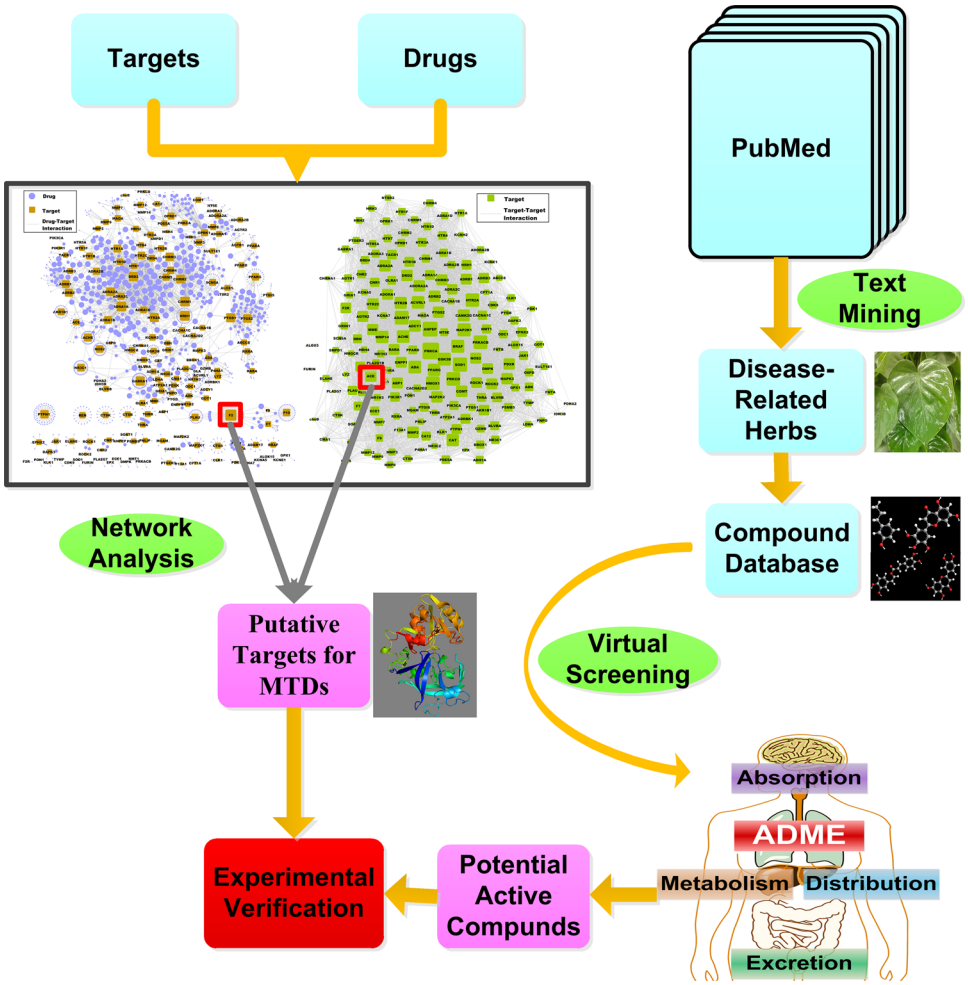


Fig. 1 Workflow for multi-target drug screening strategy

lyzed in the D–T network to screen out the most potential targets.

T–T network

To further weight the biological correlativity of the CVDs therapeutic targets, a T–T network was constructed based on the GO annotations. GO term is a means of labeling three independent wild-type gene products attributes: the molecular function, the biological processes, and their subcellular location. The process of network construction can be divided into four steps and described as follows: firstly, the CVDs therapeutic targets were mined in the same manner as labeled in the D–T network; secondly, the GO annotations of the CVDs therapeutic targets were extracted from the UniProt database (http://www.uniprot.org/); thirdly, the CVDs therapeutic targets were linked together if they are focused by at least one of the GO branches; finally, the topological parameter degree was measured to evaluate the role of targets in the network.

Potential MTDs database

To extract the available herbs for the therapy of CVDs, a wide range of text mining was performed in the PubMed with the keywords "herbal medicine" and "CVDs". And the ingredients of the obtained herbs were obtained from the Traditional Chinese Medicine Systems Pharmacology Database (TcmSP, http://sm.nwsuaf.edu.cn/lsp/tcmsp.php).

Drug targeting

The docking-based drug targeting was carried out with the software GOLD3.0.1 [21]. The starting crystal structures of the key targets were retrieved from the RCSB Protein Data Bank (http://www.pdb.org/pdb/home/home.do). CHARMM force field was employed, and hydrogen atoms were added to the proteins. The binding site was defined as a sphere encompassing protein residues within 12 Å of the original ligand. A tetrahedral geometry was applied to Zn for the zinc-containing protein. The default values were applied for other parameters, and Genetic Algorithm runs were performed for

each ligand. GOLD Score was used as the scoring function for the targets.

ADME evaluation

To evaluate the pharmacokinetic properties of the potential MTDs obtained above, an in silico ADME (absorption, distribution, metabolism, and excretion of drugs) system integrating the human oral bioavailability (OB) and "drug-likeness" (DL) models was applied. The two models were directed at predicting OB and drug-likeness to uncover candidates with promising pharmacokinetic properties.

The OB value was predicted by our previous constructed software OBioavail1.1 [22], which integrated with the metabolism information (P-glycoprotein and cytochrome P450s) and supported by 805 structurally diverse compounds (drug or drug-like molecules) with known human OB. Among the three models: multiple linear regression, partial least square, and support vector machine (SVM), the SVM provides the optimal performance (training set: $R^2 = 0.80$, SEE = 0.31; test set: $Q^2 = 0.72$, SEP = 0.22).

The DL was obtained by calculating the Tanimoto similarity [23] between herbal compounds and the drugs in DrugBank database. The database dependent drug-likeness evaluation expression is shown as:

$$T(x, y) = \frac{x \cdot y}{\|x\|^2 + \|y\|^2 - x \cdot y}, \quad (1)$$

where x is the molecular descriptor of herb compounds, y is the average molecular property of all compounds in the DrugBank database.

Potential compounds were regarded as MTDs in any of the following cases: OB $\geq$ 30 % or DL $\geq$ 0.35. And all corresponding data have been uploaded to the online database TcmSP.

Experimental validation

In an effort to assess the reliability of our method, the obtained compounds were further validated by in vitro experimental methods. Salvianolic acid A, Salvianolic acid B, and Rosmarinicacid were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China), Chengdu Sikehua Biotechnology Co., Ltd. (Chengdu, China), and Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China) respectively. Licochalcone A and Curcumin were purchased from Chengdu Biopurify Phytochemicals Ltd. (Sichuan, China). The purity of all the compounds is >98 %. All drugs were dissolved in 10 % ethanol and freshly prepared due to loss of activity under long-term storage.

The effects of all the standard compounds on ACE (Angiotensin-converting enzyme) were measured using the ACE Kit-WST Detection (Dojindo, Kumamoto, Japan) according to manufacturer's instruction. Consistently, the inhibitory effects of PTGS2/COX2 (Prostaglandin G/H synthase 2), REN (Renin), and F2 (Prothrombin) were assayed using Colorimetric COX (ovine) inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI, USA), Human recombinant Renin Inhibitor Screening Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA), and Sensolyte® 520 thrombin activity assay kit (AnaSpec, CA, USA), respectively.

Statistical analysis

Inhibitory effects on the parameters measured were compared by analyzing the means for differences using Student's t test and ANOVA test. Student's t test was applied to compare the means of two groups. ANOVA test was used to compare the means of multiple groups. Differences were considered to be significant when $p < 0.05$. Values were reported as mean $\pm$ SD of three parallel samples.

Results

Putative targets for MTDs

A catalog of 234 known target/CVDs relationships was collected from the literature. Of these, 177 human targets can be perturbed by 1,460 drugs. As shown in Fig. 2, all the 1,460 drugs and their targets generate a bipartite graph of D–T network through 2,539 edges. The results show that the average number of drugs per target is 14.3. However, merely a small portion of proteins (51/177) connect with more than 14 drugs (Table S1), indicating that proteins are targeted unequally, and different proteins may have inherently disparate disturbance ratio in the cardiovascular system. In fact, these pivotal targets are the centers of the network from the point of network topology [24]. Thus, the 51 targets play vital roles in the treatment of CVDs.

The T–T network provides a complementary, protein-centered view of polypharmacology space which consists of 177 nodes and 6,423 edges. As shown in Fig. 3, out of 177 CVDs related targets, 175 have at least one link to other targets, that is, they share the GO terms with other targets. Some targets link only a few proteins, but most are highly connected, which result in an average degree of 72.6. Interestingly, about half of the protein targets (85/177) have more interacting proteins than the average, suggesting that the 85 targets are much closer in molecular function, biological process, and subcellular location than other CVDs therapeutic targets (Table S2).

Due to the fact that a few highly connected nodes, often called hubs, in biological networks have special biological roles [25], hub proteins in the D–T network and T–T network are either vital or biological correlativity in the therapeutic of CVDs. Consequently, the 29 highly connected targets

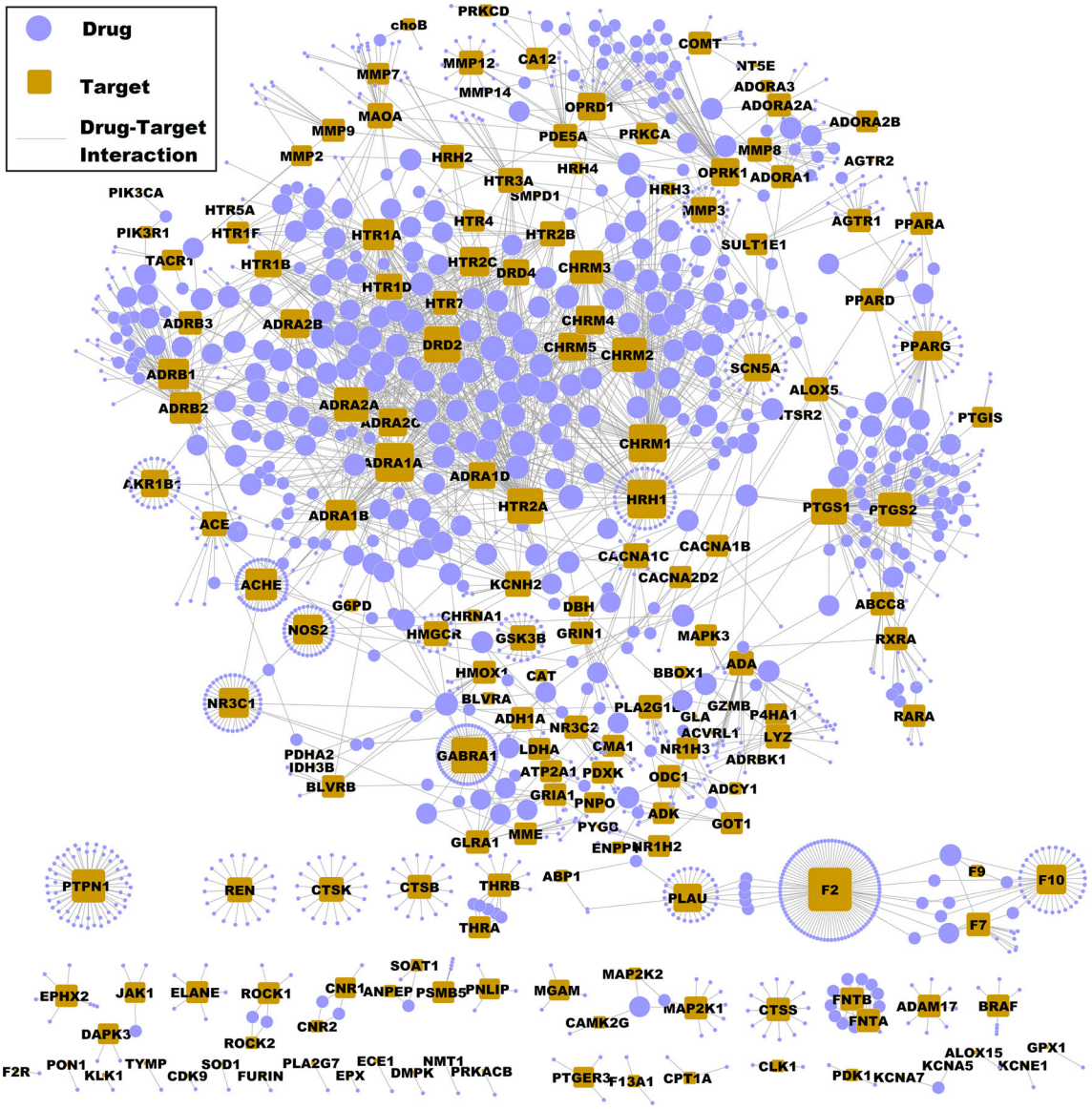


Fig. 2 The D–T network. A drug node and a protein node are linked if the protein is targeted by the corresponding drug. Node size is proportional to its degree. The letters are target labels

(Table 1) shared by the 51 targets in the D–T network, and the 85 targets in the T–T network are more inclined to act as the potential targets for MTDs than others. And the 29 targets were selected as putative targets for MTDs.

In an effort to validate the 29 selected targets indeed fit for MTDs, we applied ClueGO, a Cytoscape plug-in to address the biological interpretation of large lists of genes in the form of networks [26], and partition it into four layers: molecular function, the biological processes, subcellular locations, and the KEGG functional analysis. As shown in Fig. 4a, the main molecular functions were classified into three categories: norepinephrine binding, G-protein coupled amine receptor activity, and serotonin binding. The large majority of these targets are related to norepinephrine binding (i.e., F2 and ACHE) which is mostly responsible for the coronary vasodilatation [27]. Substantially, all the biological processes of the targets are directly boiled down to vascular process in circulatory system (Fig. 4b), indicating that the biological processes of these targets are chaste. In Fig. 4c, the principal subcellular locations were divided into two kinds: neuron projection and mitochondrion. The KEGG pie-chart (Fig. 4d) shows the functional effect of differentially targets on cellular pathways in CVDs. The prime groups are fallen into metabolic pathways, serotonergic synapse, or renin-angiotensin system.

Of the 29 putative targets for MTDs in Table 1, ACE, PTGS2, REN, and F2 were selected as the multi-target intervention solution for further molecular screening and experimental verification for the following reasons. Firstly, the

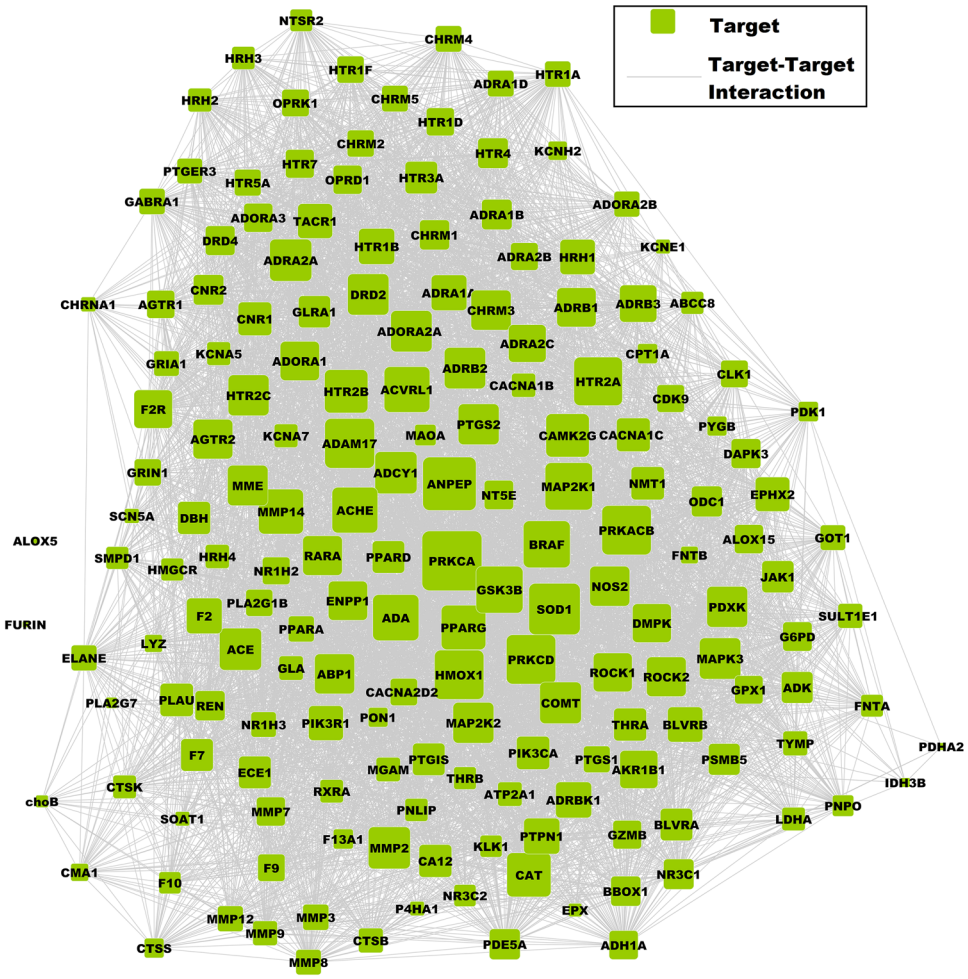


Fig. 3 The T–T network. Two neighboring targets are linked if they simultaneously focus one GO term. Node size is proportional to its degree. *The letters* are node labels

four targets distribute through the main groups of the four independent attributes. Secondly, the crystal structures are available from the PDB for further docking screening. Furthermore, these four proteins are commercially available for further experiment.

Potential MTDs

Herbs determination and compounds database building

Among the large volume of CVDs-related articles, *Ligusticum chuanxiong* (*L. chuanxiong*), *Dalbergia odorifera* (*D. odorifera*), *Corydalis yanhusuo* (*C. yanhusuo*), *Salvia miltiorrhiza* (*S. miltiorrhiza*), *Panax notoginseng* (*P. notoginseng*), *Borneolum*, *Glycyrrhizae uralensis (G. uralensis*), *Atractylodes macrocephala* (*A. macrocephala*), *Pinellia rhizome* (*P. rhizome*), *Poria cocos* (*P. cocos*), *Cinnamomi ramulus* (*C. ramulu*), *Radix astragali* (*R. astragali*), *Spatholobus suberectus* (*S. suberectus*) and *Curcuma aromatic* (*C. aromatic*) are found to be the most promising herbs for CVDs treatment. They are generally originated from herbal medicine and totally validated their therapeutic effects by animal models. Furthermore, the information for these herbs was summarized from PubMed, as shown in Table 2. And a total of 1,780 molecules (Table S3) from these herbs were deposited in the TcmSP database.

Drug targeting

The docking score in GOLD predicts potential binding affinities of compounds against the four targets at selected regions. Normally, the threshold of docking score is 60 for selecting an active ligand. In order to obtain more potential compounds for further validations, the threshold decreases to 50. As the docking scoring functions is not accurate enough to properly identify any ligand. Therefore, 380 out of 1,780 compounds were treated as hits for further evaluation. Of particular note, there is only one compound in Borneolum and Poria showed better GOLD fitness scores.

ADME screening

255 of the 380 compounds with good docking scores were predicted with favorable pharmacokinetics properties by

Table 1 Putative targets for MTDs

Target	Protein names	D–T degree	T–T degree
ACE	Angiotensin-converting enzyme	17	103
ACHE	Acetylcholinesterase	48	112
ADA	Adenosine deaminase	16	113
ADRA1A	Alpha-1A adrenergic receptor	79	87
ADRA1B	Alpha-1B adrenergic receptor	43	74
ADRA2A	Alpha-2A adrenergic receptor	60	103
ADRA2C	Alpha-2C adrenergic receptor	33	92
ADRB1	Beta-1 adrenergic receptor	44	96
ADRB2	Beta-2 adrenergic receptor	48	101
AKR1B1	Aldose reductase	29	93
CACNA1C	Voltage-dependent L-type calcium channel subunit alpha-1C	18	82
CHRM1	Muscarinic acetylcholine receptor M1	75	73
CHRM3	Muscarinic acetylcholine receptor M3	56	98
DRD2	D(2) dopamine receptor	68	101
F2	Prostaglandin F2 receptor negative regulator	100	87
GSK3B	Glycogen synthase kinase-3 beta	20	115
HRH1	Histamine H1 receptor	79	86
HTR1B	5-Hydroxytryptamine receptor 1B	27	88
HTR2A	5-Hydroxytryptamine receptor 2A	66	119
HTR2B	5-Hydroxytryptamine receptor 2B	19	106
HTR2C	5-Hydroxytryptamine receptor 2C	38	100
HTR3A	5-Hydroxytryptamine receptor 3A	18	79
NOS2	Nitric oxide synthase, inducible	39	98
NR3C1	Glucocorticoid receptor	40	74
PLAU	Urokinase-type plasminogen activator	33	81
PPARG	Peroxisome proliferator-activated receptor gamma	38	109
PTGS2	Prostaglandin G/H synthase 2	57	100
PTPN1	Tyrosine-protein phosphatase non-receptor type	55	88
REN	Renin	18	73

ADME screening (Table S4). For the 255 chemicals, 142 of them are orally available (OB $\geq$ 30 %), and 192 compounds have drug-likeness features (DL $\geq$ 0.35). Of the 255 compounds with good OB and DL values, many of them were biologically active as reported in the literature, such as Dihydrocurcumin (M192, OB = 65.49 %; DL = 0.41), Curcumin (M194, OB = 2.18 %; DL = 0.41), and Dehydrocorydaline (M206, OB = 60.36 %; DL = 0.68). Dihydrocurcumin and Curcumin are involved in the natural product *C. aromatic*. For Dihydrocurcumin with OB of 65.49 % and DL 0.35, it presents potent protective effect on anti-inflammatory [28], and thus has therapeutic effects on CVDs. Further, as one of the important ingredients of *C. aromatic*, Curcumin has relative poor OB (2.18 %). In fact, this compound can be metabolized into several products such as Dihydrocurcumin (OB = 65.49 %) under the influence of enzymes in vivo [29]. This information explains why Curcumin exhibits pharmacological activities despite its poor OB value. Dehydrocorydaline (M206, OB = 60.36 %; DL = 0.68), a natural alkaloid, is the main effective ingredient in *C. yanhusuo*. Except for the aforementioned anti-inflammatory activity, Dehydrocorydaline is able to relieve pain by inhibiting adrenergic neuron relaxation due to its blockade effect on the Taenia caecum and pulmonary artery adrenergic nerve terminals [30]. It has been reported to be one of the active compounds of *C. yanhusuo* for the treatment of coronary heart disease [31].

Among the 255 potential MTDs, five compounds, namely Salvianolic acid A, Salvianolic acid B, Rosmarinicacid, Licochalcone A, and Curcumin (Table 3) were randomly singled out for further experimental validations. Random selection is able to ensure that the five compounds could better represent the theoretical results and prove the widespread applicability of the theoretical approach applied in this study. Meanwhile, these five compounds are readily available on the market. Due to some restrictions of test conditions, this study only tested 5 of the 255 potential MTDs.

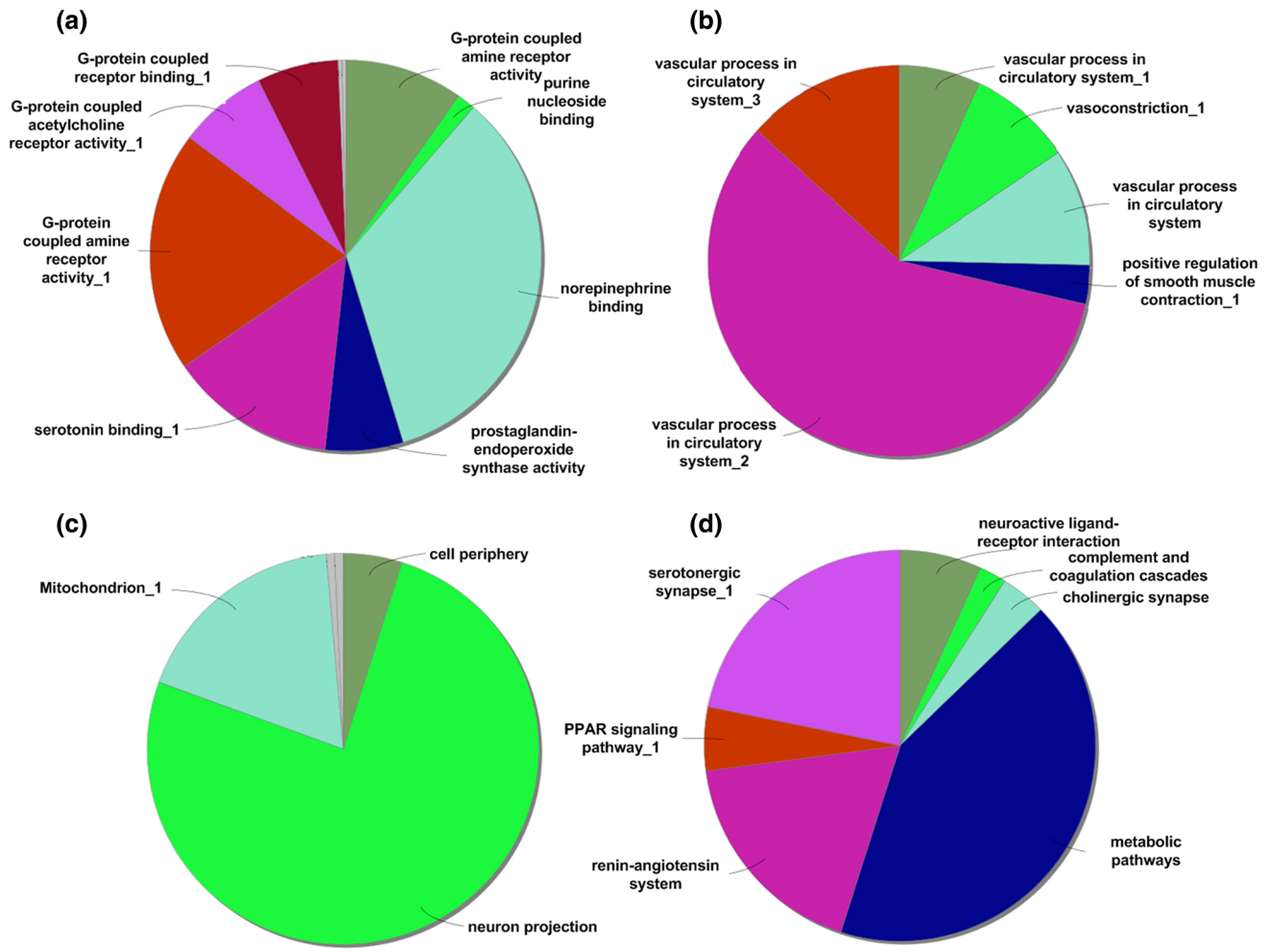


Fig. 4 ClueGO analysis of the CVDs targets. Only the label of the most significant term per group is shown. The node pie charts represent the molecular function/the biological processes/subcellular location/KEGG functional of each target have corresponding to their networks in this target set. **a** Representative the molecular function interactions among CVDs targets. **b** Representative the biological processes interactions among CVDs targets. **c** Representative subcellular location interactions among CVDs targets. **d** Representative KEGG interactions among CVDs targets

Experimental validation

The 50 % inhibition concentration (IC_{50}) values for the five selected compounds and their corresponding structures are summarized in Table 4.

Salvianolic acid A

As shown in Table 4, Salvianolic acid A exerted inhibitory activity against ACE, PTGS2, REN, and F2, with IC_{50} values of 27.3 ± 3.2, 8.3 ± 1.0, 24.4 ± 3.1, and $144.7 \pm 17.0\,\mu M$, respectively. This result demonstrates that Salvianolic acid A is the ideal master in the four targets. We note that, among them, Salvianolic acid A shows the highest inhibition effect on PTGS2 compared to those on ACE, REN, and F2 ($p < 0.05$), which indicate that PTGS2 is significantly more sensitive to Salvianolic acid A than others.

Salvianolic acid B

Salvianolic acid B is a potent compound able to inhibit ACE, PTGS2, and REN, respectively. The decreasing order of the IC_{50} values is REN (24.9 ± 3.2) > PTGS2 (28.7 ± 3.5) > ACE (86.9 ± 11.1). Actually, the statistical analysis indicated that the inhibitory activity of REN by Salvianolic acid B was significantly higher than that of PTGS2 or ACE ($p < 0.05$). Surprisingly, Salvianolic acid B did not exhibit evident activity on F2 with $IC_{50} > 500\,\mu M$. As compared to Salvianolic acid A, obtained from the same plant *S. miltiorrhiza*, Salvianolic acid B was also able to inhibit the four targets.

Rosmarinicacid

Rosmarinicacid did not exhibit significant activity on protein F2, but with good activities on ACE, PTGS2, and

Table 2 Information for CVD-related herbs

Herb name	Herb type	Species	Findings	Reference
L. chuanxiong	*Ligusticum chuanxiong*	Mice	Lactones from *Ligusticum chuanxiong Hort*. reduces atherosclerotic lesions in apoE-deficient mice via inhibiting over expression of NF-kB -dependent adhesion molecules	PMID: 24594239
D. odorifera; S. miltiorrhiza; L. chuanxiong	Guan-Xin-Er-Hao : *Salvia miltiorrhiza*, *Ligusticum chuanxiong*, *Paeonia lactiflora*, *Carthamus tinctorius* and *Dalbergia odorifera*	Rat	Guan-Xin-Er-Hao exerts significant cardioprotective effects against acute ischemic myocardial injury in rats, likely through its antioxidation and antilipid peroxidative properties and thus may be used as a promising agent for both prophylaxis and treatment of ischemic heart diseases	PMID:18951001
C. yanhusuo;	*Corydalis yanhusuo*	Rat	*Corydalis yanhusuo* exerted salutary effects on heart failure induced by myocardial infarction in rats	PMID: 17524235
S. miltiorrhiza	*Salvia miltiorrhiza*	Rat	After 2 weeks treatment with purified *Salvia miltiorrhiza* extract, survival rates of rats with experimental myocardial infarction were marginally increased (68.2 %) compared with saline (61.5 %)	PMID: 15808885
P. notoginseng	*Panax notoginseng* saponins	Rat	Saponins of *Panax notoginseng* prevent cardiac ischemia and the action is considered to be related to the inhibition of lipid peroxidation	PMID: 2403009
Borneolum; R. Chuanxiong	Suxiao Jiuxin Pill: *Rhizoma Chuanxiong* and *Borneolum*	Rat	Suxiao Jiuxin Pill plays an important role in anti-inflammation and inhibition of oxidative stress, which possibly are the mechanism of its preventing and treating atherosclerosis	PMID: 21977809
G. uralensis	*Glycyrrhiza glabra*	Rats	*Glycyrrhiza glabra* root registered a significant decline in plasma lipid profiles and an increase in high density lipoprotein -cholesterol content	PMID: 17054099
C. ramulu; P. cocos	Geiji–Bokryung–Hwan: *Cinnamomi Ramulus*, *Poria Cocos*, *Mountan Cortex Radicis*, *Paeoniae Radix* and *Persicae Semen*	Rabbits	The reduction in atherosclerosis by Geiji–Bokryung–Hwan relies not only on its cholesterol-lowering effect but also more heavily on its antioxidant potential, which prevents endothelial damage and inhibits low density lipoprotein oxidative modification in hypercholesterolemic animals	PMID: 12757741
S. suberectus	*Spatholobus suberectus*	ICR mice	*Spatholobus suberectus* has antiplatelet effects via inhibition of the glycoprotein IIb/IIIa receptor	PMID: 21211555
C. aromatic	*Curcuma aromatic*	Wistar albino rats	Significant cardioprotection and functional recovery demonstrated by *Curcuma aromatic* may be attributed to its anti-apoptotic property	PMID: 16504000
P. rhizome	Da-Chai-Hu-Tang:*Bupleuri radix*, *Pinellia rhizome*, *Scutellariae radix*, *Paeoniae radix*, *Zizyphi fructus*, *Aurantii fructus immaturus*, *Zingiberis rhizoma* and *Rhei rhizoma*	Rabbit	Da-Chai-Hu-Tang has the inhibit effect on the progression of atherosclerotic lesions	PMID: 10030725
R. astragali	*Radix Astragali*	Rat	*Radix Astragali* effectively protected against cardiac functional and morphological aberrations in experimental autoimmune myocarditis	PMID: 18782607
A. macrocephala	*Atractylodes macrocephala*	Rat	Polysaccharides extract of *Atractylodes macrocephala* may enhance immunity and improve heart function in aged rats	PMID: 22777209

REN (IC_{50} of $144.3 \pm 17.3, 152.9 \pm 15.6$, and $60.1 \pm 8.5\,\mu M$, respectively). Among them, REN showed the greatest inhibitory effect compared to the effects on ACE and PTGS2 ($p < 0.05$). Analogously, like Salvianolic acid B, Rosmarinicacid revealed much weaker inhibitory properties against the four targets than Salvianolic acid A. The data suggest that Salvianolic acid A is the strongest inhibitor against the four targets among the three compounds in *S. miltiorrhiza*.

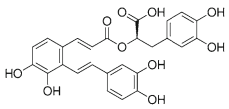
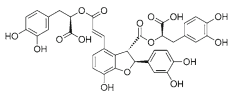
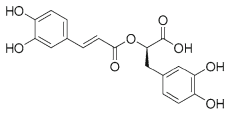
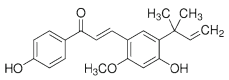
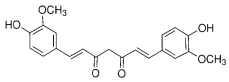
Licochalcone A

Licochalcone A exhibited relatively strong activity against ACE with IC_{50} of $46.9 \pm 6.4\,\mu M$. However, it revealed

Table 3 The information of the five selected compounds for experimental verification

Number	Compound	OB	DL	Gold Scores				Herb
				ACE	REN	F2	PTGS2	
M16	Salvianolic acid A	2.96	0.70	79.13	65.5	79.05	73.77	*S. miltiorrhiza*
M18	Rosmarinicacid	1.38	0.35	67.02	50.61	62.59	58.39	*S. miltiorrhiza*
M33	Salvianolic acid B	3.01	0.41	83.47	83.19	80.45	74.79	*S. miltiorrhiza*
M157	Licochalcone A	45.74	0.33	58.66	55.86	61.18	57.32	*G. uralensis*
M194	Curcumin	2.18	0.41	73.43	56.54	76.2	65.43	*C. aromatica*

Table 4 Structures and IC50 values for the five selected compounds

Chemical name	Structure	Targets inhibition assay (IC50 (μM): mean ± SD)			
		ACE	PTGS2	REN	F2
Salvianolic acid A		27.3 ± 3.2	8.3 ± 1.0	24.4 ± 3.1	144.7 ± 17.0
Salvianolic acid B		86.9 ± 11.1	28.7 ± 3.5	24.9 ± 3.2	>500
Rosmarinicacid		144.3 ± 17.3	152.9 ± 15.6	60.1 ± 8.5	>500
Licochalcone A		46.9 ± 6.4	>500	>500	>500
Curcumin		278.0 ± 35.4	55.8 ± 8.1	55.9 ± 7.0	>500

weaker effects against PTGS2, REN and F2 (IC_{50} > 500 μM, $P < 0.05$) than ACE, suggesting that Licochalcone A is more critically involved in inhibiting ACE than the combination of the four targets.

Curcumin. Curcumin exerted the highest inhibitory activities against both PTGS2 and REN, with IC_{50} values of 55.8 ± 8.1 and 55.9 ± 7.0 μM, respectively. As for ACE (278.0 ± 35.4 μM), Curcumin inhibited it to a weaker extent than the former two proteins ($p < 0.05$). In addition, Curcumin has also been found to inhibit F2 in a dose-independent manner (IC_{50} > 500 μM), indicating that Curcumin is a low-affinity binder to F2.

Binding mode

As shown in Fig. 5, Salvianolic acid A was selected as a template to show the docking mode of this class of inhibitors and reveal the main interactions within the enzyme active sites. The docking scores for Salvianolic acid A with ACE, REN, F2, and PTGS2 are 79.13, 65.50, 79.05, and 73.77, respectively. The interactions consisted of H-bonds, zinc-anion interactions, cation-π and $\pi-\pi$ stacking. (1) The residues VAL379 and ASP415 of ACE both formed H-bonds with the Salvianolic acid A molecule. Further, the close contact of Salvianolic acid A on the zinc-binding site (purple ball) of ACE may facilitate chelation of metal ions surrounded by ACE (Fig. 5a). (2) Salvianolic acid A interacted with F2 through forming H-bonds with ASN143, GLU203, SER262, TYR240, PHE239, and ARG233 (Fig. 5b), suggesting that extensive hydrogen bonding are important for the favorable conformation. (3) Salvianolic acid A exhibited direct interactions with PTGS2 by $\pi-\pi$ interactions at PHE487 and cation-π interaction at ARG89 (Fig. 5c). (4) In Fig. 5d, Salvianolic acid A was bounded by the formed hydrophobic interaction with ILE118, GLN16, ALA226, and TYR159. The phenyl ring of Salvianolic acid A also produced a strong $\pi-\pi$ interaction with TYR80. The implication of correct mode of ligand–protein binding is extremely important in drug discovery.

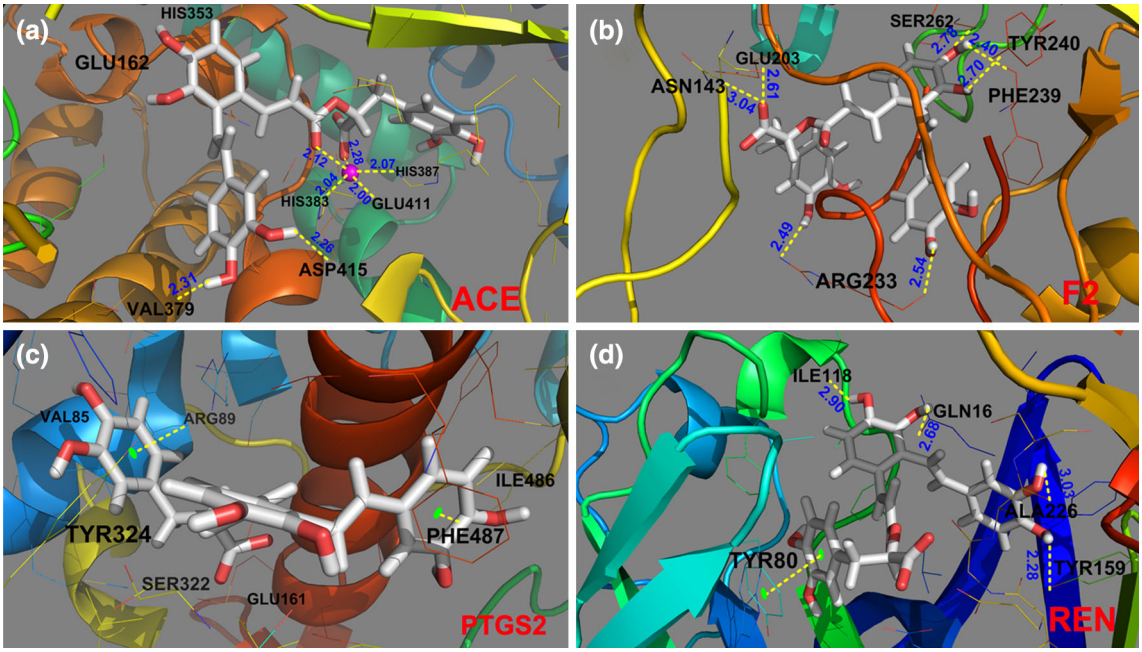


Fig. 5 Molecular models of Salvianolic acid A in the binding sites of ACE, F2, PTGS2, and REN. The *dashed lines* show the formation and distance of the hydrogen bonds. Active site amino acid residues are represented as *lines*. **a** Representative interactions between Salvianolic acid A and ACE. **b** Representative interactions between Salvianolic acid A and F2. **c** Representative interactions between Salvianolic acid A and PTGS2. **d** Representative interactions between Salvianolic acid A and REN

Discussion

Drug discovery has been subjected to evolutions through the ages, moving from one drug acting on a single receptor to computational multi-target approaches. However, the immense progress in genomic, proteomic, HTS, and rational drug design technologies tend to outstrip the increased number of approved single target drugs. It is currently evident that this process is not as effective as expected for the resulting hits often lack efficacy in vivo. In this study, we explored a new systems pharmacology method to discover MTDs for CVDs. As the technical route to the ultimate ideal of systems pharmacology, network pharmacology is changing the traditional drug discovery process by shifting from one drug–one target level to higher levels of biological systems. Thus, the first challenge is to find the set of systems targets that have desirable clinical effects.

Network analysis for identification of key targets

To find the relevant target-sets of MTDs, a network-driven approach was applied. In the notion of network, the complex system is considered as a range of node interactions linked by edges. In this study, we analyze the hubs and the centric elements of the network [24] to find the key targets. Compared to traditional target finding methods, such as experimental verification, the network-driven approach, topology analysis stresses the target action impacts on the entire network. Therefore, four therapeutic targets, i.e., PTGS2, F2, ACE, and REN were obtained, and we found that:

(1) The four targets are of high degree (Table 1), as the high-degree nodes often play more important role in a network than other low-degree nodes [32].
(2) The four targets affect the various aspects of CVDs. The treatment of CVDs usually involves anti-inflammation, anti-thrombus, and controlling high blood pressure. Of particular note, PTGS2 has been shown to play a key role in the regulation of inflammation [33], which plays a central role at all stages of atherosclerosis—the main cause of clinical CVDs events. As for F2, the main executioner of the coagulation cascade and platelet aggregation [33], it eventually promotes the formation of blood clots. ACE could convert angiotensin I into angiotensin II, which is a vasoconstrictor and thus regulates blood pressure. In addition, the substrate of ACE is the product of its upstream target REN, the first enzyme of the classic systemic renin-angiotensin system. Hence, REN also plays a key role in blood pressure regulation.
(3) The four targets locate on the complementary pathological pathways. The protein PTGS2 locates on the cyclooxygenase pathway, which could metabolize arachidonic acid into prostanoids. And there is mount-

ing evidence that some of these metabolic products play critical roles in CVDs [34]. F2 is a key protein in neuroactive ligand-receptor interaction pathway, which is well known in the development and progress of CVDs processes such as coronary heart disease. In this pathway, the F2 receptor is closely related to the cardiac function [35]. ACE sits at the downstream of the hypertrophic cardiomyopathy (HCM) pathway, which is directly related to the genetic CVD HCM [36]. REN is an important component of the renin-angiotensin system pathway, which plays a vital role in the pathogenesis of CVDs that major regulates blood pressure and fluid and electrolyte homeostasis [37].

Systems level MTDs discovery

A battery of in silico methods to prescreen the potential MTDs among the promising herbs has been broadly adopted for speeding up lead compound discovery with multi-targets [10,11,13,38]. In this work, a geometrical matching-based docking method has been employed in screening the active compound and probing the binding modes for ligands [39]. In this method, there is no need for structural features of the active compounds and thus appropriates for the comprehensive screening in our large compound library (1,780 molecules of fourteen herbs). Our results clearly show that 380 common molecules (21 %) hit the four targets in theory. To some extent, it illustrates that multi-target agents are abundant in nature in number which is consistent with the multi-target feature of herb medicines.

Pharmacokinetic properties (absorption, distribution, metabolism, and excretion of drugs) are critical processes in drug discovery and development. Two reliable in silico models were applied to filter compounds with reasonable oral bioavailability and drug-likeness properties. The wealth of potential MTDs that was obtained in our study indirectly proved the high efficiency of our approach. Of particular note, the five tested compounds all are orally bioavailable or drug-like. Of these, Salvianolic acid A, Rosmarinicacid, and Salvianolic acid B belong to *S. miltiorrhiza*, and are highly effective in facilitating microcirculation and coronary vasodilatation, inhibiting thromboxane formation and suppressing platelet adhesion and aggregation [40]. As for Licochalcone A, which has a relatively high OB value, there is evidence that the corresponding herb *G. uralensis* consumption enhances vasoconstriction action in vascular smooth muscles [41]. Curcumin, the ingredient of *C. aromatic*, is able to invigorate circulation, reduce stasis, and inhibit inflammation [42]. The effects of these three herbs are in accordance with our results described in Table 2.

Multiple targets

MTDs enhance therapeutic efficacy by collective adjusting on a primary target and the regulation on compensatory targets. In today's world, instead of the "one disease-one target-fits-all" philosophy, most people are increasingly aware of the MTDs superiority in restoring healthy state. Then, what of the roles that herbs may play in this?

Indeed, many drug candidates with much potential for further development are from natural sources as pharmacological tools by triggering the switch of multi-target states. For example, the water-soluble phenolic acid Salvianolic acid A is active against targets involved in antiplatelet and antithrombotic functions [43,44]. And Curcumin can decrease blood total cholesterol and low-density lipoprotein-cholesterol level [45]. Our results propose that herbal ingredients that normally bind to a series of functionally related/complementary targets have great potential for targeting multi-targets, which is worth more attentions for both orthodox and alternative medicine.

Low binding affinity

Low-affinity binders are molecules and drugs that interact with cellular proteins through low-affinity physical interactions. Unlike many molecules that target a single protein, MTDs usually interact with multiple targets in a weak-bonded way [2]. A fundamental question raised is whether the weak binding can regulate body system equally or worse than the high-affinity molecules? Examining the track record in biological networks for drug development over the last decade has indicated that the essentiality of the multiple nodes is determined by the inherent redundancy, multiplicity, and system regulation of the biological network. For example, Nelfinavir is a multi-target anticancer drug with weaker binding affinity than the single-target inhibitors, but shows strong positive pharmacological effects [46].

In this work, our five compounds were effective in targeting key proteins. In particular, Salvianolic acid A revealed inhibitory effects for the four targets, i.e., PTGS2, ACE, REN, and F2. Salvianolic acid B, Rosmarinicacid, and Curcumin exhibited significant inhibitory activity against the four targets except for F2. However, Licochalcone A solely showed significant activity on ACE. The results might demonstrate that the MTDs for CVDs are often low-affinity binders. However, why these low-affinity binders are effective in the therapy of CVDs? The main reasons are as follows:

(1) MTDs targeted-proteins that locate at the same pathway. Among the four combined targets, both proteins ACE and REN site on renin-angiotensin system (Fig. 6). Triggering the unilateral renal artery stenosis renin-angiotensin system is of much concern to the hyper-

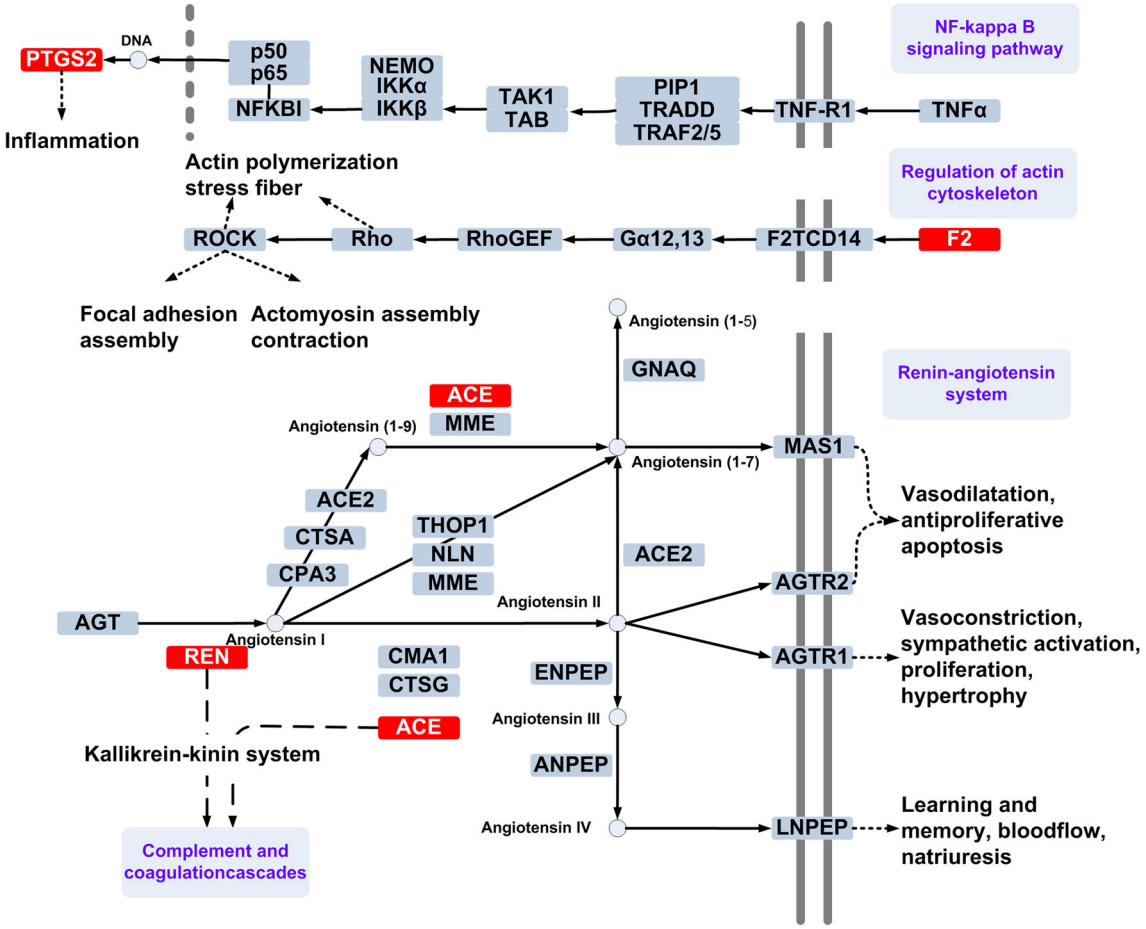


Fig. 6 Distribution of the four targets on corresponding signaling pathways

tension advance and balance disturbance [47]. Thus, compounds such as Salvianolic acid A and Salvianolic acid B that with regard to blocking the ACE and REN might allow for collective inhibition of the renin-angiotensin system pathway, which represents evidence-based mechanism for restoring physiological balance of CVDs through timely targeted interventions.

(2) MTDs targeted-proteins located at the upstream of a pathway. Normally, a drug molecule that interacts with a protein upstream of a signaling pathway perturbs the cellular network larger than that of a downstream target [48]. Protein F2 is an upstream target that regulates the actin cytoskeleton pathway (Fig. 6), which is critical for maintenance of endothelial barrier functions, and alterations the expression level [49]. Studies show that vascular endothelial factors play a crucial role in the regulation of blood pressure [50] and blood clot [51] as well as the underlying pathology of atherosclerosis [52]. Therefore, early endothelial dysfunction interventions are necessary for delaying and controlling the development of cardiovascular events. The experiment verified Salvianolic acid A with respect to disturbing F2 might improve the clinical symptoms of CVDs by this signaling cascade.

(3) MTDs targeted-proteins located in complementary pathways. Evidence supports that CVDs are closely associated with the cytoskeleton pathway and the renin–angiotensin system pathway as well as the regulation of actin cytoskeleton pathway [34]. These pathways regulate the different function modules of CVDs: the cyclooxygenase pathway closely relates to inflammation; the renin-angiotensin system pathway involves in blood pressure regulation; the regulation of actin cytoskeleton pathway strongly connects to blood clot (Fig. 6). In this study, Rosmarinicacid and Curcumin that target proteins ACE, REN, and PTGS2 help body restore balance by regulating these signal pathways. This indicates that MTDs exhibit therapeutic effects by acting on a set of complementary pathways.

Conclusion and perspective

Despite drug development is a fast-developing domain, the multi-target drug discovery is still in its infant state at

present. At present, drug design is shifting to a systems pharmacology-based approach which improves the target identification success rate. In this work, we have highlighted the principles and applications of the rNTS approach in MTDs discovery for a specific disease. The "wet" experiment proves the effectiveness of this strategy to combine targets, discover the potential agents. The novelty of our rNTS approach is reflected by the combination of network targeting and biologically active compound screening. We list a few main findings for further directions in the development of MTDs:

(1) The proposed network analysis approach is capable of selecting a set of primary targets.
(2) The molecular docking and pharmacokinetic evaluation help to summarize the interactions of a compound with the therapeutically combined targets.
(3) The rNTS approach, combining with computational and experimental analyses, offers a path to find potential combination targets for treating a specific disease.

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