Original Article Systems pharmacology-based dissection of the active ingredients and targets of Yiqi Zishen formula for application to COPD

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Abstract: In this work, a systems pharmacology model based on the pharmacokinetic analysis, drug targeting, and drug-target-disease network analyses, was applied specifically to uncover the active ingredients and therapeutic targets of Yiqi Zishen formula (YZF). Furthermore, a rat model of cigarette smoke- and bacterial infection-induced chronic obstructive pulmonary disease (COPD) was applied to evaluate the effects of YZF on COPD and its comorbidity. The expression of interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , soluble TNF- α receptor (sTNFR2), matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of MMP (TIMP)-1, endothelin (ET)-1, transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) were analyzed by immunohistochemistry. The pharmacological system efficiently generated 158 active compounds from YZF, and predicted 192 potential targets. The result showed that there was a significant target overlap between the 12 herbs in the YZF formula, which means that each herb of YZF connected with the similar targets, implying the synergistic effects among them. The target-disease network results indicated that YZF was effective in treating various pathological conditions, including respiratory tract diseases, cardiovascular disease, immune system diseases, and nervous system diseases. The therapeutic mechanisms of YZF were probably associated with modulation of inflammatory response, immune responses, matrix metalloproteinases expression, among others. In follow-up experiments, we found that YZF was effective for the treatment of COPD and its comorbidity such as ventricular hypertrophy, by inhibiting the expression of inflammatory cytokine, matrix metalloproteinases, and hypertrophic stimuli and collagen, in vivo.

Keywords: Systems pharmacology, chronic obstructive pulmonary disease, Yiqi Zishen formula, COPD rats

Introduction

Chronic obstructive pulmonary disease (COPD) is a serious public health problem and huge social burden that is the fifth leading cause of death worldwide, and its incidence is expected to increase in the next few decades [1, 2]. Although numerous drugs and medications have already been employed on COPD, patients still experience a poor prognosis because of the lack of effective therapeutic agents [3, 4]. Therefore, developing novel therapeutic drugs are still in urgent need. As one of these effective, Traditional Chinese Medicine (TCM) provided an effective treating approach to COPD [5].

Yiqi Zishen formula (YZF), a TCM recipe, is composed of thirteen medicinal herbs and has been used for the treatment of COPD. Clinical studies demonstrated that YZF provided effective relief of symptoms in COPD patients, such as alleviating clinical symptoms of stable COPD patients, reducing the exacerbation frequency, delaying acute exacerbation, and improving pulmonary function [6]. However, acceptance of YZF, a TCM, within modern biomedical practice has been restricted by the absence of knowl-

Name	Latin Name	Source	Amount (g)
Ginseng Radix et Rhizoma	Panax ginseng C.A.Mey., Radix et Rhizoma	Jiling	9
Polygonati Rhizoma	Polygonatum kingianum Collett & Hemsl., Rhizoma	Hebei	15
Schisandrae Chinensis Fructus	Schisandra arisanensis Hayata, Fructus,	Liaoning	9
Lycii Fructus	Lycium barbarum L., Fructus	Ningxia	12
Rehmanniae Radix	Rehmannia glutinosa (Gaertn.) DC., Radix,	Henan	15
Fritillariae Thunbergii Bulbus	Fritillaria thunbergii Miq., Bulbus	Zhejiang	9
Moutan Cortex	Paeonia × suffruticosa Andrews, Cortex	Anhui	12
Perillae Fructus	Perilla frutescens (L.) Britton, Fructus	Hubei	9
Stemonae Radix	Stemona tuberosa Lour., Radix	Sichuan	9
Citri Reticulatae Pericarpium	Citrus sinensis (L.) Osbeck, Pericarpium)	Guangdong	9
Ophiopogonis Radix	Ophiopogon japonicus (Thunb.) Ker Gawl., Radix	Zhejiang	15
Cinnamomi Cortex	Cinnamomum cassia (L.) J.Presl, Cortex	Guangxi	3
Pheretima	Pheretima aspergillum (E. Perrier)	Henan	12

Table 1. Thirteen medicinal herbs contained in Yiqi Zishen formula

edge of the active ingredients involved, and its therapeutic mechanisms of action.

However, studies on the active compounds and molecular mechanisms underlying the therapeutic effects of TCM, such as YZF, face various difficulties. For instance, tens of thousands active ingredients involved in the medicinal herbs make it hard to identify active compound [7]. During the past few decades, TCM studies have followed a rigid route of isolation, structure identification, and pharmacological research for exploring active compounds, which is much time-consuming and costly. Moreover, it is difficult to identify the targets of the Chinese herb medicines, because multiple ingredients contained in most formulas [8, 9]. Thus, a svstems method which could clarify the main active compounds in the herbal drugs, and identify the therapeutic targets of the compounds, is necessary.

Systems pharmacology is a promising approach to overcome these difficulties. Its technological platforms combine oral bioavailability prediction, multiple drug targets prediction and drugtarget-disease network analysis to get a global understanding of therapeutic mechanisms of TCM actions [10, 11]. In present study, a systems pharmacological model by combining active compounds identification, targets prediction, and network pharmacology techniques, was applied to shed new lights on the effects and mechanisms of YZF. Furthermore, we presented *in vivo* experimental evidence to verify the effects and mechanisms of YZF that were obtained from the computational experiment (systems pharmacology). We administered YZF to COPD rats, and investigated the effect of YZF on cigarette smoke- and bacterial infectioninduced respiratory dysfunction, pulmonary inflammatory responses, collagen deposition, protease-antiprotease imbalance and hypertrophic stimuli factor expression.

Methods

Chemicals and animals

Klebsiella pneumoniae (strain ID: 46114) was obtained from the National Center for Medical Culture Collection (CMCC, Beijing, China). Aminophylline was purchased from Shandong Xinhua Pharmaceutical Co., LTD. (Shandong, China). Antibodies against interleukin (IL)-6, IL-1 β , tumor necrosis factor (TNF)- α , soluble TNF-α receptor 2 (sTNFR2), collagen I, collagen III, collagen IV, endothelin (ET)-1, transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), matrix metalloproteinase (MMP)-2, MMP-9, and tissue inhibitor of MMP (TIMP)-1. were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Eosin alcohol solution and Mayer's hematoxylin were purchased from MUTO Pure Chemicals (Tokyo, Japan). Forty-two Sprague-Dawley rats (21 females and 21 males, 200 ± 20 g) were supplied by the Experimental Animal Center of Henan province (Zhengzhou, China). The rats were raised under controlled temperature (25 \pm 2°C), humidity $(50 \pm 10\%)$, and daily light intensity (12 h of light), and were fed with standard laboratory food and water ad libitum. All animals were handled with humane care throughout the experiment. The components of YZF were showed in **Table 1**. The herbs were identified and made in fluid extract based on the standard operating procedure of the Department of Pharmaceutics of the First Affiliated Hospital, Henan University of Traditional Chinese Medicine, Zhengzhou, China.

Dataset construction

All compounds from the 13 herbs of YZF were obtained from the Chinese Academy of Sciences Chemistry Database (http://www.organchem.csdb.cn), Chinese Herbal Drug Database, and the literature. Considering that all glucosides compounds can be easily hydrolyzed to their deglycosylation products by enteric bacteria in the intestinal tract, the glucosides and their deglycosylation products were additionally obtained as the constituents of herb drugs [11, 12]. Finally, a total of 1137 compounds were included: 190 in Ginseng Radix et Rhizoma (GRR), 38 in Polygonati Rhizoma (PR), 130 in Schisandrae Chinensis Fructus (SCF), 188 in Lycii Fructus (LF), 76 in Rehmanniae Radix (RR), 17 in Fritillariae Thunbergii Bulbus (FTB), 55 in Moutan Cortex (MC), 128 in Perillae Fructus (PF), 110 in Stemonae Radix (SR), 64 in Citri Reticulatae Pericarpium (CRP), 13 in Ophiopogonis Radix (OR), 100 in Cinnamomi Cortex (CC), 28 in Pheretima (Ph) (see Table S1, Availability of data and materials).

Oral bioavailability and drug-likeness prediction

To analyze the druggability of herbs on molecular level, this work was structured to incorporate two important properties, including oral bioavailability (OB) and drug-likeness index. Generally, the OB values, one of the most commonly used pharmacokinetic parameters in drug screening, were predicted by a robust *in silico* model OBioavail 1.1 [13]. Similarly, druglikeness was applied to investigate the structural similarity between herbal ingredients and drugs in DrugBank database, and prediction method was shown as follow:

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

Where x represents the herbal ingredients, and y represents the average molecular drug-likeness index of all drugs in DrugBank database. Then, the compounds with $OB \ge 30\%$ and drug-likeness index ≥ 0.18 were obtained as candidate compounds for further analysis. In addition, several compounds such as hesperidin, tangeretin, cinnamaldehyde, and paeonol initially were omitted based on these screening criteria, however, these compounds were supported by literature evidences, and therefore, also were obtained as candidate compounds [14-17].

Target and disease prediction

As described in the previous work, we used the systematic drug targeting tool to screen the targets of candidate compounds. We only selected target proteins with random forest score \geq 0.7 and support vector machine score \geq 0.8, which were obtained as potential targets of the candidate compounds [18].

The potential targets were projected into TTD and DrugBank to collect their corresponding diseases, and the diseases were divided into different categories based on the MeSH Browser (2014 MeSH).

Network construction

The candidate compounds, potential targets and their related diseases were applied to construct the compound-target and target-disease network. The networks were constructed by Cytoscape 3.2.1. Network Analysis plugin of Cytoscape was utilized to analyze the topological properties of the networks [18-21]. The "degree" defined as the number of links to node. Nodes with high degree can be considered the key nodes in a network.

COPD model and drug administration [22]

The rats were placed inside a closed box connected to the smoke source. Generally, rats were exposed to the tobacco (Hongqi Canal® Filter tip cigarette; tobacco type, tar: 10 mg; nicotine content: 1.0 mg; carbon monoxide: 12 mg, Henan Tobacco Industry, Zhengzhou, China) smoke of 8 cigarettes for 30 min, twice per day with 3 h smoke-free intervals during the first two weeks, and to the smoke of 15 cigarettes for 30 min, thrice per day with 3 h smokefree interval from weeks 3 to 12. In addition, *Klebsiella pneumoniae* dilution $(6 \times 10^8 \text{ CFU}/\text{mL}, 0.1 \text{ mL})$ was dropped in an alternate fashion into the rat's nostrils every 5 days from weeks 1 to 8. At the end of week 8, two COPD rats were sacrificed, and lung tissues were collected to validate whether this rat model was successful.

On week 9, COPD rats were divided into three groups with 10 rats each. Then, COPD rats were treated with normal saline (2 mL), YZF (4.84 g/kg), and aminophylline (2.3 mg/kg) by gavage every day for 12 weeks. The control rats (n = 10) also were treated with normal saline (2) mL) by gavage every day for 12 weeks. All rats were sacrificed at week 20. The heart and lung tissues were collected and stored at refrigerator temperatures. The experimental protocol was approved by the Experimental Animal Care and Ethics Committee of the First Affiliated Hospital, Henan University of Traditional Chinese Medicine. The animal experiments were conducted with the approval of the Committee on the Care and Use of Laboratory Animals of the First Affiliated Hospital (2012HLD-0001). Henan University of Traditional Chinese Medicine, China.

Pulmonary function analysis

Pulmonary function data were detected by a sealed unrestrained whole body plethysmography (Buxco Electronics, Troy, NY, USA.) connected to a transducer and computer every fourth week from weeks 0 to 20. The changes of rat respiratory function were converted to electrical signals through a pressure transducer and amplifier, and processed by computer. Finally, this work focused on three measures: tidal volume (TV), peak expiratory flow (PEF) and 50% tidal volume expiratory flow (EF50).

Histological analyses

The heart and lung tissue specimens were fixed with neutral 10% buffered formaldehyde, embedded in paraffin, sliced into 4-µm-thick slices, and supplied for histological examination. Sections were stained with Mayer's hematoxylin and 1% eosin alcohol solution (H&E staining). Samples were inspected with the aid of an Olympus BX51 microscope (Tokyo, Japan).

Alveolar number, alveolar diameter, small pulmonary vessels bronchial wall thickness

and bronchiole stenosis scores were evaluated using Image-Pro Plus® (IPP) 6.0 software (Media Cybernetics, MD, USA). Bronchia and lung injury scores were measured under an optical microscope.

Immunohistochemistry (IHC) was used to determine ET-1, TGF-β, VEGF, bFGF, MMP-2, MMP-9, TIMP-1, IL-6, IL-1β, TNF-α, sTNFR2 and collagen I, III, IV expression. Briefly, antigen retrieval was performed before staining; the primary antibodies were diluted by 1:100 and incubated overnight. After the samples were fully washed with 0.3% Triton X-100 in phosphate buffer, sections were incubated with the secondary antibody, and chromogen substrate reagent DAB were used to detect the antigen. A brown color reaction with distinct morphology was developed with DAB in the peroxidase system. Then, sections were counter-stained with hematoxylin. Finally, Samples were inspected and measured using Image-Pro Plus® (IPP) 6.0 software (Media Cybernetics, MD, USA).

Right ventricular hypertrophy index (RVHI)

After removing the arterial and adipose tissue on the epicardium, the right ventricle (RV), left ventricle (LV) and interventricular septum (S) were separated and weighed. The RVHI was calculated using the equation [23]:

RVHI = RV/(LV + S)(2)

Myocardial ultrastructure

Small right ventricle tissue samples were post fixed in buffered glutaraldehyde and osmium tetroxide, dehydrated in a series of ethanol and propylene oxide infiltration, and finally embedded in epoxy resin. Then, ultra-thin sections with 70-nm thickness were cut and stained with uranyl acetate. Changes in the muscular fibers and mitochondria of the myocardial ultrastructure were tested by transmission electron microscopy (TEM).

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using the cDNA cycle kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RT-PCR was per-



Figure 1. Compound-target network. The compound-target network was built by linking the candidate compounds of 12 herbs and all their candidate targets. The nodes represent herbs (orange squares), candidate compounds (blue rhombus) and targets (green ellipse). (GRR, Ginseng Radixet Rhizoma; PR, Polygonati Rhizoma; SCF, Schisandrae Chinensis Fructus; LF, Lycii Fructus; RR, Rehmanniae Radix; FTB, Fritillariae Thunbergii Bulbus; MC, Moutan Cortex; PF, Perillae Fructus; SR, Stemonae Radix; CRP, Citri Reticulatae Pericarpium; OR, Ophiopogonis Radix; CC, Cinnamomi Cortex).

formed using the iCycler real-time PCR amplifier (Bio-Rad, Hercules, CA, USA). Each PCR was performed in triplicate, and each experiment was repeated twice. The results were normalized with β -actin.

Statistical analysis

Statistical differences between groups were tested by one-way analysis of variance with the SPSS 19.0 software package (IBM Corporation, Armonk, NY, USA). Values are expressed as mean \pm standard error of mean. For all tests, a two-sided *p* value less than 0.05 was considered significant.

Results and discussion

Candidate compounds screening

In TCM therapy, the oral administration is the main way to deliver drugs to systemic circulation [24-26]. Furthermore, drug-like compounds contain functional groups and have physical properties that are consistent with the majority of known drugs [27]. Therefore, the evaluation of OB and drug likeness property was indispensable to determine whether a compound is pharmacologically active in TCM formula. In YZF, 138 potential compounds were predicted satisfactorily to have higher OB (\geq 30%) and drug-likeness index (\geq 0.18). In addition, some other active compounds and main ingredients of herbal drug such as paeonol, hesperidin and cinnamaldehyde shown poor OB (\leq 30%) or drug-likeness index (\leq 0.18), that were also obtained as the candidate compounds for further analysis [14, 15, 17]. We also found that all ingredients of Pheretima with relatively poor OB or drug-likeness index were removed from the candidate compounds. Based on above analyses, 178 compounds of the 12 herbs were obtained as candidate compounds, including the 138 readily absorbed compounds and the 40 pharmacologically active compounds (see Table S2, Availability of data and materials). The numbers of candidate compounds in GRR, PR, OR, SCF, LF, RR, CC, FTB, Ph, MC, PF, SR, and CRP was 22, 12, 13, 10, 45, 12, 9, 9, 12, 13, 16, 32, and 9, respectively.

Among these, ginsenoside rh2, hesperidin, cinnamaldehyde, and paeonol, the active compounds of GRR, CRP, CC, and MC, were demon-



Figure 2. ClueGO analysis of the candidate targets. Functionally grouped network with terms as nodes linked, and functionally related groups partially overlap. The node size represents the term enrichment significance. The node pie charts represent the molecular function, immunesystem processes, reactome analysis of each target have corresponding to their networks in this target set. The label of only the significant term per group was shown. A. Representative molecular function interactions among targets. B. Representative immune system processes interactions among targets. C. Representative reactome analysis interactions among predicted targets.

strated to display a broad range of pharmacological activities including antitumor, antiviral, antifungal and antiprotozoan activities [16, 17, 28, 29]. Similarly, stigmasterol and kaempferol, which are contained widely in herbs such as GRR, LF, RR, MC, PF, and SR, have been shown to possess extensive biological and pharmacological properties [30-32].

Target prediction

Generally, a broad group of active compounds contained in TCM formula can effectively prevent and treat the complex diseases in a synergistic manner. In order to clarify the molecular mechanism of such effect of TCM formula, such as YZF, it is essential to obtain the therapeutic targets of active compounds. In our study, we discovered the potential targets of candidate compounds by the pharmacophore modeling technique. The results showed that 192 candidate targets were predicted for the 158 candidate compounds, and 20 candidate compounds had no potential target according to this criterion (see Table S3, Availability of data and materials). Finally, 158 candidate compounds connected with 192 potential targets and the connections between them reached 1756.

The numbers of potential targets related to GRR, PR, OR, SCF, LF, RR, CC, FTB, Ph, MC, PF, SR, and CRP were 101, 70, 75, 26, 132, 48, 38, 58, 110, 108, 89 and 67, respectively. Although the numbers of target related to each herbal drug in YZF were different, there was a significant target overlap between the 12 herbs in YZF, which means that each herb could connect with similar targets, suggesting the potential synergistic effects among them.

For example, pelargonidin (from FTB), catechin (from MC), fumarine (from GRR), and paeonol (from MC) have been shown to attenuate the function of prostaglandin G/H synthase 2 (PTGS1/2), which plays multiple physiological roles including vascular protection and reproduction, and mediates many important patho-

logical conditions, notably inflammation and tumorigenesis [17, 33-35].

Compound-target network

In order to analyze the effects and pharmacological mechanisms of YZF at a systems level, we constructed the compound-target network. Here, we mapped the herbs, candidate compounds and potential targets onto the drugtarget networks. As shown in Figure 1, the network consisted of 362 nodes (12 herbs, 158 candidate compounds and 192 potential targets), 202 herb-compound interactions and 1756 compound-target interactions, resulting in average number of targets per active compound of 1.21. Among the 158 candidate compounds, 44 compounds possess degree (the number of targets related to the compound) larger than 12 (an average value of 11.1), which implied that most compounds bind to multiple targets. For instance, quercetin, kaempferol, and beta-sitosterol, had the highest degree distributions, and hit 87, 55 and 51 potential targets, respectively. To be specific, kaempferol had high affinities with mitogen-activated protein kinase 14, inducible nitric oxide synthase (NOS2), and PTGS1/2, which mediates a number of pathological conditions including inflammation, immunomodulatory, and oxidative stress [33, 36, 37].

On the other hand, it also suggested that lots of targets were hit by multiple compounds in the compound-target network. Take the example of estrogen receptor, the highest connected target, connected with 66% (105 of 158) of the candidate compounds. Followed by androgen receptor (degree = 96), dipeptidyl peptidase IV (degree = 72), prostaglandin G/H synthase 2 (degree = 67), thrombin (degree = 59), acetyl-cholinesterase (degree = 47), NOS2 (degree = 44), carbonic anhydrase II (degree = 40), trypsin-1 (degree = 38), peroxisome proliferator activated receptor gamma (PPARy) (degree = 35), prostaglandin G/H synthase 1 (degree = 35),



Figure 3. Target-disease network. In the target-disease network, candidate targets were connected with related diseases. 192 target proteins (green ellipse) were connected to 350 diseases (blue rhombus) which were classified into 18 groups (orange square) according to Medical Subject Headings.



Figure 4. Effect of Yiqi Zishen formula (YZF) on pulmonary function of chronic obstructive pulmonary disease (COPD) rats. COPD rats were intragastricly administered with YZF (4.84 g/kg) once daily from weeks 9 to 20. Model was treated with normal vehicle, and positive control was intragastricly administered with aminophylline (APL, 2.3 mg/kg) once daily from weeks 9 to 20. Tidal volume (TV) (A), peak expiratory flow (PEF) (B), and 50% tidal volume expiratory flow (EF50) (C) were detected every fourth week from weeks 0 to 20. Values represent the means \pm SEM. *P < .05 vs. model.

35). Among these, NOS2, PPARγ and PTGS1/2 play important pathological roles including inflammation and immune system activation [33, 37, 38]. Above results indicated that candidate compounds with high degree may play important synergistic roles in YZF pharmacological network.

In order to further clarify the therapeutic mechanism of the potential targets, ClueGO, a Cytoscape plugin, was used to analyze biological interpretation and interrelations of functional groups in biological networks [39]. As shown

in Figure 2, the results were classified into three stratums: molecular functions, immunesystem processes, and reactome analysis. The molecular functions mainly contained five categories: coenzyme binding, oxidoreductase activity, MAP kinase activity, G-protein coupled amine receptor activity, and neurotransmitter receptor activity, which implied that most potent targets were related to oxidoreductase activity and MAP kinase activity (Figure 2A). Consequently, immunesystem processes of the targets was mainly defined as positive regulation of immune effector process, toll-like receptor 10 signaling pathway, leukocyte tethering or rolling, and response to interferon-gamma (Figure 2B). Finally, the reactome of the targets are primarily related to activation of the activator protein (AP)-1 family of transcription factors, mitogen-activated protein kinase (MAPK) targets/nuclear events mediated by MAP kinase, cellular responses to stress, and activation of matrix metalloproteinases (Figure 2C). what's more, many different studies showed that above mentioned biological interpretation such as MAP kinase activity, AP-1 family of transcription factors activation and positive regulation of myeloid leukocyte differentiation participated in many pathological conditions including COPD and other respiratory diseases [40-42].

No single mechanism can account for the complex pathology in COPD, and the complex interactions likely occur between different mechanisms. Thus, a single compound or herb is probably insufficient for COPD therapy. While, TCM formulae contain many different compounds that act on multiple targets with potential synergistic effects, and has a great effect on complex diseases. From above results, common targets with multiple biological and pharmacological functions shared by the candidate compounds indicated that YZF had synergistic therapeutic effects on COPD.

Target-disease network

To gain better insight into the YZF related diseases, the diseases related to the potential targets were collected from DrugBank, TTD and PharmGkb databases, and divided into 18 groups based on the MeSH Browser (2014 MeSH) (see <u>Table S4</u>, Availability of data and materials) then built the target-disease network (**Figure 3**), where nodes represent disease cat-



Figure 5. Effects of Yiqi Zishen formula (YZF) on histological changes in stained lung sections of chronic obstructive pulmonary disease (COPD) rats. Histopathologic changes of the lung tissues were analyzed by hematoxylin and eosin staining on week 20 (magnification, $\times 100$) (A). The lung injury scores of all groups were detected (B). bronchial wall thickness (C), bronchiole stenosis (D), Small pulmonary vessels wall thickness (E), alveolar number (F) and alveolar diameter (G) were examined. Values represent means \pm SEM. *P < .05, **P < .01 vs. model.

egories (square, orange), diseases (rhombus, blue) and targets (ellipse, green), and where edges represent that interactions of herb-target-disease. The results suggested that most of target related diseases pertain to neoplasms (84/350), nervous system diseases (67/350), cardiovascular diseases (50/350), and immune system diseases (35/350) apart from respiratory tract diseases (26/350). This also demonstrated that YZF might have great effectiveness not only on respiratory diseases but also on these diseases. Furthermore, above results agreed with the age-old notion that Chinese medicines has extensive activities and being used to treat various diseases in clinic. For instance, NOS2 contribute to the pathogenesis of immunity and inflammation-associated diseases, including infections, obesity-associated metabolic imbalances, cardiovascular diseases, inflammatory bowel disease, asthma, COPD and other airway diseases. Inhibition of NOS2 has been suggested as a therapeutic strategy to treat these diseases [43-45]. In the targetdisease network, inducible nitric oxide synthase also connected with many of these diseases.

Effect of YZF on COPD and its comorbidity

The clinical research findings indicated that YZF was an effective cure for COPD [6]. In this work, the target-disease network demonstrated that YZF had great efficiency for the treatment of various diseases such as respiratory tract disease, nervous system diseases and cardiovascular disease. To provide more experimental evidences for this prediction, we investigated the effects of YZF on COPD rats and its comorbidity, ventricular hypertrophy [46].

To evaluate its anti-COPD activity *in vivo*, COPD rat model was therefore established. As shown in **Figure 4**, compared with model group, YZF and aminophylline, a classical bronchodilator, significantly increased TV, PEF and EF50 in COPD rat at week 20. Meanwhile, lung injury scores, bronchiole wall thickness, small pulmonary vessels wall thickness, bronchiole stenosis and alveolar diameter increased in the



Figure 6. Effect of Yiqi Zishen formula (YZF) on the myocardial ultrastructure and the right ventricular hypertrophy index (RVHI) changes in chronic obstructive pulmonary disease (COPD) rats. The myocardial ultrastructural changes were observed by transmission electron microscope analysis. The muscular fibers (magnification, \times 30,000) (A) and mitochondria (magnification, \times 50,000) (B) of myocardial ultrastructure images were taken on week 20. The sarcomere length (C), mitochondria density (D) and RVHI (E) were also evaluated on week 20. Values represent means \pm SEM. *P < .05, **P < .01 vs. model.



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Figure 7. Effect of Yiqi Zishen formula (YZF) on the expression of VEGF, bFGF, TGF- β , and ET-1 in the right ventricle obtained from chronic obstructive pulmonary disease (COPD) rats. Immunohistochemical analysis for the protein expression of VEGF, bFGF, TGF- β and ET-1 in right ventricle of COPD rats (magnification, ×200) (A). The expression of VEGF, bFGF, TGF- β and ET-1 was quantitatively analyzed (B). Values represent means ± SEM. **P < .01 vs. model. VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; TGF- β , transforming growth factor- β ; and ET-1, endothelin 1.

COPD rat and this increase was markedly inhibited by YZF (**Figure 5A-F**). And YZF treatment also significantly inhibited the reduction of alveolar number in COPD rat (**Figure 5G**). The results demonstrated that YZF was an effective Chinese medicine for COPD treatment.

Comorbidities, defined as other chronic medical conditions, including cardiovascular disease, diabetes mellitus, osteoporosis and muscle weakness, are common in chronic obstructive pulmonary disease (COPD). Lack of treatment of comorbidities and specific case definitions for comorbidities contributed to disease progression in COPD patients [47, 48]. In our work, we found that ventricular hypertrophy was common in COPD rats. Therefore, we investigated the effect of YZF on ventricular hypertrophy.

As shown in **Figure 6A-E**, compare with model group, YZF could significantly increase the myocardial sarcomere length and mitochondrial density of cardiocytes and reversed the RVHI at week 20. Previous researches have demonstrated that ET-1, TGF- β , VEGF, and bFGF can activate a range of hypertrophic signaling mediators, enhance myocardial angiogenesis and finally promote the development and progression of ventricular hypertrophy [49]. In present study, we found that YZF and aminophylline could significantly reduce the level of VEGF, bFGF, TGF- β and ET-1 protein (**Figure 7**).

Above results indicated that YZF treatment had obvious effects on COPD and ventricular hypertrophy, which provided the direct experimental evidences for the target-disease network prediction.

YZF inhibited the inflammatory responses in COPD rats

The inflammatory response in COPD is dominated by cytokines such as IL-6, IL-1 β and TNF- α which are of importance for macrophage activation, matrix metalloproteinase production and acute exacerbations [50, 51]. The systems pharmacology prediction showed that numerous prediction targets of YZF were associated with activation of the AP-1 family of transcription factors and MAPK targets/nuclear events mediated by MAP. Furthermore, many of the inflammatory mediators, expressed in the lung tissues of COPD patients, are controlled by MAPK/AP-1 which is upregulated in alveolar macrophages [48, 52]. Therefore, we investigated the effect of YZF on the inflammatory cytokines expression in the lung of COPD rat. As shown in Figure 8, compared with the model rat, YZF and aminophylline clearly suppressed the expression of IL-1 β , IL-6, TNF- α , and sTNFR2 at week 20. The result indicated that YZF treatment could effectively decrease the levels of inflammatory cytokines in lung, and the findings were consistent with those obtained by target protein function analysis.

YZF inhibited the collagen degradation and protease-antiprotease imbalance in COPD rats

Many studies emphasize that multiple factors are involved in the progression and pathogenesis of COPD, such as protease-antiprotease imbalance and collagen degradation [53]. For instance, proteinases such as neutrophil elastase, proteinase 3, matrix metalloproteinases (MMPs), and cathepsins cleave the components of extracellular matrix, and elastin fibers, which induce lung parenchymal destruction and inflammatory response. Additionally, the collagen degradation in COPD makes great contribution to the persistent tissue injury and has a role in the airflow obstruction characteristic of COPD [54-56]. In our study, the systems pharmacology study indicated that regulation of potential targets probably contributed to activation of MMPs. We thus investigated the effect of YZF on the expression of MMP-2, MMP-9, TIMP-1 and collagens I, III, IV in lung tissues. As shown in Figure 9A, the protein levels of MMP-2 and MMP-9 were significantly decreased by YZF treatment, and the expression of TIMP-1, endogenous inhibitor of MMP, was markedly increased by YZF. This alteration



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Active ingredients and therapeutic mechanism of YZF

Figure 8. Effect of Yiqi Zishen formula (YZF) on the expression levels of IL-1 β , IL-6, TNF- α and sTNFR2 in the lung of chronic obstructive pulmonary disease (COPD) rats. Immunohistochemical analysis for the protein expression of interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , and soluble TNF- α receptor (sTNFR) 2 in lung tissues (magnification, ×200) (A). Quantitative analysis for IL-1 β , IL-6, TNF- α and sTNFR2 expression (B). Values represent means ± SEM. *P < .05, **P < .01 vs. model.



Figure 9. Effect of Yiqi Zishen formula (YZF) on the expression of MMP-2, MMP-9 and TIMP-1 in the lung of chronic obstructive pulmonary disease (COPD) rats. Immunohistochemical and quantitative analysis for the protein expression of matrix metalloproteinase (MMP)-2, MMP-9, and tissue inhibitor of MMP (TIMP)-1 in the lung of COPD rats (magnification, \times 200) (A). RT-PCR analyses of *MMP-2, MMP-9* and *TIMP-1* mRNA levels were detected with reverse transcriptase-polymerase chain reaction (RT-PCR) (B). Values represent means ± SEM. *P < .05, **P < .01 vs. model.



Figure 10. Effect of Yiqi Zishen formula (YZF) on the protein expression of collagens I, III and IV in the lung tissues of chronic obstructive pulmonary disease (COPD) rats. Immunohistochemical staining on lung sections: collagen I, III and IV (magnification, \times 200) (A). The expression levels of collagens I, III and IV were quantitatively analyzed (B). Values represent means \pm SEM. *P < .05, **P < .01 vs. model.

was also observed at mRNA level (Figure 9B). As shown in Figure 10, collagens I, III, IV were

significantly increased in COPD rat, and this increase was clearly inhibited by YZF. Above

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results demonstrated that YZF effectively inhibited the collagen deposition and protease-antiprotease imbalance by modulating the expression of collagens I, III, IV, MMP-2/9 and TIMP-1 in COPD rat.

YZF provided a symptom improvement in patients with COPD in a randomized clinical trial [6]. However, like many other Chinese medicine, the active compounds and therapeutic mechanism of YZF are not clear. To address this important issue, we applied an integrative systems pharmacology approach to investigate the active ingredients and therapeutic targets of YZF. We identified 178 candidate compounds and their related potential targets by using systems pharmacology analytic approach. Followed by ClueGO analysis, we established the potentially functional classification of the targets. Furthermore, we also verified that YZF significantly suppressed the cigarette smoke and bacterial infection-induced COPD and its comorbidity, and achieved its anti-COPD activity by inhibition of pulmonary inflammation, collagen deposition and protease-antiprotease imbalance.

Conclusion

In summary, by using systems pharmacology methods, we identified candidate compounds, potential targets and their potential functions, and compound-target-diseases network involved in various diseases therapy. Particularly, the experimental studies further demonstrated that YZF ameliorated COPD and its comorbidity in vivo by regulating pulmonary inflammatory response, collagen deposition protease-antiprotease imbalance and hypertrophic stimuli factor expression. Overall, present study demonstrated, for the first time, the detailed mechanisms of YZF on COPD in the systems level. Our data provided more details in understanding therapeutic mechanism of TCM, which offer a molecular framework for promoting TCM modernization.

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Disclosure of conflict of interest

None.

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