Therapeutic Role and Potential Mechanism of Astragalus Membranaceus (Huangqi) and Radix Paeoniae Rubra (Chishao) in Idiopathic Pulmonary Fibrosis – Network Pharmacology and Experimental Validation

Huanyu Jiang  
Chengdu University of Traditional Chinese Medicine Affiliated Hospital

Rui Zhou  
Chengdu University of Traditional Chinese Medicine Affiliated Hospital

Liping An  
Chengdu University of Traditional Chinese Medicine Affiliated Hospital

Junfeng Guo  
Chengdu University of Traditional Chinese Medicine Affiliated Hospital

Xinhui Hou  
Chengdu University of Traditional Chinese Medicine Affiliated Hospital

Jiao Tang  
Chengdu University of Traditional Chinese Medicine Affiliated Hospital

Fei Wang  
Chengdu University of Traditional Chinese Medicine Affiliated Hospital

Quanyu Du (✉ quanydu@cdutcm.edu.cn)  
Chengdu University of Traditional Chinese Medicine Affiliated Hospital

Article

Keywords:

Posted Date: August 19th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1951370/v1

License: ☇️ This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
Abstract

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive fibrotic disease with unclear etiology and no effective treatment. This study aims to reveal the pathogenetic mechanism networks of multiple targets and pathways of IPF. Extract and metabolites of Astragalus membranaceus (AM) and Radix paeoniae rubra (RPR), two well-known traditional Chinese medicine have been proven to be effective in IPF. However, the underlying mechanisms of AM and RPR remain unclear. Based on network pharmacology analysis, differentially expressed genes (DEGs) of IPF were obtained from the GEO database. Targets of Astragalus membranaceus and Radix paeoniae rubra were identified using TCM Systems Pharmacology Database and Analysis Platform and SwissTargetPrediction. Subsequently, a protein-protein interaction (PPI) network was built and analyzed using the STRING database and Cytoscape software. Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Gene and Genomes (KEGG) analysis were performed using Metascape. Further, a component-target-pathway network and a Sankey diagram were used to obtain main active components and molecular docking was performed between the key active components and proteins encoded by key targets. Finally, in vivo studies were carried out based on network pharmacology. 117 common targets between DEGs of IPF and targets of drugs were screened out and included in the PPI network, in which AKT1, MAPK3, HSP90AA1, VEGFA, CASP3, JUN, HIF1A, CCND1, PTSG2 and MDM2 were predicted as the key targets. 117 targets were enriched in PI3K-AKT pathway, HIF-1 signaling pathway, apoptosis and MicroRNAs in cancer. Astragaloside III, (R)-Isomucronulatol, Astragaloside I, Paeoniflorin and β-sitosterol were selected as the main active components. The docking scores ranged from -4.7 kcal/mol to -10.7 kcal/mol, showing a good binding affinity between main active compounds and key targets. In vivo studies indicated that AM and RPR ameliorated pathological lung fibrotic damage caused by bleomycin and reduced mRNA level of AKT1, HSP90AA1, CASP3, MAPK3 and VEGFA. In conclusion, this study identified AM and RPR as potential therapeutic agents for IPF via regulating AKT1, HSP90AA1, CASP3, MAPK3 and VEGFA.

1. introduction

Pulmonary fibrosis (PF) is the result of a variety of interstitial lung diseases, with idiopathic pulmonary fibrosis (IPF) accounting for most of the cases.¹ IPF is a subtype of idiopathic interstitial pneumonia (IIP) and characterized by diffuse alveolitis and alveolar structural disorders, occurring primarily in adults over 50 years old.² Primary clinical manifestation includes chronic, progressive worsening of dyspnea, lung functions and functional capacities. IPF is associated with poor survival and prognosis, with 3 to 5 years median survival time after diagnosis.³ Patients may die from respiratory failure, acute exacerbation, pulmonary hypertension, lung cancer among other complications.⁴ The incidence of IPF ranges from 2 to 29/100,000 according to different studies.⁵ ⁶ At present, there is no nationwide epidemiological data of IPF in Mainland China. Regional data from several large samples indicate that the incidence of ILD is increasing significantly in China. Pirfenidone and Nintedanib are the only treatment agents for IPF recommended by ATS/ERS/JRS/ALAT.⁷ However, main effect of these agents is to reduce the decline rate
of lung functions but cannot reverse the progressive deterioration or improve the survival rate. Adverse events and high medical costs should also be considered in clinical practice.

Traditional Chinese Medicine (TCM) has been increasingly adopted as a way of complementary and alternative medicine worldwide. Chinese herb formulas are the most used TCM treatment in China and have been proven to have positive significance for improvement of quality of life and alleviation of symptoms of ILDs patients. Therefore, Chinese herbal medicines which can be traced back thousands of years are good candidates to explore and evaluate in the area IPF treatment. IPF can be classified as "the atrophic lung disease" due to its clinical characteristics as dyspnea, chronic cough, shortness of breath and expectoration.

Primary pathogenesis of IPF can be summarized as blood stasis and deficiency of qi. Therefore, the basic treatment principle of IPF in TCM is to invigorate qi and activate blood circulation. Astragalus membranaceus (AM), also known as "Huangqi", is a Chinese herb that has been used by TCM physicians for two thousands of years. More than 100 active metabolites of AM including flavonoids, saponins, polysaccharides, and amino acids have been identified. Many studies have shown that these metabolites have antiviral, immunomodulatory and anti-inflammatory properties both in vivo and in vitro. Radix paeoniae rubra (RPR), also known as Chishao in China, is the dried root of Paeonia lactiflora pall or Paeonia veitchii Lynch. Active ingredients of RPR including flavonoids, monoterpene, tanins and phenolic acids have been demonstrated a variety of biological activities such as anti-inflammation, immunoregulation, anti-viral, and antiallergic. TCM believes that AM has the function of invigorating qi and RPR has the effect of activating blood. The combination of these two herbs perfectly fits the fundamental pathogenesis of blood stasis and qi deficiency of IPF.

The compatibility of Chinese herbs is the basis of TCM for the treatment of diseases, and better pharmacological effects of the medicine pair are usually caused by the synergistic effect of different components with specific pharmacokinetic characteristics. However, the multi-ingredient and multitarget characteristics of herbs make it difficult to identify their molecular mechanisms. Therefore, computer-aided identification methods represented by network pharmacology and molecular docking were used in this study to predict potential mechanisms of AM and RPR on IPF. Then a bleomycin-induced IPF rat model was used to verify their therapeutic effects. Technical strategy of the study is shown in Fig. 1.

2. Materials And Methods

2.1 Differentially expressed genes in IPF

17 tissues (11 lung tissues with IPF and 6 control lung tissues) in GSE24206 (GPL570 platform), 10 tissues (7 lung tissues with IPF and 3 control lung tissues) in GSE101286 (GPL6947 platform) and 33 tissues (22 lung tissues with IPF and 11 control lung tissues) in GSE110147 (GPL6244 platform) were download from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The three sets of data were read and standardized by "Affy" and "Oligo" package. Differentially expressed genes (DEGs) between IPF
and control tissues were identified using the “limma” package with the threshold of $|\log FC| > 0.5$ and FDR < 0.05.

### 2.2 Bioactive Ingredients and Targets Screening

The Traditional Chinese Medicine Database and Analysis Platform (TCMSP, https://tcmspw.com/tcmsp.php) was used to determine the chemical components of AM and RPB according to two ADME parameters, namely oral bioavailability (OB) and drug-likeness (DL). Components that met the criteria of $OB \geq 30\%$ and $DL \geq 0.18$ were seen as active components and downloaded from TCMSP for further analysis. Besides, metabolites that do not meet the above screening criteria but have potential activity in the treatment of IPF were also included by searching and reading relevant literature on PubMed, CNKI and web of science. The 2D molecular structure files (.SDF) of active components were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and sent to SwissTargetPrediction (http://www.swisstargetprediction.ch/) with species setting as “Homo Sapiens” to perform the prediction of targets. Finally, potential targets of each active component with a probability of over 0.3 were kept.

The overlapping part of DEGs between IPF and control tissues and the targets of AM and RPB were obtained with a Veen diagram. Furthermore, overlapping targets were imported into the STRING database (version 11.5) (https://www.string-db.org/) with species setting as “Homo sapiens” and confidence level > 0.4, to build a protein-protein interaction (PPI) network that reflects the physical and functional interactions between proteins. Results with the .tsv format were introduced into Cytoscape v3.7.2 to visualize the data and extract the hub genes according to the degree.

### 2.3 GO/KEGG Enrichment Analyses

The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were carried out using Metascape (https://metascape.org/). Bio-enrichment results of these genes, including molecular functions (MF), cellular components, biological processes (BP), and signaling pathways, were obtained through this process. In this study, we selected the top 15 enrichment results with the $P$ value and used bubble charts to visualize the results. The component-target-pathway (CTP) network was built based on target-component results obtained from SwissTargetPrediction and target-pathway results obtained from KEGG analysis.

### 2.4 Molecular docking

Proteins encoded by the 10 key genes and DEG which was significant in three sets of data were selected as macromolecular receptors, and active components that were predicted to have a close relationship with the 11 genes were selected as ligands. We then visualized the relationship between these components and genes using a Sankey diagram. The 2D structure files of active components downloaded from PubChem were converted into PDB files using Chem3D software (version 18.0). The 3D crystal structure files of proteins were downloaded from the Worldwide Protein Data Bank (wwPDB, https://www.rcsb.org/), and all PDB files were converted to PDBQT files using AutoDock Tool (version
4.2) for further molecular docking in Autodockvina.\textsuperscript{29,30} Finally, the binding affinity, which was reflected by the docking score, was visualized using a heat map drawn with TBtools v1.09876.\textsuperscript{31}

2.5 Model Preparation and Administrations

Specific pathogen-free SD male rats (weigh, 180 ± 20g) were purchased from Chengdu Dashuo Experimental Animal Co., LTD. (Chengdu, China). All the animals were randomly divided into Blank control group (Control), Bleomycin model group (BLM) and Huangqi-Chishao group (HC) with 6 rats in each group after they were acclimated to experimental conditions for 1 week. BLM group and HC group were injected with bleomycin 5 mg/kg by endotracheal intubation to construct the rat model of pulmonary fibrosis. The control group were treated with intratracheal injection of the same volume of normal saline. Gavage treatment once a day started on the second day of bleomycin injection and continued for 28 days. The HC group was given 8.2g/kg of traditional Chinese medicine decoction (AM 30g and RPR 10g) purchased from Sichuan New Green Pharmaceutical Technology Development Co., Ltd. (Chengdu, China). The control group and the BLM group were given the same volume of normal saline. Rats were sacrificed 8 h after the final administration. The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg), and the lung tissues were collected by thoracotomy. The left lung was fixed in 4% paraformaldehyde for 24 hours and then embedded in paraffin for histological analysis and the right lung was cryopreserved for qPCR.

2.6 RNA Extraction, Reverse Transcription and qPCR

Total RNA in lung tissues was extracted using Animal Total RNA Isolation Kit purchased from Chengdu Fuji Biotechnology Co., LTD. (Chengdu, China). After a NanoDrop spectrophotometer determined RNA concentration, reverse transcription was performed to synthesize cDNA strand. The PCR amplification conditions were as follows: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, annealing and extension at 60°C for 30 s, 40 cycles. Using β-Actin as an internal reference, the relative expression of the target gene was calculated by the 2-ΔΔCt method. The primers are shown in Table 1.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>TGTCACCAACTGGGACGATA</td>
<td>GGGGTGTTGAAGGTCTCAAA</td>
</tr>
<tr>
<td>MMP7</td>
<td>GCTCTCAGAATGTGGAGTATG</td>
<td>CCCTTGCGAAGCCAATTA</td>
</tr>
<tr>
<td>AKT1</td>
<td>CTGTTCGAGCTCATCCTAATG</td>
<td>CTCTGTGTAGGGTCTTCTT</td>
</tr>
<tr>
<td>CASP3</td>
<td>CGCCATGCTGAAAACTGTA</td>
<td>CAGGGAGAAGGACTCAAATT</td>
</tr>
<tr>
<td>MAPK3</td>
<td>GGACCTCATGGAGACGGACCTG</td>
<td>CGGAGATCTGGTAGAGAAGTAC</td>
</tr>
<tr>
<td>VEGFA</td>
<td>CTACCAGCGCAGCTATTG</td>
<td>CAGGACGGCTTTAAGATAC</td>
</tr>
<tr>
<td>HSP90AA1</td>
<td>CTACTGCACCAGAATGAAGG</td>
<td>GTTCCACAAAGGCTGAGTTA</td>
</tr>
</tbody>
</table>
2.7 Histological Analysis

Fresh rat lung tissues were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and sectioned at 5 µm. Subsequently, sections were stained with hematoxylin and eosin (H&E) and masson's trichrome.

2.9 Statistical Analysis

All data were analyzed with Graphpad Prism v9.4.1 and were performed as means ± SD. Shapiro-Wilk tests were performed to determine the normality of the data distribution. One-way ANOVA was used to analyze the normalized data between groups. Kruskal-Wallis analysis was used for data not conforming to normal distribution. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 are considered significant.

3. Results

3.1 The DEGs of IPF

A total of 4638 DEGs were identified in lung tissues of IPF patients compared with normal lung tissues (Supplement Table S1). 227 genes were down-regulated and 185 were up-regulated in GSE24206 (Fig. 2A, 2D). 502 genes were down-regulated and 571 were up-regulated in GSE101286 (Fig. 2B, 2E). 1293 genes were down-regulated and 2238 were up-regulated in GSE110147 (Fig. 2C, 2F).

3.2 Bioactive Ingredients and Targets Screening

Among the 87 and 119 chemical ingredients of AM and RPR obtained from TCMSP, 15 ingredients of AM and 13 of RPR with OB ≥ 30% and DL ≥ 0.18 were screened out. Astragaloside IV (OB = 17.7, DL = 0.15), Astragaloside III (OB = 31.8, DL = 0.10), Astragaloside II (OB = 46.1, DL = 0.13) and Astragaloside I (OB = 46.8, DL = 0.11) were included in this study for their bioactivities reported in previous studies though they did not meet the inclusion criteria. Finally, 32 bioactive ingredients were included, as shown in Table 2. A total of 728 genes were identified as potential targets of AM and RPR from SwissTargetPrediction after deleting the duplicates (Supplement Table 2). Matching DEGs of IPF and targets of AM and RPR, 171 genes were selected as potential targets in the therapeutic effect of AM and RPR in IPF.
<table>
<thead>
<tr>
<th>Mol. ID</th>
<th>Molecular name</th>
<th>OB</th>
<th>DL</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOL000354</td>
<td>Isorhamnetin</td>
<td>49.6</td>
<td>0.31</td>
</tr>
<tr>
<td>MOL000354</td>
<td>Quercetin</td>
<td>46.4</td>
<td>0.28</td>
</tr>
<tr>
<td>MOL000409</td>
<td>Astragaloside IV</td>
<td>17.7</td>
<td>0.15</td>
</tr>
<tr>
<td>MOL000407</td>
<td>Astragaloside</td>
<td>31.8</td>
<td>0.1</td>
</tr>
<tr>
<td>MOL000403</td>
<td>Astragaloside II</td>
<td>46.1</td>
<td>0.13</td>
</tr>
<tr>
<td>MOL000401</td>
<td>Astragaloside I</td>
<td>46.8</td>
<td>0.11</td>
</tr>
<tr>
<td>Radix paeoniae rubra</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOL001921</td>
<td>Lactiflorin</td>
<td>49.1</td>
<td>0.8</td>
</tr>
<tr>
<td>MOL001924</td>
<td>Paeoniflorin</td>
<td>53.9</td>
<td>0.8</td>
</tr>
<tr>
<td>MOL007004</td>
<td>Albiflorin</td>
<td>30.3</td>
<td>0.8</td>
</tr>
<tr>
<td>MOL000449</td>
<td>Stigmasterol</td>
<td>43.8</td>
<td>0.8</td>
</tr>
<tr>
<td>MOL004355</td>
<td>Spinasterol</td>
<td>43.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Mol. ID</td>
<td>Molecular name</td>
<td>OB</td>
<td>DL</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>MOL002776</td>
<td>Baicalin</td>
<td>40.1</td>
<td>0.8</td>
</tr>
<tr>
<td>MOL000358</td>
<td>β-sitosterol</td>
<td>36.9</td>
<td>0.8</td>
</tr>
<tr>
<td>MOL007003</td>
<td>Benzoylpaeoniflorin</td>
<td>31.1</td>
<td>0.5</td>
</tr>
<tr>
<td>MOL001002</td>
<td>Ellagic acid</td>
<td>43.1</td>
<td>0.4</td>
</tr>
<tr>
<td>MOL007016</td>
<td>Paeoniflorigenone</td>
<td>65.3</td>
<td>0.4</td>
</tr>
<tr>
<td>MOL000492</td>
<td>Cianidanol</td>
<td>54.8</td>
<td>0.2</td>
</tr>
<tr>
<td>MOL002714</td>
<td>Baicalein</td>
<td>33.5</td>
<td>0.2</td>
</tr>
<tr>
<td>MOL002883</td>
<td>Ethyl oleate</td>
<td>32.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

### 3.3 Protein-Protein-Interaction Network Construction

A Veen diagram was used to obtain intersections between targets of AM, RPR and DEGs of IPF (Fig. 3). 171 common targets were identified and based on these targets, a PPI network was constructed. The network consisted of 166 nodes and 1030 edges after setting confidence level > 0.4 (Fig. 4). In this network, each node represents a target, and each edge represents the interaction between two targets. The larger the size of the node, the brighter the color, and the closer to the center of the circle, the higher the degree value of the node, which means that there are more nodes connected to it; the thicker the edge and the brighter the color, the higher the betweenness of the edge. AKT1, MAPK3, HSP90AA1, VEGFA, CASP3, JUN, HIF1A, CCND1, PTSG2 and MDM2 were predicted as the key targets according to the descending order of degree values (Supplement Table S3).

### 3.4 GO/KEGG Enrichment Analysis and Component-Target-Pathway Network

GO and KEGG enrichment analysis were performed on 171 common targets using Metascape platform (Supplement Table S4). 173 KEGG pathways were significantly enriched in pathways involving inflammation, apoptosis, and cell survival such as PI3K-AKT signaling pathway, HIF-1 signaling pathway, apoptosis and MicroRNAs in cancer (Fig. 5A). Additionally, GOBP analysis revealed that AM and RPR could affect the response to drug, response to nutrient levels, cellular response to external stimulus, cellular response to lipid metabolic process and so on (Fig. 5B). According to the ascending order of \( p \) values, the top 15 GOMF and GOCC items were also shown in Fig. 5C and Fig. 5D.

The CTP network contained 116 nodes and 502 edges (Fig. 5E). In this network, the stress of Astragaloside III, (R)-Isomucronulatol, Astragaloside I, Paeoniflorin and β-sitosterol was 19660, 17304, 5622, 5112 and 3910 respectively. They were considered as key ingredients in the anti-IPF effect of AM and RPR.
3.5 Molecular Docking

In the Venn diagram, MMP7 was the only overlapping target of all parts. As a result, 10 key targets and MMP7 were selected as ligand in the process of molecular docking. The one-to-one correspondence between the 11 targets and active components was visualized with a Sankey diagram, which was the basis for further molecular docking between the five most important components and 11 targets (Fig. 6A). The molecular docking scores are shown in the heat map in Fig. 6B. The docking scores ranged from -4.7 kcal/mol to -10.7 kcal/mol, indicating a good binding affinity between main active compounds and key targets. The docking processes of each component and the protein encoded by the key genes with a docking score less than -9 kcal/mol were visualized (Fig. 6C).

3.6 The Potential AM and RPR Alleviated Pulmonary Fibrosis in BLM Rats

*In vivo* experimental validation was performed to further explore the effect of AM and RPR on IPF. Results indicated that the alveolar structure of rat lung tissues was intact in the control group. Disrupted lung tissues, pronounced diffuse fibrosis, and more deposited collagen were observed in the BLM group with significantly thickened alveolar septa, some ruptured alveolar walls, and more inflammatory cells infiltration in some locations compared with the control group. The disruption of alveolar structures of the HC group was reversed by treatment to some extent and was more intact than that of the BML group. Fibrosis showed symptomatic relief in the HC group compared with the BML group (Fig. 7A). These results suggested that AM and RPR alleviated BML-induced pulmonary fibrosis.

In network pharmacology, the PPI network and Veen diagram suggested that AKT1, HSP90AA1, CASP3, MAPK3, VEGFA and MMP7 may play vital roles in the therapeutic effect of AM and RPR on IPF. To investigate the underlying molecular mechanism of AM and RPR on IPF, we evaluated the expression of these 6 targets using qPCR. As shown in Fig. 7B, when compared with the control group, significantly higher expression of AKT1, HSP90AA1, CASP3, MAPK3, VEGFA and MMP7 was seen in bleomycin-treated rats. However, treatment with AM and RPR attenuated the rise of the expression of AKT1, HSP90AA1, CASP3, MAPK3 and VEGFA. The expression of MMP7 showed a downward trend after treated with AM and RPR.

4. Discussion

IPF, the most severe subtype of interstitial lung disease, is characterized by chronic inflammatory, deposition of extracellular matrix (ECM), dysfunction of epithelial–mesenchymal transition (EMT), and remodeling of abnormal lung tissue structure. There is no accepted effective therapeutic approach currently. Therefore, a novel, effective and safe therapeutic method for IPF is urgently needed. Natural products have increasingly been one of the most important resources for pharmaceutical research and development due to their unique biological functions. AM and RPR are two main ingredients of Buyang Huanwu Decoction (BYHWD), a well-known Chinese formula for supplementing Qi and activating blood
circulation. Recent study has demonstrated the protective effect of BYHWD in pulmonary fibrosis model in vitro and its mechanism is related to inhibit pulmonary inflammation, collagen deposition, and EMT by suppressing the TGF-β1 signaling pathway.\textsuperscript{33} AM extract injection has been reported to ameliorate pathological lung fibrotic damage caused by BLM in rats and improve lung function by reducing the expression levels of TGF-β1 and collagens I and III whereas elevating those of MMP-3, MMP-9, TIMP-1, CXCL12, and CD90.\textsuperscript{34}

Therefore, we used network pharmacology analysis to investigate the common transcription factor regulatory network in IPF and identified AM and RPR as candidate drugs for IPF. The therapeutic effect and mechanism of AM and RPR were verified \textit{in vivo}, and the network pharmacology analysis revealed their molecular functions and pharmacological targets for treating IPF.

The diverse and heterogeneous etiology and pathogenesis of IPF make it suitable to identify pathways and targets by comparing samples from IPF patients and control tissues based on microarray screening. First, 171 overlapped targets were screened out from targets of AM, RPR and DEGs in GSE110147, GSE101286, and GSE24206. Based on the 171 targets, we revealed a regulatory network and key targets in regulating IPF. Afterward, the GO and KEGG enrichment analysis were performed, and the results indicated a combination of multiple biological processes and pathways involved in the anti-IPF effect of AM and RPR. Multiple pathways are associated with inflammation, autophagy, apoptosis, and cell survival, including PI3K/AKT pathway, MicroRNAs in cancer and apoptosis etc. The PI3K/AKT pathway is involved in multiple pathological changes of IPF, including alveolar epithelial cells damage, ECM overproduction, EMT and apoptosis.\textsuperscript{35–38} Targeting PI3K/AKT pathway has already shown benefits in the treatment of IPF.\textsuperscript{39} However, numerous crosstalk and interactions of PI3K/AKT with multiple pathways including TGF, VEGF, WNT and Notch pathway suggest a complex network of pathogenesis of IPF.\textsuperscript{40} Blocking a single node in this network will not be sufficient or effective.

Astragaloside III, (R)-Isomucronulatol, Astragaloside I, Paeoniflorin and β-sitosterol were selected as the main active components according to the CTP network and Sankey diagram. Many metabolites of AM and RPR have already been proven to be promising drugs to alleviate fibrosis progression in multiple organs. Astragaloside IV could significantly inhibited BLM-induced EMT in BLM-induced pulmonary fibrosis via targeting PI3K/AKT pathway.\textsuperscript{41} Quercetin could resist ROS damage and inflammation in the process of IPF.\textsuperscript{42} Paeoniflorin could inhibit the early stages of TGF-β mediated EMT in alveolar epithelial cells by decreasing the expression of the transcription factors Snail via the up-regulation of Smad7.\textsuperscript{43}

Molecular docking validation was performed and revealed that all main active components had good binding affinity with key targets. Based on the above results, we further investigated the therapeutic effect of AM and RPR on IPF. The results showed that AM and RPR treatment significantly reversed the rise of the expression of AKT1, HSP90AA1, CASP3, MAPK3 and VEGFA caused by BLM \textit{in vivo}.

Mitophagy and apoptosis manifest a cell type specific feature in the progress of pulmonary fibrosis. Apoptosis and mitophagy-induced type II alveolar epithelial cells and apoptosis resistant and mitophagy-
impaired macrophages promote the development of pulmonary fibrosis synergistically. Several transcription factors contribute to this. AKT1 activation plays a vital role in the regulation of pulmonary fibrosis as it is strongly associated with regulating survival and differentiation of myofibroblasts. The function of AKT1 in the progression of pulmonary fibrosis is strongly related to TGF-β. In alveolar macrophages, TGF-β induces AKT1 activation, which promotes mitochondrial reactive oxygen species (ROS) and mitophagy. This AKT1-mediated mitophagy leads to apoptosis resistance and prolonged survival of macrophages and is required for pulmonary fibrosis progression. However, apoptosis acts oppositely in alveolar epithelial cells. Apoptosis of alveolar cells contributes to early fibrosis and lung injury. Increased levels of pro-apoptotic factors Caspases-3, Bax, PARP have been observed in lipopolysaccharide-induced pulmonary fibrosis model and induction of alveolar cell apoptosis can exacerbate pulmonary fibrosis. In addition, activation of non-Smad signalling pathways including MAPK and AKT by TGF-β1 also promotes aspects of EMT. The activation of PI3K/AKT pathway has been identified to inhibit the function of FOXO3 and the repressed expression of FOXO3 in normal fibroblasts contributes to IPF fibroblast phenotype. HSP90, a member of stress-inducible proteins, is composed of two isoforms, HSP90A and HSP90B, and participate in the regulation of TGF-β1 by inhibiting the activity of CHIP (carboxyl terminus of Hsc70-interacting protein) to ubiquitinate and degrade Smad3. HSP90 inhibition has been proven to abrogate TGF-β-induced fibroblast activation and ECM production. Several MAPKs including ERK, JNK and p38 have been proven to activate activation protein 1 (AP-1), which is involved in the phenotypic transformation of human lung fibroblasts into myofibroblasts induced by TGF-β. VEGFA is a permeability and angiogenic factor. A recent study has demonstrated that the level of VEGFA correlated with TGF-β level and can be raised by BLM which was consistent with our result.

Matrix metalloproteinases (MMPs) are metalloendopeptidases that can degrade components of the ECM and non-matrix proteins. Most MMPs have been demonstrated to promote the development of IPF and are up-regulated in IPF blood and/or lung samples. MMP7 is not the key target predicted by the PPI network though, it is the DEG of three series and potential target of AM and RPR. The role of MMP7 in IPF includes promoting EMT and increasing lung levels or activity of profibrotic mediators or reducing lung levels of antifibrotic mediators.

qPCR revealed an escalation of AKT1, HSP90AA1, CASP3, MAPK3, VEGFA and MMP7 after BML treatment, which was consistent with results of other studies. The downward trend of these factors after treatment implies a multi-target and multi-pathway characteristic of AM and RPR in the treatment of anti-IPF. Thus, we speculate that AM and RPR suppressed TGF-β1/PI3K/Akt, MAPK, VEGF signaling pathways by targeting these pharmacological targets, subsequently ameliorating ECM deposition and inflammation in IPF.

Some limitations lie in this study. Some of the active components of Chinese medicine will also change during the decoction process, while some other compounds with therapeutic effects that may be generated during the decoction process cannot be included in this study. In addition, we investigated
several nodes in the complex network of multiple targets and pathways and further studies could link the upstream and downstream transcription factors of key targets to reveal more clearly the regulatory role of AM and RPR in the signaling pathway in IPF. It is expected that in the future, researchers will be able to overcome challenges and convert the extensive recognition of the pathogenetic mechanisms of IPF into effective therapeutic approaches.

5. Conclusion

In this study, the potent pharmacological targets and the therapeutic mechanism of AM and RPR on IPF were investigated by network pharmacology and verified by in vivo experiment. In conclusion, this study identified AM and RPR as potential therapeutic agents for IPF via regulating AKT1, HSP90AA1, CASP3, MAPK3 and VEGFA.

Declarations

Data availability

The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) (accession number: GSE24206, GSE101286 and GSE 110147), and the Worldwide Protein Data Bank (wwPDB, https://www.rcsb.org/) (accession number: 1UNQ, 4JJE, 2W96, 5L9B, 4BQG, 5VPB, 6GES, 4IPF, 2Y6D, 1PPX and 1mjv). The original data presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author Contributions

Conceptualization, H.J. and Q.D.; methodology, R.Z. and L.A.; supervision, F.W. and Q.D.; visualization, H.J. and J.G.; writing—original draft, H.J.; writing—review and editing, H.J., J.T. and X.H. All authors have read and agreed to the published version of the manuscript.

Acknowledgement

This study was supported and funded by the Natural Science Foundation of Sichuan Province (NO. 2022NSFSC1277) ; Science and Technology Development Foundation of the Hospital of Chengdu University of Traditional Chinese Medicine (NO. 19YY01; NO. 20YY11) and Chengdu University of traditional Chinese medicine graduate research innovation practice project (NO. CXZD2021010).

Conflicts of Interest

The authors have declared no conflict of interest. They have no known competing financial interests or personal relationships that could have appeared to influence the research reported in this publication.

References


Figures
Figure 1

Technical strategy of the study.
Figure 2

(A) Heatmap of DEGs in GSE24206. (B) Heatmap of DEGs in GSE101286. (C) Heatmap of DEGs in GSE110147. (D) Volcano plot of DEGs in GSE24206. (E) Volcano plot of DEGs in GSE101286. (F) Volcano plot of DEGs in GSE110147.
Figure 3

Common targets between DEGs of IPF and target of drugs
Figure 4

PPI network. The node size and color are proportional to its degree value. The larger and brighter the node, the more important the target in the network.
Figure 5

(A) KEGG analysis. (B) GO-BP analysis. (C) GO-MF analysis. (D) GO-CC analysis. (E) CTP network. The outer circle of the network represents the top 15 pathways; the inner circle represents components of AM and RPR that are related to genes enriched in these pathways; the innermost grid layout represents each target gene. Greater size of nodes represents higher degree value.
Figure 6

(A) Sankey diagram of showing the correspondence between 2 Chinese medicines, 24 components, and 11 targets. AM: astragalus membranaceus; RPR: Radix paeoniae rubra. (B) Heatmap of molecular docking score. (C) The molecular docking complexes. The binding site of the ligand and the protein residue has been identified in 2D form.
Figure 7

(A) Representative images of three groups stained with H&E and masson's trichrome (magnification, 200x) (B) The mRNA expression of 6 targets screened out by network pharmacology.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTables.xlsx