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Original Article

Synergistic Effects of Chuanxiong-Chishao Herb-Pair on Promoting Angiogenesis at Network Pharmacological and Pharmacodynamic Levels*

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ABSTRACT **Objective**: To investigate the synergistic effects of Chuanxiong-Chishao herb-pair (CCHP) on promoting angiogenesis *in silico* and *in vivo*. **Methods**: The mechanisms of action of an herb-pair, Chuanxiong-Chishao, were investigated using the network pharmacological and pharmacodynamic strategies involving computational drug target prediction and network analysis, and experimental validation. A set of network pharmacology methods were created to study the herbs in the context of targets and diseases networks, including prediction of target profiles and pharmacological actions of main active compounds in Chuanxiong and Chishao. Furthermore, the therapeutic effects and putative molecular mechanisms of Chuanxiong-Chishao actions were experimentally validated in a chemical-induced vascular insufficiency model of transgenic zebrafish *in vivo*. The mRNA expression of the predicted targets were further analyzed by real-time polymerase chain reaction (RT-PCR). **Results**: The computational prediction results found that the compounds in Chuanxiong have antithrombotic, antihypertensive, antiarrhythmic, and antiatherosclerotic activities, which were closely related to protecting against hypoxic-ischemic encephalopathy, ischemic stroke, myocardial infarction and heart failure. In addition, compounds in Chishao were found to participate in anti-inflammatory effect and analgesics. Particularly, estrogen receptor α (ESRα) and hypoxia-inducible factor 1-α (HIF-1α) were the most important potential protein targets in the predicted results. *In vivo* experimental validation showed that post-treatment of tetramethylpyrazine hydrochloride (TMP•HCl) and paeoniflorin (PF) promoted the regeneration of new blood vessels in zebrafish involving up-regulating ESRα mRNA expression. Co-treatment of TMP•HCl and PF could enhance the vessel sprouting in chemical-induced vascular insufficiency zebrafish at the optimal compatibility proportion of PF 10 μmol/L with TMP•HCl 1 μmol/L. **Conclusions**: The network pharmacological strategies combining drug target prediction and network analysis identified some putative targets of CCHP. Moreover, the transgenic zebrafish experiments demonstrated that the Chuanxiong-Chishao combination synergistically promoted angiogenic activity, probably involving ESRα signaling pathway.

KEYWORDS tetramethylpyrazine, paeoniflorin, angiogenesis, network pharmacology

Chinese medicine (CM) is a medical system characterized by the concept of organic wholeness as its principal theory and treatment based on syndrome differentiation as its diagnostic and therapeutic features.[1] CM has attracted considerable attention and acceptance in many countries due to its satisfactory therapeutic action.[2] Recent work involving CM has put forward the holistic philosophy of CM sharing much with the main ideas of emerging network pharmacology and network biology.[3] However, CM formula normally contains many active constituents which generally act upon multiple targets.[4] The complexity of the chemical constituents and the therapeutic targets

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pose big challenge to modern analytical chemistry and pharmacology methods.[5,6] The present work attempts to discover the candidate rule of CM from a systematic perspective at molecular level and pharmacodynamic level to interpret the abstract theory of CM through relatively simple two kinds of botanical drugs (Figure 1).

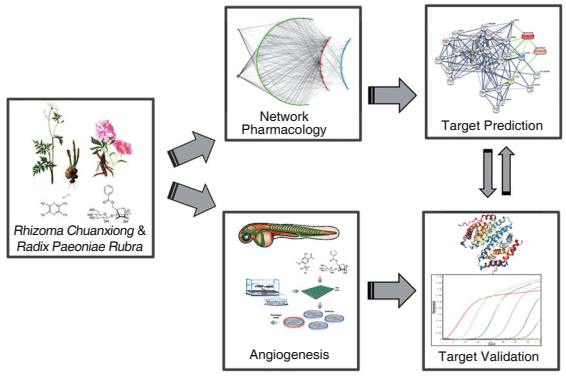

Figure 1. Schematic Diagram of Network Pharmacological and Pharmacodynamic Strategies

Chuanxiong (*Rhizoma Chuanxiong*) and Chishao (*Radix Paeoniae Rubra*) are two classical activating blood circulation herbs in China. Chuanxiong is one of the most popular herbal medicines in the World.[7] Ligustrazine (2,3,5,6-tetramethylpyrazine, TMP, Figure 2A) and Paeoniflorin (PF, Figure 2B) are two representative bioactive compounds of the two herbs. TMP, a natural alkaloid and the predominant bioactive ingredient of Chuanxiong, had been demonstrated antioxidant, anti-platelet aggregation, anti-apoptosis, calcium-homeostasis and anti-inflammatory effect, which might contribute to protecting vascular diseases.[8] PF as a principle bioactive components of Chishao, had been widely studied as analgesic, antipyretic, anti-inflammatory, anti-oxidant, anti-hyperlipidemia, anti-hyperglycemia, anti-thrombotic and platelet-inhibitory agent.[9] In previous study, we had demonstrated that PF could promote angiogenesis in zebrafish *in vivo* and human umbilical vein endothelial cells (HUVECs) *in vitro* in a dose-dependent manner.[10]

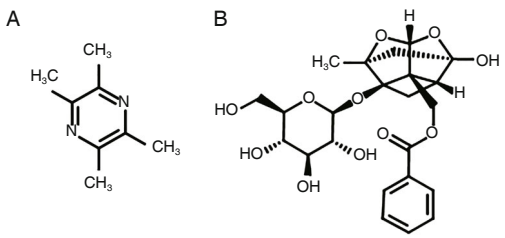

Figure 2. Chemical Structures of TMP (A) and PF (B)

Angiogenesis, an important natural process for healing and reproduction, refers to the establishment of a mature blood vessel network through expansion and remodeling of the pre-existing vascular primordium,[11] which had been continuously investigated as an innovative therapeutic approach for ischemic cardio-cerebrovascular diseases.[12]

Former experimental and clinical evidence had demonstrated the cardioprotective effects of TMP and PF. For example, TMP injection, which could prevent atherosclerosis as well as ischemia-reperfusion injury, had been extensively used in clinics in China for nearly 30 years; PF had been reported to protect against ischemic stroke with good permeation through the blood brain barrier[13] and exhibit pro-angiogenic action in zebrafish *in vivo* and HUVECs *in vitro* model. However, further exploration is still needed, and limited information of TMP and PF had been exported from target prediction platform. In this study, we tried to use target prediction of these two ingredients to make a complementary understanding of Chuanxiong-Chishao herb-pair (CCHP).

METHODS

Data Collection

The data of chemical constituents, targets and diseases used for network pharmacological analysis in Chuangxiong and Chishao were collected from Traditional Chinese Medicine Systems Pharmacology Database (TCMSP).[14] In this platform, target information was obtained from DrugBank;[15] herb-target mappings were obtained from two sources: HIT[16] for experimentally validated herb-target pairs and the SysDT model[17] for compounds without validated targets. The disease information was obtained from TTD[18] and PharmGKB (https://www.pharmgkb.org/). The knowledge about interactions between proteins and small molecules is essential for the understanding of molecular and cellular functions. We applied different database of known and predicted interactions between chemicals and proteins. To facilitate access to this data, Search Tool for Interactions of Chemicals (STITCH) database (http://stitch.embl.de/), SwissTargetPrediction (http://www.swisstargetprediction.ch/), Similarity Ensemble Approach (SEArch, http://sea.bkslab.org/search/) and Chemmapper (http://lilab.ecust.edu.cn/chemmapper/) were used to predict interact relations between TMP and PF, based on the concept that compounds sharing high 3D similarities may have relatively similar target association profile.

Establishment of Herb-Compound-Target-Cardiovascular Disease Network

To delineate the difference and similarity of chemicals between Chuanxiong and Chishao, we performed herbal compound comparison based on chemical constituents. Based on the Compound-Target-Disease Network of each herb, the cardiovascular disease system was selected manually and the related targets and compounds information of both herbs were extracted for further analysis. This extracted information was used to construct an Herb-Compound-Target-Cardiovascular Disease Network.

Analysis and Visualization of Network

The bipartite graphs were constructed by Cytoscape (version 3.4.0 Boston, MA, USA).[19] In the network, the compounds, targets and diseases were represented by nodes, and each interaction between two nodes was represented by an edge. Since the therapeutic effectiveness of a CM formula was achieved through collectively modulating the molecular network by its active compounds, two crucial topological parameters, i.e., degree and betweenness centrality[20] were analyzed to specify the importance of each node in the network. The "degree" of a node was the number of edges connecting to the node, and the highly connected nodes (half of the maximum degree of nodes) were referred to as hubs. And the betweenness centrality of a node refers to its capacity located in the shortest communication paths between different pairs of nodes in the network. All the topological properties of these networks were analyzed using Network Analyzer of Cytoscape.[19]

Molecular Docking for Target Validation

To validate the compound-target associations, the molecular docking simulation was further performed on TMP and PF combined with their predicted targes by AutoDock software (version 4.2). AutoDock Tools-1.5.6 was used to cluster the conformations. The program AutoGrid was used to generate the grid maps. The docking area of this grid was written by $60 \times 60 \times 60$ with a 0.375 Å grid space. Lamarckian genetic algorithm (LGA) was employed in this simulation process. The binding free energy ($\triangle G_{bind}$) $\leqslant -5.0$ kcal/mol indicated a high binding affinity of compound with their receptor.[21]

Chemicals and Reagents

Vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitor Ⅱ (VRI) was obtained from Merck KGaA (Germany). Tetramethylpyrazine hydrochloride (TMP•HCl) were purchased from Chengdu Biopurify Phytochemicals Ltd. (Sichuan, China). PF was purchased from National Institute for Food and Drug Control (Beijing, China). Dimethyl sulfoxide (DMSO) was acquired from Sigma (St Louis, MO, USA). Stock solution of TMP•HCl (300 mmol/L in DMSO) and PF (300 mmol/L in DMSO) were prepared and appropriately diluted as required.

Maintenance of Zebrafish and Embryo Collection

The *Tg*(*fli-1a:EGFP*)*y1* zebrafish, in which endothelial cells (ECs) express enhanced green fluorescent protein (EGFP), were maintained as described in the Zebrafish Handbook.[22] Briefly, the zebrafish was maintained in standard conditions at the temperature of 28 ℃ with a 14 h:10 h light/dark cycle. The zebrafish was fed twice daily with brine shrimp and also with general tropical fish food occasionally. Zebrafish embryos were generated by natural pair-wise mating (3–12 months old) and were raised at 28.5 ℃ in E3 medium.

All animal experiments were conducted in accordance with the ethical guidelines of Institute of Chinese Medical Sciences, University of Macau and the protocol was approved by Institute of Chinese Medical Sciences-Animal Ethics Committee of the University of Macau.

Drug Treatment of TMP•HCl and PF

Healthy embryos were selected at 21 h post-fertilization (hpf) and pretreated with 500 ng/mL VRI for 3 h. Afterwards, VRI was washed out and embryos were distributed into 24-well microplate (8 embryos in each well) containing 0.1% DMSO (v/v) or different concentrations of TMP•HCl (0.1–100 μmol/L), PF (0.1–100 μmol/L) and PF 10 μmol/L with different concentrations of TMP•HCl (0.1–10 μmol/L) for a treatment of 24 h at 28.5 ℃. Embryos receiving 0.1% DMSO (v/v) only served as vehicle control and were equivalent to no treatment. All experiments were repeated 3 times.

Morphological Observation of Zebrafish

At 48 hpf, Zebrafish embryos were removed from microplates and observed for viability and gross morphological changes under a fluorescence microscope (Olympus IX81 Motorized Inverted

Microscope, Japan) equipped with a digital camera (DP controller, Soft Imaging System, Olympus). Images were analyzed with Adobe Photoshop 7.0.

Target Validation of TMP•HCl and PF in Zebrafish Using Real-Time Polymerase Chain Reaction

The 21 hpf healthy *Tg*(*fli-1a:EGFP*)*y1* zebrafish embryos were collected and distributed into 4 groups: vehicle control, VRI, TMP•HCl 1 μmol/L and PF 10 μmol/L, thereafter treated with 500 ng/mL VRI for 3 h apart from vehicle control group. Afterwards, VRI was washed out and embryos were exposed to 1 mL of E3 medium containing different drugs in 24-well plates (n=30). Total RNA was extracted from zebrafish embryos using RNeasy Mini Kit (Qiagen, USA) in accordance with the manufacturer's instructions, and converted into single-strand cDNA using SuperScript™ Ⅲ First-Strand Synthesis System followed by real-time polymerase chain reaction (RT-PCR, Invitrogen™, USA) using the TaqMan® Universal PCR Master Mix (Branchburg, USA), corresponding probe from Universal Probe Library (Roche, Swiss). Then RT-PCR was performed in the ABI ViiA™ 7 PCR System (Applied Biosystems) with the following amplification profile: hold at 50 ℃ for 2 min, hold at 95 ℃ for 10 min and 40 cycles at 95 ℃ for 15 s, 60 ℃ for 1 min. The expression of estrogen receptor α (ESRα) and hypoxia-inducible factor 1-α (HIF-1α) mRNAs was normalized to the amount of β-actin, using the relative quantification method described by the manufacturer.

Statistical Analysis

All data were expressed as the mean ± standard deviations ($\bar{x} \pm s$). Data were analyzed with one-way ANOVA followed by Tukey's multiple comparison test with the following statistical criteria. P value less than 0.05 (P<0.05) was considered significant. Chart was made with GraphPad Prism 6.0 software (San Diego, CA). Each experiment has been repeated at least 3 times independently.

RESULTS

Chuanxiong-Chishao-Compound-Target-Cardiovascular-Disease Network

Herb-Compound-Target-Disease interaction (Figure 3) was built by candidate compounds and their related targets, and the Target-Disease Network linking potential targets and diseases was constructed for exploring the protein interactions and the therapeutic targets for diseases.

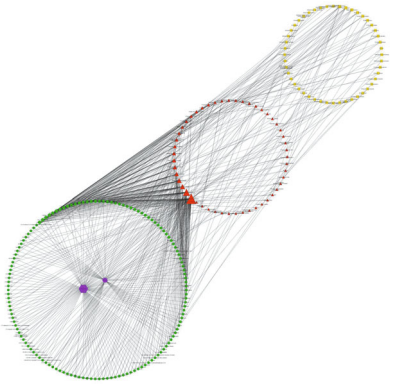

Figure 3. Cardiovascular Diseases Related Compound-Target-Disease Network of Chuanxiong-Chishao

Notes: The targets of Chuanxiong were concentrated in the whole cardiovascular system; Chishao placed emphasis on the anti-inflammatory effect and analgesics. Herb (hexagon, purple), compounds (ellipse, green), targets (triangle, red), cardiovascular diseases (round rectangle, yellow)

In this construction of network, 183 target proteins of Chuanxiong were predicted, most of which were related to the above mentioned diseases, such as ESR related to myocardial infarction;[23] cell division protein kinase 2, nitric-oxide synthase endothelial and β1 adrenergic receptor related to cardiovascular disease;[24,25] adenosine A2A receptor and mitogen-activated protein kinase 14 possibly involved in inflammation treatment.[26] Moreover, many compounds (i.e. Isobutyrophenone) have been predicted to target at α-1A adrenergic receptor; Chuanxiongol and (Z)-ligustilide have been predicted to target at adrenergic receptors, which are related to cardiovascular diseases.

The current work predicted 190 targets for Chishao, many of which showed relationships with inflammation and other diseases. For example, the main compounds, paeonin and baicalein, were highly connected with trypsin-1, which was involved in various pathological processes including inflammation, abnormal blood coagulation, tumor invasion, and atherosclerosis.

Among the targets, the protein with the highest degree was prostaglandin G/H synthase 2, followed by γ-aminobutyric acid receptor subunit α-1, prostaglandin G/H synthase 1, muscarinic acetylcholine receptor M2, muscarinic acetylcholine receptor M1, sodium-dependent noradrenaline transporter, etc.

Target Prediction of TMP and PF

A list of potential protein targets was ranked by the inference of chemical–protein association network whose

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edges weighed by the bioactivity of the similar compounds to the query. The biological annotations for each target, including name, species, function and involved pathway, were also displayed (Appendix 1, Figure 4).

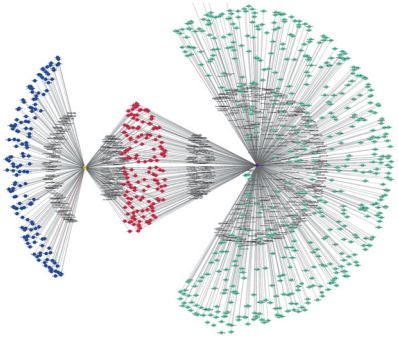

Figure 4. Target Prediction of TMP and PF

Notes: TMP (purple) and its targets (aqua), PF (yellow) and its targets (cobalt), mutual targets (magenta).

Target Validation

In docking analysis, TMP was able to bind ESR α (PDB code 3erd) and HIF-1 α (PDB code 5jwp), with the binding free energies were –5.67 and –6.94 kcal/mol, respectively. PF was also able to bind ESR α and HIF-1 α, with –6.94 and –5.59 kcal/mol, respectively (Figure 5).

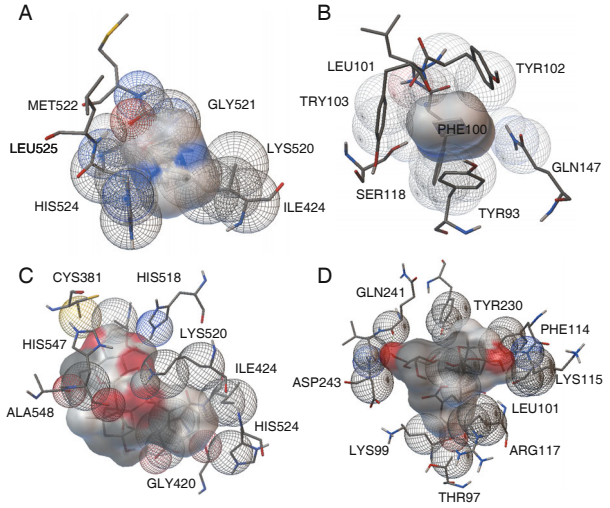

Figure 5. Ligands (TMP and PF) and Important Interactions in the Ligand-Binding Pocket of Receptors

Notes: A: TMP with ESR α; B: TMP with HIF-1 α; C: PF with ESR α; D: PF with HIF-1 α

Pro-angiogenic/Restorative Effect of TMP•HCl in Zebrafish Embryos

In vehicle control group, the intersegmental vessels (ISVs) sprouted and elongated from the dorsal aorta (DA) and posterior cardinal vein (PCV) to dorsal longitudinal anastomotic vessel (DLAV).[27] VRI displays anti-angiogenic properties and strongly inhibits the kinase activity of VEGF receptors.[28] VRI was demonstrated to induce significant blood vessel loss in ISVs, including a lack of physiological vessel formation and a loss of pre-existing blood vessels in ISVs and DLAV in zebrafish embryos. After the treatment of TMP•HCl, several ISVs sprouted from DA or PCV, but did not reach DLAV to form intact ISVs. Moreover, VRI-induced vascular insufficiency in ISVs could be significantly rescued by TMP•HCl (10, 30 and 100 μmol/L) post-treatment for 24 h in a dose-dependent manner (P<0.01). TMP•HCl at 100 μmol/L restored ISVs sprouting close to normal level (Figure 6).

Pro-angiogenic/Restorative Effect of PF in Zebrafish Embryos

In vehicle control group, ISVs sprouted and elongated from DA and PCV to DLAV. After the

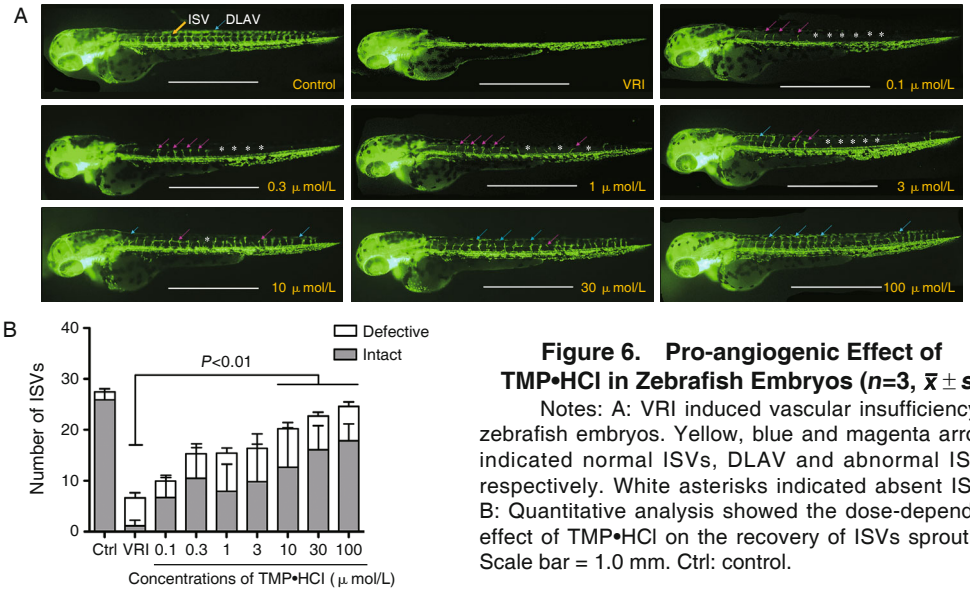

Figure 6. Pro-angiogenic Effect of TMP•HCl in Zebrafish Embryos (n=3, $\bar{x} \pm s$)

Notes: A: VRI induced vascular insufficiency in zebrafish embryos. Yellow, blue and magenta arrows indicated normal ISVs, DLAV and abnormal ISVs, respectively. White asterisks indicated absent ISVs. B: Quantitative analysis showed the dose-dependent effect of TMP•HCl on the recovery of ISVs sprouting. Scale bar = 1.0 mm. Ctrl: control.

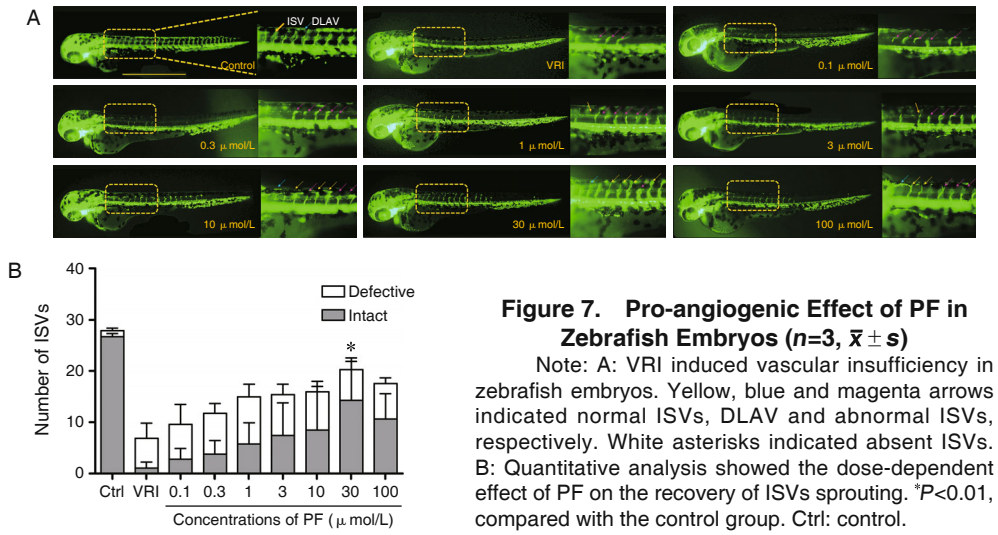

Figure 7. Pro-angiogenic Effect of PF in Zebrafish Embryos (n=3, $\bar{x} \pm s$)

Note: A: VRI induced vascular insufficiency in zebrafish embryos. Yellow, blue and magenta arrows indicated normal ISVs, DLAV and abnormal ISVs, respectively. White asterisks indicated absent ISVs. B: Quantitative analysis showed the dose-dependent effect of PF on the recovery of ISVs sprouting. $^{*}P$<0.01, compared with the control group. Ctrl: control.

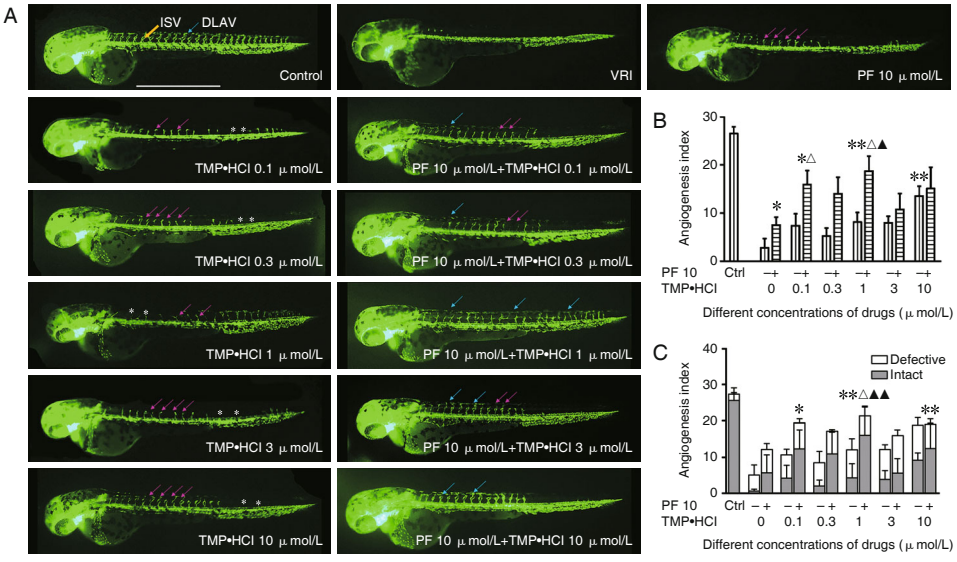

Figure 8. Pro-angiogenic Effect of Co-treatment of PF and TMP•HCl in Zebrafish Embryos (n=3, $\bar{x} \pm s$)

Notes: A: Morphological observation of zebrafish embryos. B: After treatment of PF 10 μmol/L (with or without TMP•HCl), angiogenesis index (number of intact ISVs+0.5 × number of defective ISVs) of ISVs sprouting was higher than without PF 10 μmol/L. PF 10 μmol/L paired with TMP•HCl 1 μmol/L co-treatment group showed significant activity on rescuing the VRI-induced blood vessel loss in zebrafish. $^{*}P$<0.05, $^{**}P$<0.01 compared with VRI group; $^{\triangle}P$<0.05, compared with group treated with PF 10 μmol/L; $^{\blacktriangle}P$<0.05, $^{\blacktriangle\blacktriangle}P$<0.01 compared with corresponding group treated without PF 10 μmol/L. Ctrl: control. Yellow, blue and magenta arrows indicated normal ISVs, DLAV and abnormal ISVs, respectively. White asterisks indicated absent ISVs.

treatment of PF (0.1–100 μmol/L) for 24 h, ISVs at various concentrations sprouted from DA or PCV, but did not reach DLAV to form intact ISVs. VRI-induced vascular insufficiency in ISVs could be significantly rescued by PF at 30 μmol/L (P<0.01, Figure 7).

Synergistic Pro-angiogenic/Restorative Effect of Co-treatment of PF Paired with Different Concentrations of TMP•HCl in Zebrafish Embryos

The capacity of PF to protect against blood vessel loss in zebrafish embryos when treated at low dose (0.1–10 μmol/L) was limited. However, this insufficiency of blood vessels could be significantly rescued by co-treatment of a lower concentration of PF (10 μmol/L) paired with different concentrations of TMP•HCl (0.1–10 μmol/L) for 24 h (Figure 8). The pro-angiogenic effect of cotreatmnet of PF 10 μmol/L and TMP•HCl 1 μmol/L was better than single use (P<0.05). Quantitative analysis showed the PF 10 μmol/L paired with TMP•HCl 1 μmol/L co-treatment group might be of the optimal compatibility. TMP•HCl and PF post-treatment could promote angiogenic activity by activating ESR α (Figure 9).

DISCUSSION

In order to disclose the combination principle

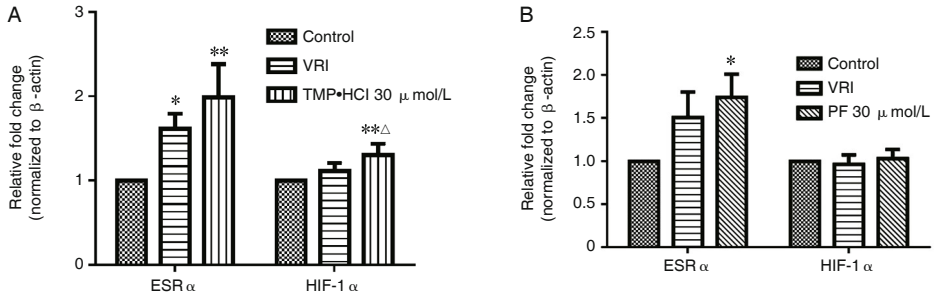

Figure 9. Effect of TMP•HCl and PF Post-treatment on ESR α Gene Expression

Notes: A and B: TMP•HCl and PF post-treatment could promote angiogenesis by activating ESR α in zebrafish embryos, respectively. $^{*}P<0.05$, $^{**}P<0.01$, compared with control group; $^{\triangle}P<0.05$, compared with VRI group.

of CM, it is essential building the compound-target interaction profiles.[29] In this study, we applied Chuanxiong and Chishao, a representative herb-pair of activating blood circulation, to analyze the network pharmacology. Subsequently, a set of CM network pharmacology methods were created to study the herbs in the context of targets and diseases networks, including predicting target profiles and pharmacological actions of main active compounds of Chuanxiong and Chishao, to prioritize disease-associated genes, and to reveal herb-gene-cardio-cerebrovascular disease co-module associations. The result showed that the targets of Chuanxiong were concentrated in the whole cardiovascular system, indicating that Chuanxiong might have antithrombotic, antihypertensive, antiarrhythmic, antiatherosclerotic effects, and be used for hypoxic-ischemic encephalopathy, ischemic stroke, myocardial infarction or heart failure. In addition, Chishao placed emphasis on the anti-inflammatory effect and analgesics. These data support the reasonability of the CM theory in construction of a formula (Appendix 2).

As an empirical system of multicomponent therapeutics, CM might have the potential of addressing a relationship between multicompound and drug synergistic effects, which is capable of systematically controlling various diseases such as the angiogenic disorders.[30,31] The application of network theory would be a very useful tool to visualize and analyze the interaction data among different components in the mixtures to capture the complexity in a simple, compact, and illustrative manner.

HIF-1 α is considered as the master transcriptional regulator of cellular and developmental response to decrease in available oxygen in the cellular environment, or hypoxia.[32,33] Recently, Nakada, et al[34] observed that exposure to hypoxaemia 7 days after myocardial infarction operation induced a robust regenerative response and highlighted the potential therapeutic role of hypoxia in regenerative medicine. Martínez-Lara, et al [35] demonstrated that HIF-1 α accumulation could lead to the expression of angiogenic proteins, which was ascribed to the up-regulation of ESR α. There is evidence suggesting that ESR α is expressed in ECs, and that ESR α agonists, such as estrogen, could induce ECs proliferation, migration and vascular remodeling.[36,37] The molecular mechanisms of cardiovascular protection exerted by estrogens may be related a VEGF-A-delta-like ligand 4-notch1 axis signalling-mediated modulation of angiogenesis.[38] Previous study in our laboratory had demonstrated that ESR α-enhanced ROCK-Ⅱ signaling pathway activation was the critical mechanism of phytoestrogenic compounds in the promotion of angiogenesis.[39]

Previous studies had shown that TMP could inhibit neovascularization, fibrosis, thrombosis, and suppress angiogenesis and tumor growth of lung cancer.[40,41] On the contrary, the results of *in vivo* zebrafish experiment showed that VRI-pretreatment induced blood vessel loss in developing zebrafish; post-treatment of TMP•HCl and PF promoted the regeneration of new blood vessels involving up-regulating ESR α mRNA expression. The pharmacology results showed that co-treatment of TMP•HCl and PF could enhance the ISVs sprouting in VRI-induced vascular insufficiency zebrafish. Taken together, the compatibility treatment of TMP•HCl and PF might be a promising therapeutic strategy for ischemic disease.

The network pharmacological strategies

combining drug target prediction and network analysis identified some putative targets of CCHP. Moreover, the transgenic zebrafish experiments demonstrated that the Chuanxiong-Chishao combination synergistically promoted angiogenic activity, probably involving ESR α signaling pathway. The discovered mechanisms of botanic drug pairs will be not only helpful to optimize the drug combinations in multi-component and multi-targets therapeutics, but also critical for developing novel drug combinations that can lead to more efficient treatments of complex diseases.

Conflict of Interests

The authors claimed no potential conflicts of interest relevant to this article.

Author Contributions

Wang Y and Guo G contributed equally to this work. Chen KJ, Lee SM, Cong WH, Yang BR, Xin QQ and Wang Y conceived and designed the experiments; Yang BR, HU YJ, Liao QW performed the network pharmacology analysis; Wang Y, Guo G, Xin QQ performed the zebrafish experiments. Wang Y and Guo G contributed significantly to analysis and manuscript preparation. Chen KJ, Lee SM and Cong WH helped perform the analysis with constructive discussions.

Electronic Supplementary Materials: Supplementary materials (Appendixes 1-2) are available in the online version of this article at http://dx.doi.org/10.1007/s11655-017-2408-x

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