Systems pharmacology-based investigation of Sanwei Ganjiang Prescription: related mechanisms in liver injury

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[ABSTRACT] Liver injury remains a significant global health problem and has a variety of causes, including oxidative stress (OS), inflammation, and apoptosis of liver cells. There is currently no curative therapy for this disorder. Sanwei Ganjiang Prescription (SWGJP), derived from traditional Chinese medicine (TCM), has shown its effectiveness in long-term liver damage therapy, although the underlying molecular mechanisms are still not fully understood. To explore the underlining mechanisms of action for SWGJP in liver injury from a holistic view, in the present study, a systems pharmacology approach was developed, which involved drug target identification and multilevel data integration analysis. Using a comprehensive systems approach, we identified 43 candidate compounds in SWGJP and 408 corresponding potential targets. We further deciphered the mechanisms of SWGJP in treating liver injury, including compound-target network analysis, target-function network analysis, and integrated pathways analysis. We deduced that SWGJP may protect hepatocytes through several functional modules involved in liver injury integrated-pathway, such as Nrf2-dependent anti-oxidative stress module. Notably, systems pharmacology provides an alternative way to investigate the complex action mode of TCM.

[KEY WORDS] Systems pharmacology; Traditional Chinese Medicine; Sanwei Ganjiang Prescription; Liver injury; Oxidative stress


Introduction

Owing to its anatomical location and inimitable function for clearing exogenous chemicals (i.e., drugs) and endogenous toxins like cholesterol, liver is the primary target of gut-guided pathogens, chemicals, and their products, which may lead to the entire organism dysfunction and even systemic disorders with important implications [1-2]. Oxidative stress (OS) is regarded as a conjoint pathological mechanism, thus contributing to initiation and progression of liver injury [3-4]. Therefore, antioxidants represent a curative strategy to prevent and cure liver diseases from OS.

As a comprehensive system of medicine practice characterized by a holistic therapeutic philosophy, traditional Chinese medicine (TCM) has been used in combating various diseases successfully for more than 3000 years, which has attracted an increasing interest worldwide for its fewer adverse effects and lower toxicity [5-6]. Traditional Tibetan medicine (TTM), derived from TCM, also has achieved notable success in diseases prevention and treatment, such as the Sanwei Ganjiang prescription (SWGJP) in the liver diseases treatment [7]. It has been created for the treatment of liver disease in the Xizang district of China and consists of Ganji-
ang (GJ, Zingiberis Rhizoma), Doukou (DK, Alpinia katsu-madai) and Roudoukou (RDK, Myristica fragrans Houtt) in a 6 : 5 : 4 ratio [8]. Our previous work has demonstrated that SWGJP has a hepatocytic protective effect [7-8], partly through reducing OS [9]. However, the targets and corresponding action modes involved in SWGJP therapeutic effects are still ambiguous, despite promising effects on liver treatment.

Currently, systems pharmacology [10-12] is an effective approach to facilitate drug discovery and clinical practice for the treatment of diverse complex diseases and is receiving increasing attention. As a novel strategy to identify candidate compounds and corresponding targets for herbs, systems pharmacology has been used to elucidate the therapeutic mechanism of Chinese herbal medicine [13-14]. Thus, the increasing prosperity of systems pharmacology may provide an alternative way to explore the detailed pharmacological mechanisms of SWGJP in liver treatment.

In the present study, we explored the mechanism of SWGJP in liver injury treatment based on systems pharmacology approach. Briefly, as displayed in Fig. 1, we first collected the chemical ingredients for SWGJP from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) database and then obtained the putative targets of these compounds via known target mapping and target prediction with a network-based inference method. Subsequently, we mapped the putative targets into relevant databases to assess their corresponding biological processes and pathways. Finally, network construction and pathway integration analyses were conducted to illuminate the molecule mechanisms of SWGJP in the treatment of liver injury in a holistic view.

Materials and Methods

Data preparation

The structural information, simplified molecular-input line-entry system and international chemical identifier (SMILES and InChIKey), based on chemical ingredients for each herb in SWGJP, were extracted from the TCMSP, a comprehensive systems pharmacology database for TCM [15]. The InChIKey of each molecule was retrieved to obtain the corresponding PubChem CID information with the python script “PubChemPy” (https://pypi.python.org/pypi/PubChemPy).
Target identification for SWGJP

Target identification is critical for systems pharmacology-based drug discovery. In the present study, we carried out the target identification of SWGJP through mapping known drug-target interaction (DTI) and predicting DTIs, using a balanced substructure-drug-target network-based inference (bSTDNB) method. We mapped all the chemical ingredients in the formula into a known drug-target interaction network of natural products to obtain experimental validated DTIs [12]. This network consisted of 18008 DTIs, linking 2988 unique natural products to 3546 targets.

In a recent study, we developed predictive network models to predict new targets of natural products via bSTDNB [16], which utilized resource-diffusion processes in the substructure-drug-target network to prioritize potential targets for known drugs and new chemical entities (NCEs). Two parameters, \( \alpha \) and \( \beta \), were imported to balance the initial resource allocation of different node types and the weighted values of different edge types, respectively. The third parameter \( \gamma \) was used to balance the influence of hub nodes. Four parameters (\( \alpha = \beta = 0.1 \), \( \gamma = -0.5 \), and \( k = 2 \)) of bSTDNB were adopted from our previous study [17]. Among the four network models generated with different types of fingerprints, bSTDNB_KR achieved optimal performance with the highest value of \( P (0.049) \), R (0.752), eP (27.02), eR (27.24), and AUC (0.959). Thus, bSTDNB_KR was utilized to predict the new targets of natural products in the global network. Finally, the top 20 predicted target candidates for each natural product were obtained.

Oxidative stress (OS)-associated target collection

The National Center for Biotechnology Information (NCBI) gene database is a common gene-centered information resource, which integrates gene-specific information from multiple sources. In the present study, we collected 800 human OS-associated genes from this database.

Networks construction

In this section, compound-target (C-T) and target-function (T-F) networks were constructed to understand the complex interactions between herb ingredients, targets, and functions. In the networks, the nodes denoted compounds or targets or functional modules, while the edges represented links between them.

The networks were generated and analyzed by Cytoscape 3.2.1 [18] with the quantitative property “degree” of a node that referred to the number of edges linked to it, suggesting node importance in the network.

Gene ontology (GO) terms of enrichment and analysis

To comprehensively decipher the protein targets involved in the biological interpretation of the network, we performed a biological process interpretation based on the targets (their “degree” are greater than 2) of SWGJP, with a widely used Cytoscape plugin ClueGO [19].

Pathway construction and analysis

To investigate the biological effects of protein targets and how they contribute to the liver injury via regulating certain pathways, a “liver injury-integrated pathway” was constructed, based on our present understanding of liver injury pathology and pathway analysis. Briefly, the gained protein targets were mapped to Kyoto Encyclopedia of Genes and Genomes database (KEGG, http://www.genome.jp/kegg/) to distribute them to potential pathways. Among these pathways relative to liver injury, we selected and incorporated them into a “liver injury-integrated pathway” for pathological and clinical data.

Results and Discussion

Collection of chemical ingredients for SWGJP

In the present study, a total of 249 chemical ingredients in SWGJP were extracted from TCMSP after removing duplicated structures. The numbers of ingredients for the herbs were 71 (Doukou, DK), 148 (Ganjiang, GJ), and 64 (Roudoukou, RDK), respectively. Among the 249 chemicals, 30 ingredients existed in more than two herbs. All of three herbs in the formula contained the four ingredients (CID6549, CID6654, CID7460, and CID14896). Candidate compounds were defined if there were known targets or putative targets via bSTDNB for a certain compound. Finally, 43 candidate compounds were obtained.

Putative target identification for candidate compounds of SWGJP

After merging known C-T interactions and predicted DTIs by bSTDNB, we identified 408 potential targets for the 43 candidate compounds. We further identified 144 OS-associated targets for SWGJP via overlapping 408 potential targets into the curated human OS-associated genes (800 genes).

Analysis of synergy of potential targets in SWGJP

SWGJP was based on the “Jun-Chen-Zuo-Shi” or “sovereign-minister-assistant-courier” principle, a theory of TCM that guides physicians to formulate herbal medicine [20]. Significant progress has been made in herbal synergism investigation with clinical trials and preclinical reports, as well as mechanisms of similar actions of herbal ingredients, which are implicated by their molecular interactions [21]. To explore the related mode at the molecular level of SWGJP, we investigated the distribution of targets (OS-labeled targets only) for the three herbs (127 from GJ, 51 from DK, and 39 from ROK, respectively) using Venny.

In Fig. 2, the three herbs covered 144 targets associated with OS. Moreover, Seventy-seven (53.5%) were specific targets derived from DK, indicating that DK was the “sovereign” herb in SWJP in treating OS of liver injury [20]. Twenty-two of them (15.3%) were common targets for the three herbs, suggesting that SWGJP could exert its magnifying effects using common targets. These targets included cyclin-dependent kinases (CDK5, CDK5R1, and CDKN2A), glutathione S-transferases (GSTM1, GSTM2, and GSTP1), metalloproteinases (MMP2, MMP9, and MMP3), lipoxigenases (ALOX12 and ALOX15), and peroxisome proliferator-activated...

Fig. 2 Overlaps of potential targets among three herbs in SWGJP. The corresponding numbers of targets for the three herbs are 127 for Doukou, 51 for an Ganjiang, and 39 for Roudoukou

Fig. 3 Global compound-target (C-T) network of candidate compounds in SWGJP. Nodes represent molecular modules (compound or target) and the size of them are proportional to degree. Edges represent the interactions that targets are connected by compound.
genes and 264 non-OS genes. This network consisted of 679 predicted C-T interactions and 351 experimentally validated ones. Network analysis revealed the average connectivity of targets for each candidate in this network was 23.95, while the average connectivity of candidates for each target was 2.52.

Among the 43 candidate compounds, 14 had OS-labeled target degrees (N) greater than 8, e.g., CID5280343 (Quercetin, N = 104), CID5280445 (Luteolin, N = 52), CID3314 (Eugenol, N = 18), CID638278 (Isoliquiritigenin, N = 14), 637566 (Gernanol, N = 11), and CID445070 ((2E, 6E)-Farnesol, N = 9). Meanwhile, among the 144 target proteins encoded by OS-associated genes, 8 were targeted by more than 8 candidate compounds (D): LMNA (Prelamin-A/C, D = 25), MAPT (Microtubule-associated protein tau, D = 24), ALDH1A1 (Retinal dehydrogenase 1, D = 23) and ALOX15 (Arachidonate 15-lipoxygenase, D = 21), MAPK1 (Mitogen-activated protein kinase 1, D = 18), HIF1A (Hypoxia-inducible factor 1-alpha, D = 14), NFKB1 (Nuclear factor NF-kappa-B p105 subunit, D = 8), and PTGS2 (Prostaglandin G/H synthase 2, D = 8). Previous studies have shown that these targets encoded by OS play crucial roles in the pathogenesis of liver injury. For example, LMNA mutation and pre-lamin A accumulation are involved in the pathological process of OS, and mitochondrial dysfunction alters the level of antioxidant enzymes and induces cell fates [29-30]. ALDH1A1, one member of the retinal dehydrogenases family with detoxification effects [31-32], serves as a protector against OS damage to metabolize reactive products of toxic lipid peroxidation [33-34]. Taken together, targeting these aspects makes a major contribution to the effect of SWGJP on liver injury.

The aforementioned network (Fig. 3) gives a global overview of the C-T interactions of SWGJP. We next selected three typical natural products (6-Shogaol, Cineole, and Myristicin) in this formula to illustrate their hepatoprotective profiles with new mechanisms of actions.

6-Shogaol (CID: 5281794), a predominant pungent constituent form GJ [35], has been reported to protect against the liver-related diseases [35-36] such as alcohol-induced liver damage [37]. As shown in Fig. 4, 6-Shogaol interacted with 8 known and 7 predicted targets (including 13 OS-associated proteins as well as 2 non-OS associated proteins). For example, the two OS-associated proteins (PTGS1 and MTOR) were predicted as the targets of 6-shogaol. Recent studies have suggested that 6-shogaol is a strong inhibitor of PTGS1 (IC50 = 4 ± 1 μmol·L−1) [38], indicating the potential mechanism action of 6-shogaol in treating liver injury.

Cineole (Eucalyptol, CID: 2758), the bicyclic monoterpene rich in DK [39], has been reported to exert protective bioactivity on liver against 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) [40], D-galactosamine/lipopolysaccharide (GalN/LPS) [41], and steatosis [42] in vivo. Fig. 4 indicates that cineole binds to 7 known and 4 predicted targets (including 8 OS-associated proteins as well as 3 non-OS associated proteins). For example, NFKB1, playing crucial roles in the development of hepatocellular injury [43], was predicted to interact with cineole. A recent study has demonstrated that cineole could inhibit NF-κB translocation, resulting in decreased levels of its target genes [44].

Fig. 4  A bipartite compound-target network for 3 representative ingredients in SWGJP. This network includes 16 experimentally validated (also containing the Known & Predicted interaction) and 21 computationally predicted compound-target interactions connecting 3 representative ingredients in SWGJP (6-Shogaol, Cineole and Myristicin), and 28 targets (22 oxidative stress-associated targets and 6 non-oxidative stress targets)
Myristicin (CID4276), one of the major essential oils of RDK \[^{[45]}\], was found to possess extraordinarily potent hepatoprotective activity \[^{[46]}\]. The exact hepatoprotective mechanism of myristicin remains unclear. As shown in Fig. 4, myristicin interacts with 10 OS-associated proteins and only 1 non-OS protein, consisting of 1 known and 10 predicted ones. Previous data have demonstrated that absence of CYP1A2 \[^{[47]}\], and CYP1A1 \[^{[48\text{-}49]}\] plays a role in repression 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) or benzo[a]pyrene (BaP)-induced hepatocellular injury, indicating that CYP1A1 and CYP1A2 enzymes have roles in toxicant-induced liver damage. Interestingly, CYP1A2 and CYP1A1 are the two predicted OS-associated proteins for myristicin in our predictive network model. Recent studies have shown that myristicin acts as an inhibitor of CYP1A2 \[^{[50]}\] and increases CYP1A1 transcription and protein expression in a dose-dependent manner \[^{[51]}\], indicating a potential hepatoprotective mechanism of myristicin.

**Target-Function network (T-F network)**

The T-F network displays liver injury-associated biological processes and related targets based on the functional annotation bioinformatics microarray (DAVID 6.8) analysis. As shown in Fig. 5, this network consisted of 806 target-function pairs connecting 315 targets with 7 liver injury-related functional modules. These modules included hepatocyte functions and activities associated with OS, enzymes activities, metabolism processes, biosynthetic processes, immune- and inflammatory activities, and cell death. On average, a target was involved in 2.59 function modules, with 50 out of 315 targets being associated with more than 5 function modules. Fig. 5 suggests that SWGJP regulated OS via the reduction of reactive oxygen species and regulation of monooxygenase activity. Liver injury is associated with OS, while anti-OS is a strategy for drug discovery in liver injury treatment \[^{[3\text{-}4]}\].

**Gene ontology (GO) terms of enrichment analysis**

GO term enrichment analysis was performed in the present study. The biological processes modulated by these targets included regulation of reactive oxygen species metabolic processes, negative regulation of extrinsic apoptotic signaling pathways, and negative regulation of cell death. The detailed GO terms analysis data are shown in Supplementary Table S6. Most functional models were involved in liver injury-associated pathological processes. For example, in liver injury, apoptotic or necrotic mechanisms depending on the signaling pathways activation, can result in the death of hepatocytes as a ubiquitous consequence \[^{[52\text{-}53]}\], such as diclofenac-induced apoptotic hepatocyte cell death \[^{[54]}\].
injury were incorporated into a “liver injury integrated-pathway.” The integrated pathway included the mitogen-activated protein kinases (MAPK) signaling pathway, the phosphatidylinositol 3-kinases-protein kinase b (PI3K-AKT) signaling pathway, the tumor protein p53 (P53) signaling pathway, apoptosis signaling pathway, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, and so forth. As seen in Fig. 6, several functional modules were involved in this integrated pathway (pro-survival, apoptosis, anti-OS, and detoxification). Herein our discussion will be mainly focused on representative modules to explore the underlying therapeutic mechanisms of SWGJP for liver injury.

Fig. 6  Liver injury integrated pathway and therapeutic modules. The orange nodes indicate the potential protein targets for candidate compounds in SWGJP prescription

Nrf2-dependent anti-oxidative stress module
OS is regarded as one of the critical pathological action modes of liver damage. Nrf2 (nuclear factor erythroid 2-related factor 2), a basic leucine zipper (bZIP) protein, mediates the expression of cytoprotective proteins, such as hemeoxygenase-1 (HO-1), glutathione-S-transferases (GST), and NAD(P)H. Quinone oxidoreductase 1 (NQO1) protects against oxidative damage induced by toxin insults, OS, and electrophilic stress [55-57]. As shown in Fig. 6, Nrf2 was predicted as a protein target of CID5280343 (Quercetin). Previous study has shown that activation of Nrf2 could increase antioxidant proteins expression to prevent cisplatin-caused hepatic damage [58], indicating that SWGJP could directly stimulate Nrf2 to prevent liver injury from OS. In addition, HO-1 and NQO1, the downstream proteins of Nrf2, are also the predicted targets of CID5280445 (Luteolin) and CID445070 (Farnesol). Recent research has confirmed that upregulation of HO-1 and NQO1 through activation of Nrf2 signaling pathways can protect against CCl4-induced liver injury [59-60]. Therefore, modulating the Nrf2/HO-1/NQO1 signal pathway may be an important mechanism action of SWGJP in the treatment of liver damage.

Mitochondrial-based anti-apoptosis module
Apoptosis is considered as one of the most common forms of cell death in liver injury. The mitochondria-mediated intrinsic apoptosis pathway, regulated by Bcl-2 family proteins-including proapoptotic (Bid, Bax, Bak) and anti-apoptotic (Bcl-2, Bcl-xL) [61], plays a significant role in amplifying and integrating apoptotic signals in hepatocytes, completing the effector caspase (CASP) cascade [62-63]. Indeed, liver damage can be initiated by the intrinsic pathway, which induces the expression of pro-apoptotic mitochondrial proteins and the release of cytochrome c to the cytoplasm, leading to activation of initiator CASP9 and eventually CASP3, resulting in executing apoptosis [64]. As shown in Fig. 6, proteins involved in apoptosis pathway were targeted by many candidate compounds in SWGJP. For instance, CID637566 (Geraniol) modulates BAK and CID638278 (Isoliquiritigenin)
regulated Bax, which is an essential gateway to mitochondrial dysfunction for cell death in response to various stimuli [65]. Additionally, compounds CID5281794 (6-Shogaol) and CID5280343 (Quercetin) modulated Bcl-2 and Bcl-XL simultaneously, which blocks cytochrome c release from mitochondria and insures the prevention of the CASP cascade and apoptosis [66]. In the CASP cascade, an apoptotic execution [67], the initiators CASP9 and CASP8 as well as the effectors CASP7 and CASP3 were predicted as targets of compounds such as 6-shogaol and quercetin, suggesting that SWGJP could regulate CASP cascade to alleviate the apoptotic effect. All the data demonstrated that SWGJP might regulate the homeostasis mitochondria-dependent pathway via the Bcl-2 protein family, and modulate the CASP cascade repression to amplify anti-apoptotic effects synergistically.

**NF-κB-mediated pro-survival module**

As outlined above, a common consequence of liver injury is the death of hepatocytes, as this involves activation of specific pathways to counteract damaging effects, resulting in hepatic toxicity and liver integrity [68]. Correspondingly, NF-κB plays a pivotal role in the modulation of survival responses to insults, ensuring that hepatocytes bypass cell death [69].

As seen in Fig. 6, target proteins involved in NF-κB signaling were regulated by herbal compounds to achieve the modulation effect of pro-survival. For example, CID6184 (Hexanal) and CID8181 (Methyl palmitate) were predicted to modulate NF-κB signaling were regulated by herbal compounds to achieve the regulatory function of IκB (IKK2–), IKK2–/–, while previous data have suggested that activation of NF-κB can induce transcription of cytoprotective genes in hepatocytes despite elevated levels of TNF [69-71]. The results from genetic experiments have emphasized the important function of IκB (IKK2–) subunits, activators of NF-κB activity, for regulation of NF-κB in IKK2–/– mice, resulting in massive hepatocytes survival in the liver with TNF [72]. We observed that compounds CID27588 (Eucalyptol) and CID5280343 (Quercetin) could regulate NF-κB and IKK to protect hepatocytes apoptosis, suggesting that NF-κB might be a promising therapy for liver injury and that SWGJP could promote cell survival via NF-κB-dependent signaling pathways in liver injury treatment.

**Conclusions**

At present, there are no western allopathic therapies to help patients transcend liver injury induced by chemicals, thereby creating an urgent need for a novel and efficient curative approach (from a fresh perspective). The efficacy of TCM in medical practice has been demonstrated for thousands of years. The concept of TCM for diseases treatment can be regarded as a philosophy of equilibrium, which mainly emphasizes the regulation of the interactions among all illness-associated elements within the body toward a balance point. However, the detailed mechanisms of action of herbs at protein and pathway levels are still difficult to demonstrate.

SWGJP, a TCM prescription for liver disease treatment in China, can ameliorate hepatic dysfunction in animal models. In the present study, we developed an integrative systems pharmacology approach to exploring the mechanisms of SWGJP in liver injury treatment from a systematic view. For the first time, we identified 43 candidate compounds and 408 of their corresponding target proteins, based on known target mapping and in silico target prediction via network-based method. We further proposed the molecular mechanism of SWGJP to treat liver injury through multilevel data integration, including compound-target network analysis, target-function network analysis, and integrated pathways analysis. The analytical results showed that SWGJP might protect hepatocytes from death through multiple pathways, such as Nrf2/HO-1 signaling pathway. Collectively, these findings showed that systems pharmacology could provide an alternative way to investigate the complex action mode of TCM.

**References**


