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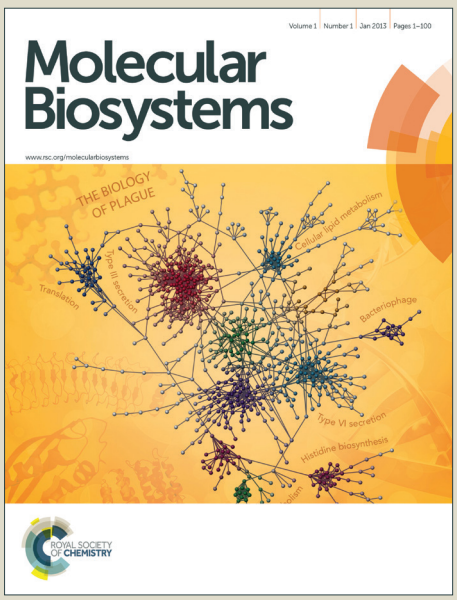

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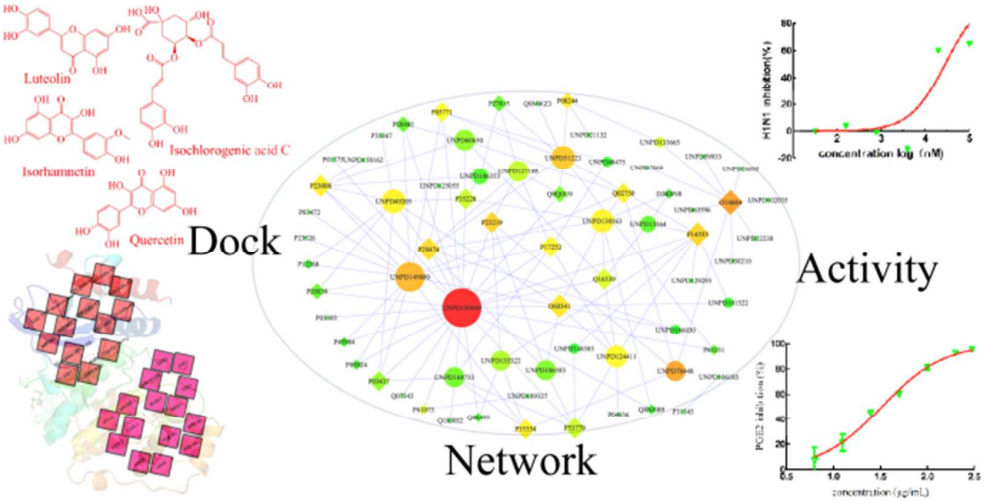
The mechanism of RDN on URTIs is to inhibit virus-host intercation and to regulate signaling pathways by combination of multi-target ingredients



Network Pharmacology Study on Mechanism of Traditional Chinese Medicine for Upper Respiratory Tract Infection†

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Abstract: Traditional Chinese medicine (TCM) is a multi-component and multi-target agent and could treat complex diseases in a holistic way, especially, infection diseases. However, the underlying pharmacology remains unclear. Fortunately, network pharmacology by integrating system biology and polypharmacology provides a strategy to this issue. In this work, Reduning Injection (RDN), a well-used TCM in clinic for upper respiratory tract infections (URTIs), was exemplified to interpret the molecular mechanism and predict the new clinical indications by integrating molecular docking, network analysis and cell-based assay. 32 active ingredients and 38 potential targets were identified. And *in vitro* experiments confirmed the bioactivities of the compounds against lipopolysaccharide (LPS)-stimulated PGE_2 and NO production in RAW264.7 cells. Moreover, network analysis showed that RDN could not only inhibit viral replication but also alleviate the sickness symptoms of URTIs through directly targeting the key proteins in respiratory viral life cycle and indirectly regulating host immune systems. In addition, other clinical indications of RDN such as neoplasms, cardiovascular diseases and immune system diseases were predicted on the basis of the relationships between targets and diseases.

Keywords: network pharmacology, drug-target network, Reduning Injection, virtual screening, *in vitro* validation

Introduction

Upper respiratory tract infections (URTIs) are syndromes of familiar clinical symptoms including fever, headache, malaise, sore throat, rhinorrhea, nasal congestion and sneezing, which mainly caused by the host immune response to respiratory viral infection such as influenza viruses, rhinovirus (RV), respiratory syncytial virus (RSV)[1-3]. URTIs are known to be the most common illnesses[4, 5] and result in an enormous economic burden on health care systems due to high frequency of infections (2-6 times per year for people of all ages)[6]. There are current two important treatment strategies: antiviral drugs were limited to use in clinic due to drug safety and resistance, whereas the other agents (e.g. decongestants, analgesics and antihistamines) were also found to be ineffective because of the limited efficacy on

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].

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specific symptoms[7]. Therefore, a combination therapy of antiviral drugs and anti-immunity drugs has been proposed as an alternative approach to control respiratory infection[6].

Traditional Chinese medicine (TCM), as a complementary and alternative medicine, is mainly consisted of herb medicines, of which many had effects on immune system regulation[8] and viral infection resistance[9]. Therefore, TCM has been an attractive area for researcher to develop multi-target combination drugs for prevention and treatment of URTIs[10-12]. For example, *Lonicera Japonica* Thunb. (Jinyinhua), *Gardenia Jasminoides* Ellis (Zhizi) and *Artemisia Annua* L. (Qinghao) have been widely used in many formulae that could clear heat, dispel wind and detoxicate[13, 14], such as Shuanghuanglian, lonicerae and forsythiae powder, Huanglian jiedu decoction. These herbs were also reported to possess potent antiviral, anti-inflammatory and immunomodulatory activities[15-17]. Reduning Injection (RDN) containing the three herbs was developed by Kanion pharmaceutical corporation (Lianyungang, China). It was widely used for treating URTIs. Previous works[18, 19] showed that RDN contained various ingredients, and had antiviral and significant anti-inflammatory activities[20-23]. In addition, it was recommended to treat influenza infection (such as H1N1, H7N9) in "diagnostic and treatment protocol for human infections with avian influenza A" (http://www.nhfpc.gov.cn/). Thus, it was reasonable to infer that RDN could treat URTIs by suppressing virus and modulating immune response to viral infection. However, the essential compounds and the mechanisms of RDN was unclear and needed to be further explored, because of its complicated characteristics.

Network pharmacology by integrating network biology and polypharmacology was considered as the next paradigm in drug development[24]. It has revealed that biological systems, as strongly interconnected networks, could be perturbed by multi-target drug. Network pharmacology also provided a system-level approach to understand of the pathogenesis of disease[25], and could be used for lead discovery, target identification and indication-prediction[26]. Moreover, the holistic and systematic approach would also help to understand the action mechanism and to identify essential compounds of TCM. For example, network pharmacology has been used to interpret the molecular mechanism of TCM[27, 28]. In this work, molecular docking and network analysis were employed to identify key compounds and potential targets, and to uncover the molecular mechanism of RDN on URTIs. New clinical indications for RDN were also predicted by target-disease network.

Materials and methods

Collection of molecules and chemical space analysis

The 3D structures of 46 ingredients separated from RDN, as reported (Table S1, ESI†) [19], were download from the Universal Natural Product Database (UNPD) [29]. Meanwhile, 38 FDA-approved drugs were collected from DrugBank[30] according to the terms related URTIs such as fever, infection and cold. Molecular descriptors of the molecules and drugs (Table 1) were calculated by Discovery Studio. Then, principal component analysis (PCA) was conducted in library analysis module of Discovery Studio to visualize the distributions of molecules and drugs in chemical space. The

input parameters were listed in Table 1, and the variances of PC1, PC2 and PC3 in Figure 1 were 0.521, 0.229 and 0.125, respectively.

Molecular docking

According to the pathogenesis of URTIs such as systematic infections and their associated inflammatory response, 45 protein targets, including 15 targets of viral origins, were carefully collected from DrugBank[30] and Therapeutic Target database (TTD) [31]. The X-ray or NMR structures of the proteins for docking were downloaded from RCSB Protein Data Bank[32] (Table S2, ESI†) by the following criteria: (A) the structure should contain original ligand to define the active site for docking; (B) the resolution of the structure of protein-ligand complex was below 2.5Å[33]. Molecular docking was then carried out by AutoDock 4.0[34] and DOVIS 2.0[35] according to the protocol described in previous work[29].

Network construction and analysis

The drug-target network (DTN) and target-disease network (TDN) for a specific herb could assisted in identifying active compounds, understanding the action mechanism, and exploring of new clinical application[29, 36-38]. The interaction between molecules and target proteins (docking scores higher than 5.0) were chosen to generate DTN in which nodes represented molecules or target proteins. The DTN could derive two biologically relevant networks: drug-drug network (DDN) by linking the molecules which shared one or more target proteins, and target-target network (TTN) in which each pair nodes (target proteins) shared one or more molecules. The relevant diseases were collected by mapping potential targets into TTD. They were then projected into different categories according to the medical subject headings (MeSH, http://www.nlm.nih.gov/). Based on these relationships, TDN (Figure 4) was constructed to investigate new indications of RDN. All networks were constructed by Cytoscape 3.0.2[39], and the topological properties were measured by the NetworkAnalyzer plugin[40].

Experimental validation

The anti-inflammatory effects of the predicted compounds were investigated by the productions of prostaglandin E_2 (PGE_2) and nitric oxide (NO) in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. In the experiment, the compounds were obtained from "National Institutes for Food and Drug Control" (purity, ⩾98%), and the powder of RDN was prepared by lyophilized the liquid of RDN using vacuum freeze drying system. The murine macrophage RAW264.7 cell lines were purchased from Cell Culture Center of the Chinese Academy of Medical Sciences (CCC, Beijing, China). RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sijiqing, Deqing, Hangzhou, China) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 ℃ under 5% CO_2. The cell viability was measured by a conventional MTT assay, as reported previously[41]. After pre-incubation of RAW264.7 cells (1×10^5 and 2×10^6 cells/mL for

PGE_2 and NO assays, respectively) for 24h, cells were pretreated by various concentrations of the compound (containing 0.1% dimethyl sulfoxide) for 1 h, and were further incubated with LPS (1.0 μg/mL) for 18~20h. The supernatant was collected, and the inhibitory activities of compounds were determined by analyzing NO and PGE_2 levels through Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrocholide in deionized water) and enzyme-linked immunosorbent assay kits (Enzo Life Sciences, Farmingdale, NY, USA), respectively, as described previously[42]. The absorbance was measured using SpectraMax M2e microplate reader (Molecular Devices, Menlo Park, USA) at 550 nm or 405/570 nm for NO and PGE_2, respectively. The inhibitory potency (IC_{50}) of each compound was calculated by GraphPad Prism 5.0.

Results and discussion

Physicochemical property analysis

Physicochemical properties of a molecule were inherent to its structure, and were essential to understand its biological activities, pharmacokinetics, metabolism, toxicity, and pharmaceutical properties[43]. Table 1 showed that most molecules in RDN obeyed the Lipinski's rule of five[44]. The number of rotatable bond (nRBs) and polar surface area (PSA) were below 10 and 140 $Å^2$, respectively. It implied that the compounds would have good oral bioavailability with no less than 20%[45]. As the important predictors of absorption, distribution, metabolism, excretion and toxicity (ADMET) profiles, molecular weight (MW, less than 400), AlogP (less than 3) and PSA (more than 75 $Å^2$) displayed that the molecules had remarkablely short half life and low toxicity [45], which were verified in our previously study[46]. The distributions of the molecular properties of drugs and molecules, except for PSA, were similar. And the large overlap between molecules and drugs in chemical space (Figure 1) demonstrated that the active compounds in RDN would have similar therapeutic and pharmacological actions on URTIs[30].

Table 1 Statistics of molecular descriptors of molecules in RDN and drugs

Descriptors	Molecules				Drugs			
	Mean	Min	Max	Median	Mean	Min	Max	Median
ALogP [a]	1.0	-3.8	3.1	1.7	1.7	-3.4	9.2	2.7
MW [a]	309.7	138.1	696.7	309.2	271.8	126.0	547.7	258.8
nRBs [a]	3.9	0.0	13.0	4.0	4.3	0.0	20.0	4.0
nRings [a]	2.3	0.0	5.0	2.0	2.0	0.0	4.0	2.0
nAroRings [a]	0.9	0.0	2.0	1.0	1.4	0.0	3.0	2.0
nHBAs [a]	6.7	1.0	17.0	7.0	3.8	1.0	10.0	3.0
nHBDs [a]	3.7	0.0	10.0	3.0	1.9	0.0	7.0	2.0
SA	297.1	133.3	638.9	278.7	262.7	109.8	514.8	250.9
PSA	111.6	9.2	265.5	111.0	76.3	3.2	200.7	60.8

FractionalPSA [a]	0.3	0.1	0.5	0.4	0.3	0.0	1.0	0.3
SASA	485.0	292.3	901.9	477.0	459.8	236.4	796.1	444.5
PSASA	190.4	22.7	428.7	174.1	136.3	11.8	347.7	113.2
FractionalPSASA	0.4	0.1	0.6	0.4	0.3	0.0	0.7	0.3
SAVol	426.3	260.7	788.8	425.8	406.5	211.5	691.7	396.2
Rule of 5 violations	0.9	0.0	4.0	0.0	0.2	0.0	2.0	0.0

[a] the descriptors were used to build the PCA model for 46 molecules in Reduning Injection and 38 drugs related on URTIs from DrugBank. The descriptor abbreviations used in this table include: MW, molecular weight; nRings, number of rings; nAroRings, number of aromatic rings; nRBs, number of rotatable bonds; nHBAs, number of hydrogen acceptors; nHBDs, number of hydrogen donors; SA, molecular surface area; PSA, molecular polar surface area; SASA, molecular solvent-accessible surface area; PSASA, molecular polar solvent-accessible surface area.

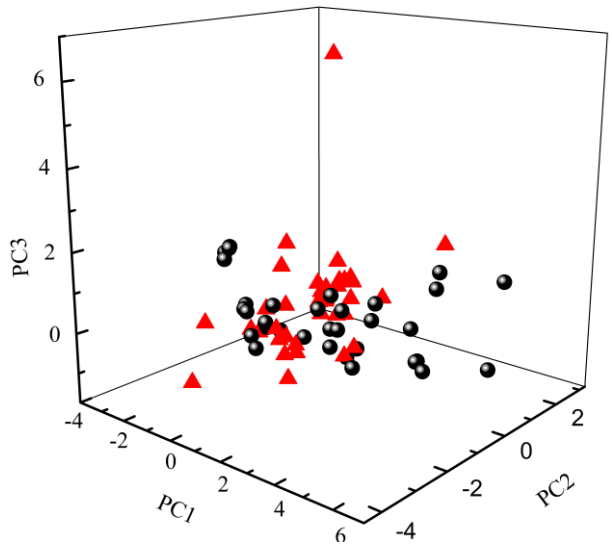


Fig. 1 The distributions in chemical space between molecules in RDN and drugs from DrugBank according to PCA. Red triangles and black circles represented drugs and molecules in RDN, respectively.

Active compounds and target proteins identification based on DTN

Generally, a herbal medicine contained hundreds of pharmacological compounds and would interact with several cellular targets to treat complex diseases[47, 48]. And the underlying mechanism was not clear. As a newly emerged field, network pharmacology was a systems biology-based methodology, and could help us understand the mechanism of multiple actions of drugs across multiple scales from molecular and cellular levels to tissue and organism levels by analyzing the features of biological networks[49].

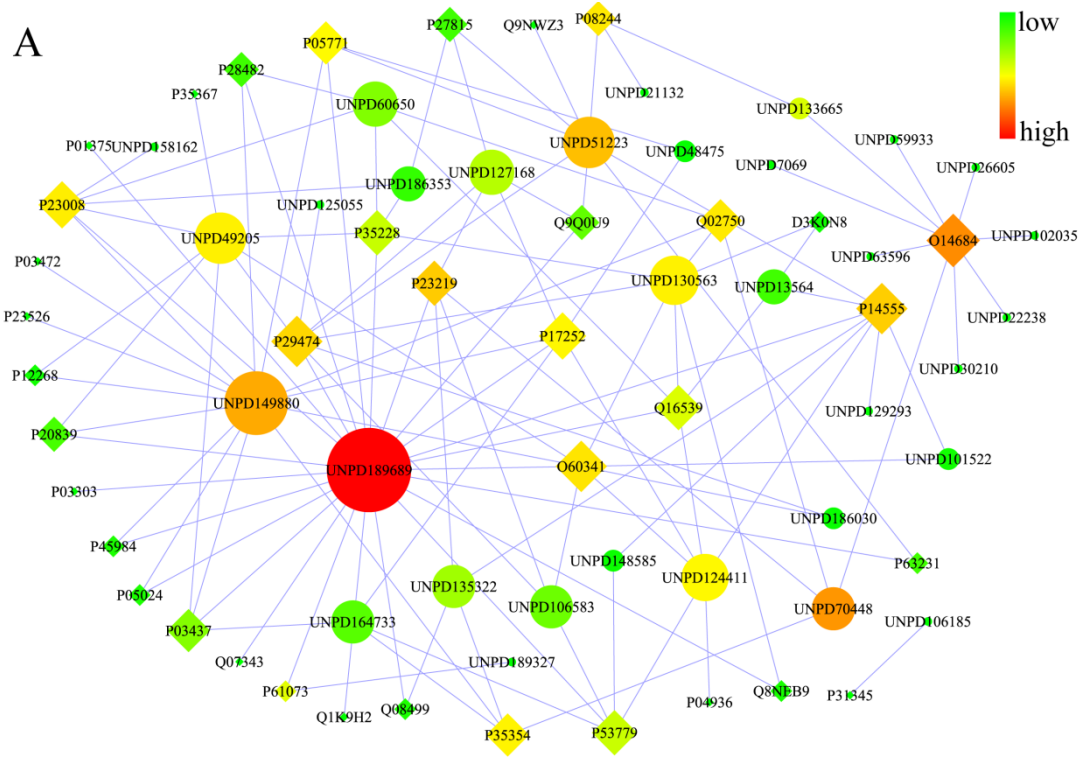
According to the molecular docking results, DTN including 69 nodes (32 molecules and 37 proteins) and 122 edges was constructed in Fig. 2A. The association between molecules and target proteins were listed Table S3 (ESI†). The global topological

features (Table 2) revealed that the target could interact with multiple molecules (3.81 molecules per target on average), and a molecule could also target several proteins related to URTIs. The results demonstrated that RDN would be a multi-component and multi-target agent, and there may be synergistic therapeutic efficacies on URTIs.

Table 2 Topological features of the DTN, DDN and TTN

network	average degree	avg.shortest path	network density	network centralization	network heterogeneity	clustering coefficient
DTN	3.54	3.28	0.05	0.31	1.01	0.00
DDN	10.19	1.85	0.34	0.39	0.43	0.78
TTN	21.50	1.39	0.61	0.29	0.39	0.86

Two key topological parameters, degree and betweenness centralities that could characterize the most influential nodes in a network[50] were employed to quantify the importance of a node (molecules or target proteins) and the extent of the influence of the node on the spread of information through the network. The degree and betweenness of each node (molecules or proteins) were listed in Table S3 (ESI†). It showed that the degree of the nodes with more interactions (hubs) played more important roles in the organization and integrity of the drug-target network, indicating that they would have important pharmacological functions in RDN.



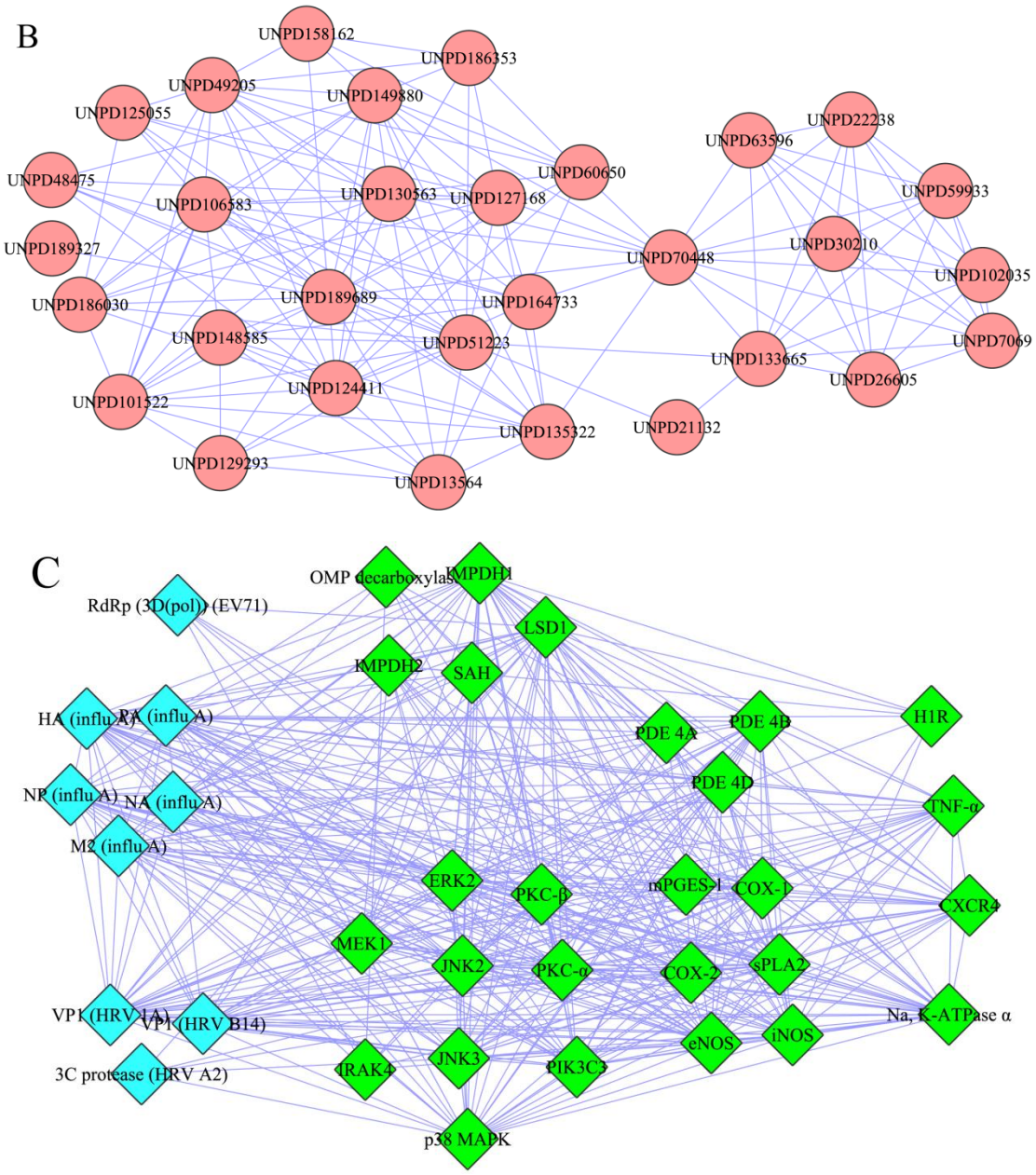
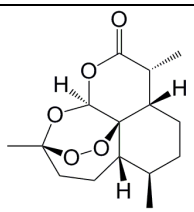
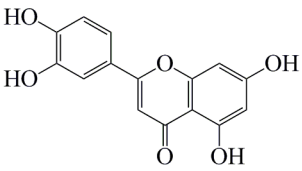
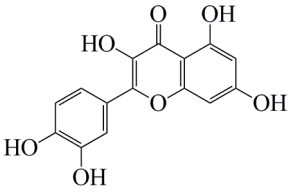
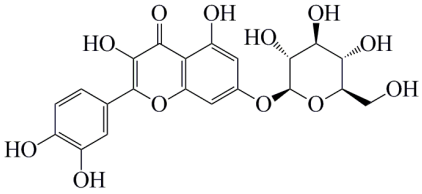
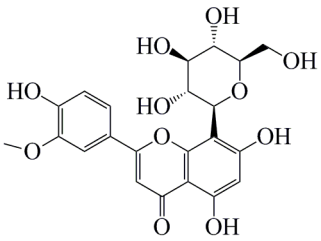


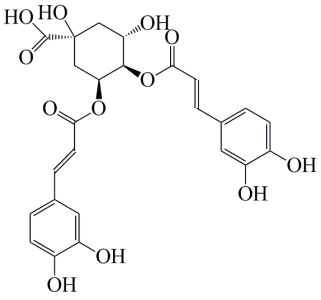
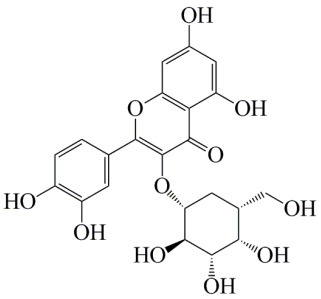
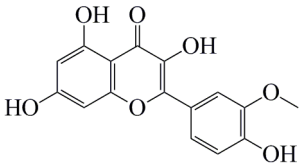
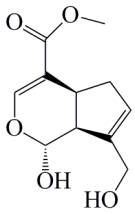
Fig. 2 The drug-target network (A), drug-drug network (B) and target-target network(C). Gray edges represented the interaction two nodes. Ellipses and diamonds corresponded to molecules in RDN and target proteins related on URTIs, respectively. For DTN, the size and bright color of each node were proportional to degree and betweenness centrality, respectively. For TTN, blue diamonds represented targets of viral origin, and green diamonds represented human origin.

Among 32 candidate active compounds (Fig. 2A and Table S3, ESI†), several molecules (Table 3) may be essential. For example, Artemisinin, which had the most potential targets and highest betweenness centrality, has been officially used as an indicator compound to control the quality of *Artemisia Annua*. And Luteolin, a typical flavonoid, had a wide variety of pharmacological effects such as antiviral[51], anti-inflammatory[52] and antioxidant activities[53]. Whereas its glycoside (Luteoloside),

as an indicator to evaluate the quality of *Lonicera Japonica* in "Pharmacopoeia of People's P.R. China", also had high degree and betweenness centrality. Interestingly, as a similar structure of Luteolin, Quercetin can relieve the symptoms and severity of URTIs in clinical trials[54]. In addition, Genipin which located on between two modules in DDT (Fig. 2B) and shared common targets with other molecules could exert synergistic therapeutic effects on URTIs. The results indicated that these molecules (Table 3) could play the major role in the pharmacological effects of RDN. Meanwhile, Fig. 2B showed the significant interactions between the active compounds, indicating that they may interact with intracellular targets in holistic way. Thus, it implied the multi-component property of RDN.

Table 3 Compounds with high degree and betweenness centrality in RDN

UNPD ID	Name	Structure	Degree [a]	BW [a]
UNPD189689	Artemisinin		24	0.428
UNPD149880	Luteolin		14	0.393
UNPD49205	Quercetin		8	0.055
UNPD51223	Luteoloside		8	0.133
UNPD130563	Scoparin		7	0.054

UNPD124411	Isochlorogenic acid C		6	0.044
UNPD127168	Hyperoside		5	0.024
UNPD60650	Isorhamnetin		5	0.017
UNPD70448	Genipin		4	0.197

[a] the two parameters of the nodes (molecules) in DTN were calculated by the NetworkAnalyzer plugin. BW represented betweenness centralities.

On the other hand, 37 potential targets (Table S4, ESI†) from virus (10) and human (27) were concerned in various pathogenic processes of URTIs including viral replications, inflammatory response and immune suppression. Among these targets, the hub nodes in the DTN (Fig. 2A) and TTN (Fig. 2C) should be paid more attention. For example, microsomal prostaglandin E_2 synthase-1 (mPGES-1) with the highest degree and betweenness has been considered as a novel drug target for anti-inflammatory drugs to reduce side effects of current cyclooxygenase-2 (COX-2) inhibitors[55]. And the secreted phospholipase A_2 (sPLA2) enzyme and nitric oxide synthase (NOS) were the main target against inflammatory disorders[56-58]. In addition, rhinovirus coat protein (VP1) with high degree and betweenness participating in viral attachment was also an important target for the identification of anti-rhinovirus drugs because rhinovirus was the most common cause for human URTIs[59].

Action mechanism of RDN on URTIs

The above DTN and TTN (Fig. 2) showed that RDN could not only directly inhibit

some critical viral proteins for various respiratory viral replications, but also regulate host immune system to resist viral infection. Respiratory viruses are the most reason for URTIs, especially influenza and rhinovirus[1, 2]. Currently, antiviral treatment is a main strategy in clinic, such as neuraminidase (NA) inhibitors, M2 ion channel blockers and capsid-binding inhibitors[60]. Notably, there were 13 molecules in RDN that could target 10 viral proteins involving in viral cellular replication, viral attachment and viral release, especially influenza virus (6/10) (Fig. 2C). For example, our previous study has identified Luteolin and Quercetin as inhibitor of NA[61] *in vitro*. Similarly, Isochlorogenic acid A was reported to exert potent anti-RSV activity via the inhibition of virus-cell fusion[62]. On the other hand, various host cell factors played the major role in the replication of respiratory virus. Interestingly, some compounds could inhibit the cellular factors such as inosine 5'-monophosphate (IMP) dehydrogenase, S-adenosylhomocystein hydrolase (SAH) and orotidine 5'-monophosphate (OMP) decarboxylase[63], to disrupt the replication machinery of the respiratory virus. Thus RDN would treat URTIs by directly inhibiting viral replication.

Meanwhile, the symptoms of URTIs in which the changes was the main parameter of efficacy for new treatments, were mainly attributed to the host immune response to viral infection[3]. Inflammation is one of the first host defense systems to infection. Thus, drugs against inflammation may be beneficial to alleviate these symptoms. Network analysis showed that these candidate active molecules could directly interact with the key synthases of pro-inflammatory cytokines. Moreover, several potential targets (e.g. PI3KC, JNK, ERK, p38) participated in phosphatidylinositol 3-kianse (PI3K) and mitogen activated protein kinase (MAPK) pathways, which were important to regulate the expression of chemokines, cytokines and the key enzymes after respiratory virus infection[64]. Similarly, Respiratory virus could also take advantage of the cell signaling pathways to ensure efficient replication[65]. For example, MEK1-ERK signaling cascade was reported to be an essential role in Enterovirus 71 (EV71) and influenza A replication[66, 67]. Thus, the results described another mechanism that the compounds could target the host biological pathways to inhibit respiratory viral replication and to relieve the clinical symptoms of URTIs.

Therefore, RDN could treat URTIs by two ways: directly suppressing replication of viruses via targeting different key proteins in respiratory viral life cycle; regulating the host defense system to indirectly resist viral infection and to alleviate the symptoms of URTIs. Meanwhile, it interpreted the broad and non-specific anti-pathogen action of RDN on respiratory virus.

In vitro validation

The phenotype (biomarker) of cells could reflect the effects of multi-target compounds to some extent[68]. Therefore, a macrophage cell-based assay was applied to validate the anti-inflammatory activities of the compounds with high degree (Table 3). LPS was often used as a stimulus in inflammatory models. Thus, the model of LPS-induced PGE_2 and NO production in RAW264.7 cells was employed to investigate the anti-inflammatory activities of the compounds. Table 4 showed that four ingredients could inhibit PGE_2 and NO production in concentration-dependent

manner. Similarly, both *in vitro* and *in vivo*[21] experimental results (Fig. 3) also validated that RDN had inhibitory effect on production of PGE_2 and NO. Thus, it indicated that the prediction model based on combination of molecular docking and network analysis had a good performance, and could identify the active compounds and potential targets of RDN. The results suggested that the high degree of the compounds could be more potent to modulate the biological pathways by interacting with more protein targets to lead phenotypic responses. In addition, the approach can be used to evaluate the efficacy of a compound on other biosystems or diseases if the corresponding biological pathway can be constructed, such as blood clotting cascade and platelet aggregation[68,69].

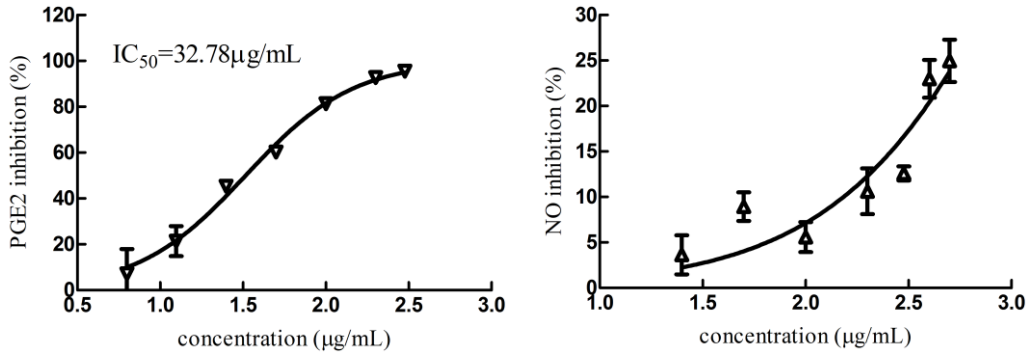


Fig. 3 the dose-response curves of RDN against the production PGE_2 and NO.

Table 4 the inhibitory potency of the compounds with high degree on PGE_2 and NO production

UNPD ID	IC_{50} (μM)		UNPD ID	IC_{50} (μM)	
	PGE_2	NO		PGE_2	NO
UNPD189689	25.6	20.6	UNPD49205	51.8	145.7
UNPD149880	2.6	6.9	UNPD60650	11.0	32.8
UNPD51223	- [a]	- [a]	UNPD124411	ND [b]	ND [b]
UNPD130563	- [a]	- [a]	UNPD130563	ND [b]	ND [b]

[a] the inhibitory activities of the compounds on PGE_2 and NO were less than 30% at the concentration of 500 μM.

[b] the compounds were not available to purchase for wet experiments.

Indication prediction

Systems biology and network analysis showed that many diseases were generally caused by malfunction of multiple genes[70]. Their products would further perturb different biological processes, leading to different diseases[71]. As a result, the diseases shared biological processes could be treated by the same drug[72]. Similarly, this also offered an opportunity to explore new indications for RDN by TDN (Fig. 4). The results showed that there were 124 relationships between 26 potential targets and 99 diseases in TTD. Among the diseases, most of them were classified into neoplasms (26), pathological conditions, signs and symptoms (19), cardiovascular diseases (18), immune system diseases (13), respiratory tract diseases (9) and virus diseases (7)

(Table S5, ESI†). It suggested that RDN could be used in clinic for treating the manifestations of these diseases to some extent. For example, RDN was reported to treat chronic obstructive pulmonary disease (COPD)[73] and infantile Rotavirus enteritis[74] in clinical practices. This would be possible to provide new information on clinical uses for RDN and promote drug discovery from TCM.

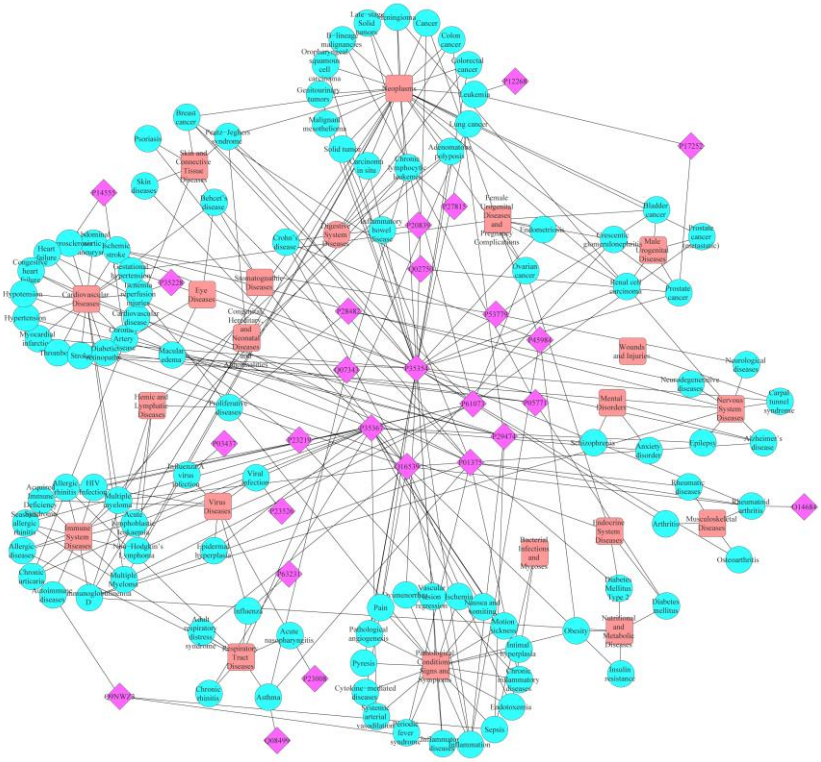


Fig. 4 Target-disease network. Cyan ellipses and purple diamond represented diseases and potential targets, respectively. 99 disease terms were organized into 21 categories (pink round rectangle) according to MeSH.

Conclusions

Traditional Chinese Medicine has accumulated a large number of clinical practices, and exhibited broad pharmacologic effects, but the underlying mechanism was still unclear. Due to complicate interactions between compounds and cellular targets, network pharmacology would offer an alternative way to illustrate the multi-component and multi-target characteristics of TCM. In this work, a network pharmacology approach was employed by integrating molecular docking, network analysis and bioactivity validation to uncover the molecular mechanism of RDN on URTIs, that is inhibiting virus-host cell interaction and regulating cell signaling pathways by combination of multi-target ingredients. The results demonstrated that herbal products could have unique characteristics with regard to its use as antiviral agents. Meanwhile, this work could provide a new therapeutic strategy to prevent emergence of viral resistance. However, the work only offered some hints on the therapeutic mechanism of RDN, and there still need to be more wet experiments in the future work.

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