Efficacy of active compounds of Chanqin granules on airway neurogenic inflammation induced by PM2.5 in vivo

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Abstract

OBJECTIVE: To investigate the efficacy of active compounds of Chanqin (CQ) granules on PM2.5-induced airway neurogenic inflammation in vivo, and to elucidate the underlying mechanisms of action.

METHODS: The Traditional Chinese Medicine systems pharmacology (TCMSP) database was searched, and the results were combined with oral bioavailability and drug analysis to identify the compounds in CQ granules. The pharmacophore modeling approach was used to predict the compound targets, and the diseases corresponding to the targets were obtained by searching the therapeutic target database (TTD), pharmacogenomics knowledgebase (PharmGKB) and DrugBank databases. Cytoscape software was used to construct the network pharmacological charts for Component-Target and Target-Disease interactions of the CQ granules. Then, the mechanisms of action and effectiveness of CQ granules for the treatment of PM2.5-induced airway neurogenic inflammation were analyzed.

RESULTS: A total of 195 compounds and 171 targets were obtained from the analyses. A total of 569 corresponding diseases were identified for these targets. Component-target and target-disease networks were constructed. The possible mechanisms and effective components in CQ granules for treating airway neurogenic inflammation were analyzed. Quercetin, kaempferol and isorhamnetin, beta-sitosterol and sitosterol, which are typically found in the formulation, have extensive pharmacological activities, including anti-inflammatory, antioxidant and antiviral actions and neuroprotective properties. Among these targets, androgen receptor, estrogen receptor, prostaglandin G/H synthase 2, and inducible nitric oxide synthase play important pathological roles, including the induction of neurogenic inflammation. CQ granules may have therapeutic effectiveness for numerous diseases in addition to respiratory diseases, including neoplasms, digestive system diseases, cardiovascular diseases, respiratory tract diseases and nervous system diseases. In vivo, CQ granules are effective in treating pulmonary inflammation and downregulate neuropeptides in the bronchoalveolar lavage fluid after PM2.5 exposure. CQ granules significantly decreased the levels of neurokinin A, neurokinin B and calcitonin gene-related peptide in the lung and dorsal root ganglia. CQ also significantly suppressed the upregulation of p-extracellular regulated protein kinase 1/2 and p-methyl ethyl ketone 1/2 induced by PM2.5 exposure.

CONCLUSION: CQ granules have potential for the
treatment of neurogenic inflammation induced by PM$_2.5$ in vivo, and the mechanism might involve downregulation of neuropeptides in the BALF, lung and dorsal root ganglia.

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**Keywords:** Neurogenic inflammation; Pharmacology; Neuropeptides; MAP kinase signaling system; Ganglia, spinal; PM$_2.5$; Chan Qin granules

**INTRODUCTION**

In recent years, the impact of air pollution on human health has attracted increasing attention worldwide. The latest WHO report indicates that 3.7 million deaths were caused by air pollution in 2012. Current research on the mechanism of action of Traditional Chinese Medicines (TCMs) for treating diseases caused by PM$_2.5$ has mainly focused on two pathological processes: peroxidative injury and inflammatory injury. Research on tachykinins in the respiratory tract has substantially advanced our understanding of the key role of neurogenic inflammation in the pathogenesis of airway diseases, including that caused by PM$_2.5$, which is presently a hot research topic. Drugs that target tachykinin could therefore be useful for treating neurogenic inflammation caused by PM$_2.5$. Chan Qin (CQ) granules, a TCM, are composed of 11 different herbs, and have been shown to have various pharmacological effects in airway injury, including an antiinflammation effect. Furthermore, they can relieve cough, alleviate early inflammation post-infection, and reduce neurogenic inflammation. However, the therapeutic mechanisms of action of the active substances in CQ remain poorly understood.

Most Chinese herbs exert a therapeutic action by targeting a number of molecules in the human body. However, it is difficult to identify these multiple targets. Additionally, thousands of compounds are present in most TCM formulations, making it difficult to identify the active constituents. Therefore, there is an urgent need for a new strategy to identify the active ingredients and therapeutic targets of TCMs. Systematic pharmacology is an emerging field combining oral bioavailability (OB) prediction, multidrug target prediction and network analysis to understand effective components and therapeutic targets of TCMs. In this study, we investigate the therapeutic efficacy of the active compounds of CQ granules on PM$_2.5$-induced pulmonary neurogenic inflammation in vivo. We use a systematic pharmacological approach to predict the therapeutic value of the active compounds, and we examine the underlying mechanisms of action. Furthermore, network prediction is used to identify drug targets.

**MATERIALS AND METHODS**

**Chemicals and animals**

PM$_2.5$ was purchased from the National Institute of Standards and Technology (SRM #1649b, NIST, Boulder, CO, USA). Antibodies against SP, neuropeptide B (NKA), neuropeptide B (NKB), calcitonin gene related peptide (CGRP), extracellular regulated protein kinase (ERK) 1/2, p-ERK1/2, methyl ethyl ketone (MEK) 1/2 and p-MEK1/2 were purchased from Abcam, Cambridge, UK. Thirty-two Sprague-Dawley rats (male), weighing 180-220 g, were purchased from the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine (Shanghai, China). The rats were housed under controlled temperature (26-28 °C), humidity (50% ± 10%) and a 12/12-h light/dark cycle, and fed standard food and water. Throughout the experiment, the health of the animals was monitored. The animal experiments were carried out with the approval of the Experimental Animal Care and Ethics Committee of the Shanghai University of Traditional Chinese Medicine. The methods were in accordance with the guidelines of the Experimental Animal Care and Ethics Committee of the Shanghai University of Traditional Chinese Medicine.

**Dataset construction**

All components in CQ granules were extracted from the TCM pharmacology database (http://tccmspwn.com) and added to the CQ component database. A total of 1145 chemicals were identified in the 11 constituent herbs, as follows: 280 in Gancao (Radix Glycyrrhiza, GC), 102 in Jiegeng (Radix Platycodi, JP), 17 in Zhiqiao (Fructus Aurantii Submaturus, ZQ), 58 in Huangqin (Radix Scutellariae Baicalensis, HQ), 288 in Chaihu (Radix Bupleuri Chinensis, CH), 116 in Banxia (Rhizoma Pinelliae, GC), 56 in Shegan (Rhizoma Belamcandae, BX), 56 in Shegan (Rhizoma Belamcandae, SG), 91 in Ziwan (Radix Asteris Tatarici, ZW), 28 in Baijuan (Rhizoma et Radix Cynanchi Stauntonii, BQ), 101 in Qianhu (Radix Peucedani, QH), and 8 in Chantui (Periostracum Cryptotympanae, CT).

**OB and drug-likeness screening**

Screening for OB, i.e. the oral dose of drug reaching the bloodstream, is an important stage in drug discovery and development. In addition, drug-likeness is a qualitative concept used in drug screening for evaluating the structural similarity between compounds and drugs in the DrugBank database, which is performed at the early stage of drug discovery. The drug-likeness prediction formula is as follows:

$$f(a, b) = \frac{a \cdot b}{\|a\|^2 + \|b\|^2 - a \cdot b}$$

where a represents the herbal ingredients, and b represents the average molecular drug-likeness index of all drugs in the DrugBank database. OB ≥ 30% and drug-likeness index ≥ 0.18 were set as the threshold to select candidate compounds.
Target and diseases analysis
Target recognition is a key step in drug development. The systematic drug targeting method is designed to identify potential targets for drugs and natural products. Chemical, genomic and pharmacological information is integrated for target prediction. In the current study, the goal was to use systematic drug targeting methods to identify the targets. We searched databases associated with the candidate targets which were extracted from the Therapeutic Target (TTD), DrugBank and PharmGKB databases, and the diseases were segregated further into different groups based on the MeSH Browser (2017 MeSH).

Network construction
To understand the complex relationship among the compounds, targets and diseases, networks were constructed. Herbs, candidate compounds, potential targets and diseases were used to construct the compound-target and target-disease networks. The networks were produced and analyzed with Cytoscape 3.2.1. The degree of a node is defined as the number of edges connected to it, reflecting the importance of the node in the network.

PM2.5 model and drug administration
The PM2.5 rat model was prepared as described in the previous study. We chose a PM2.5 exposure dose of 25 mg/kg (0.1 mL/100 g body weight). Briefly, 125 mg of PM2.5 (SRM#1649b, NIST) was suspended in 5 mL of phosphate buffered saline (PBS) for 24 h and agitated for 20 min. Before use, farinose solid was diluted with 0.9% sterilized saline to the concentration needed. Exposure was performed by intratracheal instillation with 0.9% saline or PM2.5 suspension, once a day for 4 weeks. On week 5, 30 rats exposed to PM2.5 were randomly divided into three groups and intratracheally administered normal saline (2 mL), CQ (9.36 g/kg, 2 g/mL) or dextromethorphan chlorpheniramine pseudoephedrine (DCP) solution (8 mL/kg) every day for 2 weeks. The control rats were administrated normal saline (2 mL). After exposure, on week 8, all the rats were killed, and the lung and dorsal root ganglia (DRG) tissues were collected. The components in CQ granules were as follows: SG 15 g, ZW 15 g, JG 9 g, CH 15 g, QH 10 g, GC 9 g, BQ 15 g, BX 10 g, HQ 15 g, ZQ 9 g, CT 6 g.

Histological analysis
Paraformaldehyde-fixed lung and DRG tissue samples were paraffin-embedded, and then cut into 4-μm sections and stained with Mayer’s hematoxylin and 1% eosin alcohol solution (HE staining). Morphological changes were examined by light microscopy. Lung injury was scored by an investigator blinded to the study protocol. For immunohistochemical analysis, after dewaxing, the lung and dorsal root ganglion tissue slices were microwaved with ethylene diamine tetracetic acid (EDTA) antigen retrieval buffer (pH 8.0), and then treated with 3% hydrogen peroxide solution to block endogenous peroxidases. The sections were thereafter incubated with normal serum for 30 min, followed by blocking reagent, and then incubated with primary antibodies against SP, NKA, NKB and CGRP (Abcam, Cambridge, UK). The sections were incubated with secondary antibodies at room temperature for 2 h, and analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

Western blotting
For Western blotting, 50 μg total protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes using a wet transfer system (Bio-Rad, Hercules, CA, USA). The membranes were then blocked with 5% nonfat milk in TBST buffer [2.42 g/L Tris-HCl, 8 g/L NaCl, and 1 mL/L Tween 20 (pH 7.6)], incubated overnight at 4 °C with primary antibodies suspended in TBST buffer, and then incubated with secondary antibody conjugated with horseradish peroxidase. Finally, the protein bands were detected on a ChemiDoc XRS + system (Bio-Rad, Hercules, CA, USA).

ELISA
The concentrations of neuropeptides (SP, NKA, NKB, CGRP) in whole protein extracts from alveolar lavage fluid were detected with a commercial enzyme linked immunosorbent assay (ELISA) kit (R&D Systems, Emeryville, CA, USA). Absorbances were measured with a microplate reader at 490 nm.

Statistical analysis
One-way analysis of variance (ANOVA) was conducted to test the differences between groups with SPSS 22.0 (IBM Corp., Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, IL, USA). Results are expressed as mean ± standard deviation. For all tests, a two-sided P-value less than 0.05 was considered significant.

RESULTS
Candidate compound screening of CQ granules
Identifying the active compounds in medicinal herbs is commonly used to uncover the mechanisms of action of TCMs. CQ is prepared from 11 medicinal herbs, including RB, RAT, RPL, RBC, RP, RG, RRCS, RHP, RSB, FAS and PC. All 11 herbs were submitted to the TCM Systems Pharmacology (TCMSp) database. Then, we combined OB screening with drug-likeness evaluation to identify the active substances in CQ granules. A total of 195 potential compounds with OB ≥ 30% and drug-likeness index ≥ 0.18 were harvested from the herbal constituents of CQ granules. All com-
pounds of one constituent, PC, were excluded because they showed a low OB or drug-likeness index. The 195 compounds from the 10 herbs were considered candidate compounds. QH, BQ, ZW, SG, BX, CH, HQ, ZQ, GC and JG contributed 24, 4, 19, 17, 13, 17, 24, 5, 92 and 7 candidate compounds, respectively. Among these, quercetin, kaempferol and isorhamnetin, which are typically found in CH, ZW and GC, have extensive pharmacological activities, including anti-inflammatory, antioxidant and antiviral actions. Similarly, beta-sitosterol and sitosterol, which are found in HQ, BQ and QH, have been reported to have antioxidant, anti-inflammatory and neuroprotective properties.

**Target prediction**
We explored the therapeutic targets of the ingredients. The pharmacophore modeling approach was used to predict potential targets based on the active compounds. A total of 171 potential targets were predicted for 177 candidate compounds, while the remaining 18 had no corresponding targets using this method. The 177 candidate compounds had interactions with 171 potential targets, and the connections between them totaled 3593. The number of potential targets of QH, BQ, ZW, SG, BX, CH, HQ, ZQ, GC and JG were 120, 54, 121, 84, 88, 118, 84, 75, 141 and 54, respectively. Although the number of targets differed among the herbs, there was substantial target overlap among them.

**Compound-target network**
To gain insight into the complex interaction of compounds and their corresponding targets at a systems level, we constructed a compound-target network based on the candidate compounds of CQ granules and their potential targets. As shown in Figure 2, the compound-target network contained 348 nodes (10 herbs, 177 candidate compounds, and 171 potential targets) and 3593 compound-target interactions. The mean degree value (the number of associated targets) of the candidate compounds was 12, and 129 compounds possessed a degree greater than 13, suggesting that most compounds impacted multiple targets. Specifically, four compounds, namely, quercetin, kaempferol, 5, 7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)chroman-4-one and beta-sitosterol, have 86, 55, 52 and 51 targets, respectively, and are therefore important pleiotropically-active compounds in CQ granules owing to their key positions in this network. Additionally, numerous compounds share the same target in the compound-target network. For example, the androgen receptor, estrogen receptor, prostaglandin G/H synthase 2, inducible nitric oxide synthase and peroxisome proliferator-activated receptor gamma are targeted by 166, 162, 145, 137 and 131 compounds, respectively.

To investigate the mechanisms of action of the 171 potential targets, ClueGO, a widely used Cytoscape plugin, was employed to identify interactions between functional groups and biological interpretations in biological networks. As shown in Figure 3, the results...
were divided into two strata: molecular functions and reactome analysis. The molecular functions were mainly categorized into four groups, acetylcholine receptor activity, monoxygenase activity, oxidoreductase activity and neurotransmitter receptor activity, suggesting that most potential targets were related to neural signal transmission and oxidoreductase activity (Figure 3A). The reactome of the targets were mainly related to JAKs associate with IL6RB, G alpha (z) autoinactivates by hydrolyzing GTP to GDP, thrombin activation, M2 and M4 receptors bind acetylcholine, and ERK1/2-activated AP1 complex binds the KDM6B promoter (Figure 3C).

**Target-disease network**

To identify diseases associated with CQ granules, the potential targets were uploaded to DrugBank, Therapeutic Target Database (TTD) and PharmGkb database. A total of 569 diseases were classified into 21 groups according to the MeSH Browser (2017 MeSH). Finally, a target-disease network was constructed based on potential targets and their corresponding diseases. As shown in Figure 4, multiple collected diseases belonged to neoplasms (179/569), digestive system diseases (58/569), cardiovascular diseases (54/569), respiratory tract diseases (44/569) and nervous system diseases (36/569), suggesting that CQ granules may have therapeutic effectiveness for numerous diseases in addition to respiratory diseases. These results indicate that this TCM formulation has a wide range of pharmacological activities, and could successfully be applied in the treatment of various diseases. For example, we found that prostaglandin G/H synthase 2, an important target of CQ granules, is linked to inflammation-associated diseases.
cases, cancer, and nervous and respiratory diseases, including pain, asthma, post-infectious cough and other airway diseases. Intriguingly, prostaglandin G/H synthase 2 has been linked to the pathogenesis of neurogenic inflammation and is a new therapeutic target for this disorder.\(^{18,22}\)

**Effect of CQ granules on airway inflammation and pulmonary neuropeptides**

Previous studies have shown that CQ granules can significantly reduce airway neurogenic inflammation caused by coughing after infection.\(^{3}\) In this study, we predicted that CQ granules are effective treatment for a variety of diseases, including respiratory diseases, nervous system diseases, inflammation-related diseases, and cancer. To test this, we studied the effect of CQ granules on airway neurogenic inflammation induced by PM\(_{2.5}\) in rats.

To evaluate the effect of CQ granules on airway neurogenic inflammation, a rat model of PM\(_{2.5}\) exposure was used. Lung injury, histopathology and neuropeptides in the bronchoalveolar lavage fluid (BALF) were assessed. As shown in Figure 5, compared with the model rats, CQ granules suppressed the increase in neuropeptide levels in the BALF of rats exposed to PM\(_{2.5}\), including substance P (SP), NKA, NKB and CGRP. DCP, widely used for the treatment of post-infectious cough, also decreased SP, NKA, NKB and CGRP levels in PM\(_{2.5}\)-exposed rats (Figure 5C-F). In addition, the increased lung injury scores in the model rat were dramatically mitigated by CQ granules (Figure 5B). These results suggest that CQ granules are effective in treating pulmonary inflammation and downregulate neuropeptides in the BALF after PM\(_{2.5}\) exposure.

Next, we investigated the effect of CQ granules on neuropeptides in lung tissue. As shown in Figure 6, compared with the model rats, CQ granules significantly decreased the levels of NKA, NKB and CGRP. Both CQ granules and DCP significantly downregulated pulmonary neuropeptides.

**Effect of CQ granules on neuropeptides in the DRG**

PM\(_{2.5}\) is a major hazard to the respiratory system. The mechanisms of PM\(_{2.5}\)-induced injury are not clear, but appear to involve oxidative stress, immune-mediated injury, and perturbed calcium homeostasis.\(^{23-26}\) Given the widespread distribution of tachykinin, TCM formulations may be better suited than single-target drugs for treating multiple tissues, such as the DRG. Here, we found increased expression of neuropeptides in the DRG of the rats exposed to PM\(_{2.5}\). We thus investigated the effect of CQ granules on this tissue. As shown...
and sitosterol, which are found in HQ, BQ and QH, dant and antiviral actions.

quercetin, kaempferol and isorhamnetin, which are found in CH, ZW and GC, have extensive pharmacology prediction analysis suggested that numerous potential targets of CQ granules are associated with the ERK1/2-activated AP-1 complex. Therefore, we evaluated the effect of CQ granules on the levels of ERK1/2 pathway components in the lung tissues and DRG of PM2.5-exposed rats. As shown in Figure 8, compared with the model rats, CQ granules significantly suppressed the upregulation of p-ERK1/2 and p-MEK1/2 induced by PM2.5 exposure.

We identified 177 compounds and 171 targets of CQ granules from the database. Among the compounds, quercetin, kaempferol and isorhamnetin, which are found in CH, ZW and GC, have extensive pharmacological activities, including anti-inflammatory, antioxidant and antiviral actions.3,11 Similarly, beta-sitosterol and sitosterol, which are found in HQ, BQ and QH, have been shown to possess antioxidant, anti-inflammatory and neuroprotective properties.13,25 We also found that different compounds in CQ granules regulate common or similar targets to exert synergistic effects. For example, many constituents, including beta-sitosterol, stigmasterol, vestirol, medicarpin and cavidine, can activate the 5-hydroxytryptamine 2A receptor, and can thereby provide a synergistic therapeutic effect for patients. Four compounds, namely, quercetin, kaempferol, 5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl) chroman-4-one and beta-sitosterol, which have a maximum number of targets, are key pleiotropically-active compounds in CQ granules owing to their crucial positions in the network. For example, several studies show that quercetin can inhibit inflammation, enhance immune function and promote apoptosis by mediating the activation of prostaglandin G/H synthase 2, peroxisome proliferator-activated receptor gamma, interleukin-2 and caspase-9.15,16 Additionally, many proteins are the targets of multiple compounds in the compound-target network, including androgen receptor, estrogen receptor, prostaglandin G/H synthase 2, and inducible nitric oxide synthase. These targets play important pathological roles, including the induction of neurogenic inflammation.17,18 Most of the related targets were related to the activation of neurotransmitter receptors, AP-1 family of transcription factors and thrombin activation. These bio-
Figure 7 Effect of CQ granules on SP, NKA, NKB and CGRP levels in the DRG of PM2.5-exposed rats

Normal group: the rats were intragastrically treated only with normal saline; model group: the PM2.5-exposed rats were intragastrically treated with 9.36 g/kg of CQ granules once daily; DCP group: the PM2.5-exposed rats were intragastrically treated with 8 mL/kg of DCP once daily. A1-A16: levels of SP, NKA, NKB and CGRP in the DRG were quantitatively analyzed. A1-A4: SP; A5-A8: NKA; A9-A12: NKB; A13-A16: CGRP. B1-B4: The levels of SP, NKA, NKB and CGRP in the DRG were quantitatively analyzed. B1: SP; B2: NKA; B3: NKB; B4: CGRP. Results are given as means ± standard deviation. \( P < 0.01 \), versus normal group; \( P < 0.01 \), versus model group (n = 8).


Figure 8 Effect of CQ granules on ERK1/2 and MEK1/2 levels in the lung and DRG tissues of PM2.5-exposed rats
Normal group: the rats were intragastrically treated only with normal saline; Model group: the PM2.5-exposed rats were intragastrically treated with 9.36 g/kg of CQ granules once daily; DCP group: the PM2.5-exposed rats were intragastrically treated with 8 mL/kg of DCP once daily. A: p-ERK1/2, ERK1/2, p-MEK1/2 and MEK1/2 expressions in lung tissues were detected by Western blotting. GAPDH was used as internal control. B: the relative expression of pERK1/2 in lung tissues. C: the relative expression of pMEK1/2 in lung tissues. E: the relative expression of pERK1/2 in DRG. F: the relative expression of pERK1/2 in DRG. Results are given as means ± standard deviation. *P < 0.01 versus normal group; **P < 0.01 versus model group (n = 8). CQ: Chanqin; DCP: dextromethorphan chlorpheniramine pseudoephedrine solution; ERK: extracellular regulated protein kinase; MEK: methyl ethyl ketone; SP: substance P; NKA: neuropeitin A; NKB: neuropeitin B; CGRP: calcitonin gene-related peptide; DRG: dorsal root ganglia.


