Discovery of the covalent SARS-CoV-2 M pro inhibitors from anti-viral herbs via integrating target-based high-throughput screening and chemoproteomic approaches

Guang-Bo Ge[1], Ya-Ni Zhang[1], Guang-Hao Zhu[1], Wei Liu[2], Xi-Xiang Chen[1], Yuan-Yuan Xie[1], Jian-Rong Xu[1], Mei-Fang Jiang[1], Xiao-yu Zhuang[1], Wei-Dong Zhang[1], and Hong-Zhuan Chen[1]

[1]Shanghai University of Traditional Chinese Medicine
[2]Shuguang Hospital

July 11, 2023

Abstract

The main proteases (M ^{pro}) are highly conserved cysteine-rich proteins that can be covalently modified by numerous natural and synthetic compounds. Herein, we constructed an integrative approach to efficiently discover covalent inhibitors of M ^{pro} from complex herbal matrices. This work begins with biological screening of sixty clinically used antiviral herbal medicines, among which *Lonicera japonica* (LJ) demonstrated the strongest anti-M ^{pro} effect (IC $_{50}$ = 37.82 μg/mL). Mass spectrometry-based chemical analysis and chemoproteomic profiling revealed that LJ extract contains at least 50 constituents, of which 22 exhibited the capability to covalently modify M ^{pro}. We subsequently verified the anti-M ^{pro} effects of these covalent binders. Gallic acid and quercetin were found to potently inhibit SARS-CoV-2 M ^{pro} in dose- and time- dependent manners, with the IC $_{50}$ values below 10 μM. The inactivation kinetics, binding affinity and binding mode of gallic acid and quercetin were further characterized by fluorescence resonance energy transfer, surface plasmon resonance, and covalent docking simulations. Overall, this study established a practical approach for efficiently discovering the covalent inhibitors of M ^{pro} from herbal medicines by integrating target-based high-throughput screening and mass spectrometry-based assays, which would greatly facilitate the discovery of key anti-viral constituents from medicinal plants.

Research article

Discovery of the covalent SARS-CoV-2 M^{pro}inhibitors from anti-viral herbs *via* integrating target-based high-throughput screening and chemoproteomic approaches

Ya-Ni Zhang[a], Guang-Hao Zhu[a], Wei Liu[b], Xi-Xiang Chen[a], Yuan-Yuan Xie[c], Jian-Rong Xu[c], Mei-Fang Jiang[a], Xiao-Yu Zhuang[a,**], Wei-Dong Zhang[a], Hong-Zhuan Chen[a,c], Guang-Bo Ge[a,*]

[a] *Shanghai Frontiers Science Center of TCM Chemical Biology, Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, Shanghai, China.*

[b] *Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai, China.*

[c] *Academy of Integrative Medicine, Shanghai University of Traditional Chinese Medicine, 1200 Cailun Road, Shanghai, 201203, China.*

*Corresponding author:

E-mail address: geguangbo@shutcm.edu.cn (G.-B. Ge)

Phone: +86-024-51323184

**Corresponding author:

E-mail address: xyzhuang59@foxmail.com (X.-Y. Z)

Abstract

The main proteases (M^{pro}) are highly conserved cysteine-rich proteins that can be covalently modified by numerous natural and synthetic compounds. Herein, we constructed an integrative approach to efficiently discover covalent inhibitors of M^{pro} from complex herbal matrices. This work begins with biological screening of sixty clinically used antiviral herbal medicines, among which *Lonicera japonica* (LJ) demonstrated the strongest anti-M^{pro} effect (IC_{50} = 37.82 μg/mL). Mass spectrometry-based chemical analysis and chemoproteomic profiling revealed that LJ extract contains at least 50 constituents, of which 22 exhibited the capability to covalently modify M^{pro}. We subsequently verified the anti-M^{pro} effects of these covalent binders. Gallic acid and quercetin were found to potently inhibit SARS-CoV-2 M^{pro} in dose- and time- dependent manners, with the IC_{50} values below 10 μM. The inactivation kinetics, binding affinity and binding mode of gallic acid and quercetin were further characterized by fluorescence resonance energy transfer, surface plasmon resonance, and covalent docking simulations. Overall, this study established a practical approach for efficiently discovering the covalent inhibitors of M^{pro} from herbal medicines by integrating target-based high-throughput screening and mass spectrometry-based assays, which would greatly facilitate the discovery of key anti-viral constituents from medicinal plants.

Keywords: SARS-CoV-2 M^{pro}, *Lonicera japonica* (LJ), anti-viral agents, covalent inhibitors, inhibitory mechanism

1. Introduction

The Coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus has a significant impact on global health and the economy[1]. Emerging evidence highlights the potential value of herbal medicine in the treatment of viral infections including COVID-19, due to their potentials to block viral replication and relieve virus-induced systemic inflammation. For instance,*Lonicera japonica, Pomegranate peel, and Bergenia ligulata* have been shown to be capable of impede influenza virus replication[2-4],*Isatis tinctoria, Laggera pterodonta* and *Forsythia suspensa* have been reported to both inhibit virus replication and suppress inflammatory responses[5-7]. Herbal medicines are undoubtedly a rich source of tool compounds and drug leads for developing novel antiviral drugs[8,9]. However, unlike Western medicines that commonly formulated with a single active ingredient, herbal medicines contain complex mixtures of chemically diverse natural compounds[10]. This complexity poses a challenge when it comes to identifying and isolating the specific active constituents responsible for their therapeutic effects. Furthermore, the availability of commercially purified compounds for certain constituents is often limited, further hindering in-depth characterization of their pharmacological effects and mechanisms of action.

Several virus-based and host-based targets have been validated for the discovery and development of antiviral agents to combat COVID-19. Among these therapeutic targets for β-coronaviruses (CoVs), the M^{pro} has garnered significant attentions from medicinal chemists due to its critical role in the viral replication and assembly[11]. There is increasing evidence indicating that inhibiting or disrupting M^{pro}can prevent the formation of replication-essential enzymes, thus impeding viral multiplication and replication[12,13]. The SARS-CoV-2 M^{pro}, also known as 3-chymotrypsin-like proteases ($3CL^{pro}$), is a canonical cysteine protease that contains 12 cysteine residues per monomer[14]. Among these, Cys145 and Cys44, located in the catalytic pocket of the enzyme, are particularly important for its function[15-17]. They form the catalytic dyad that cleaves the viral polyproteins into smaller functional proteins necessary for viral replication. In addition, Cys156 and Cys300 play important roles in forming enzymatically active dimeric forms of the protein[17-19]. Targeting these cysteine residues is a promising avenue of research for developing effective inhibitors against SARS-CoV-2 M^{pro}. The scientific community has dedicated to discovering new covalent inhibitors for M^{pro}. Commonly employed approaches in this endeavor include high-throughput screening and structure-based vir-

tual screening, both relying on known compound libraries or databases[20,21]. In this context, several natural compounds derived from traditional medicinal plants, such as myricetin, oridonin and isojacareubin, have been identified. These compounds possess catechol or Michael receptors that have the ability to covalently bind to the cysteine residues in SARS-CoV-2 M^{pro}[16,17,22,23].

In this work, a practical strategy for rapidly discovering irreversible inhibitors of SARS-CoV-2 M^{pro} from complex components (such as herbal extracts or compound prescriptions) was demonstrated. Our approach integrates target-based high-throughput screening and mass spectrometry (MS)-based approaches, enabling efficient screening of the herbal medicines with potent time-dependent inhibition on SARS-CoV-2 M^{pro} and to rapidly identify the covalent M^{pro} inhibitors from crude herbal extract (**Fig. 1**). Firstly, fluorescence resonance energy transfer (FRET) technique was employed for screening the anti-M^{pro} effects of clinically used anti-viral herbal medicines and the results showed that*Lonicera japonica* (LJ) significantly inhibited SARS-CoV-2 M^{pro} in a time-dependent manner. After then, ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) was used for global chemical profiling of the constituents in LJ extract, while the M^{pro}-compound conjugated were carefully characterized, which provided key information to decipher the key constituents in LJ extract with the capability to covalently modify M^{pro}, as well as the key modified site(s) of M^{pro} by small molecules. This strategy enables both multiplexed screening and direct identification of small molecule binders from complex herbal mixtures, eliminating the need for fractionation. Furthermore, inactivation kinetics, binding kinetics and covalent docking simulations were conducted to gain further insights into the interactions between the target protein and the newly identified M^{pro}inhibitors from LJ extract.

2. Material and methods

2.1. Reagents

Herbal products with potential antivirus activity were obtained from Tianjiang Pharmaceutical Co., Ltd. (Jiangsu, China). Ammonium bicarbonate (NH_4HCO_3), dithiothreitol, and hydrochloric acid (HCl) were provided from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Urea was purchased from Dalian Meilun Biotechnology Co. Ltd. (Dalian, China). Iodoacetamide, chymotrypsin and trypsin were provided by Sigma-Aldrich (St. Louis, MO, USA). The peptide Dabcyl-KNSTLQSGLRK-Edans was synthesized from GenScript biology science and technology Co. Ltd. (Nanjing, China).

2.2 Screening of the anti-SARS-CoV-2 M^{pro} effects of herbal products

This study screened 60 herbs with potential antiviral effects for their inhibitory effects against SARS-CoV-2 M^{pro}. The proteolytic activity of M^{pro} was evaluated through a fluorescence resonance energy transfer (FRET) assay utilizing a fluorescently tagged substrate (Dabcyl-KNSTLQSGLRK-Edans). The procedures were conducted in a 96-well black plate, following the steps outlined in the reported study[24,25]. The inhibitory activities of LJ extract and individual constituents in LJ anti-SARS-CoV-2 M^{pro} were evaluated using identical methods mentioned above.

2.3 Global chemical analysis of LJ by UHPLC-Q-Exactive Orbitrap HRMS

The comprehensive analysis of the components in LJ was performed using an ultra-high performance liquid chromatography system (Dionex UltiMate 3000, Thermo Fisher Scientific) coupled to a Q-Exactive Orbitrap high resolution mass spectrometer (Q-Exactive HRMS, Thermo Fisher Scientific)[26]. The liquid chromatography and mass spectrometry conditions are detailed in the supplementary material. To differentiate isomers and reduce false positive results, component identification was based on their accurate molecular weights and comparison of retention times and tandem mass spectrometry fragmentation patterns with reference standards.

2.4 MS-based proteomics

Natural compounds that capable of covalently modify cysteine residues on SARS-CoV-2 M^{pro}, as well as the modification site, were elucidated using a MS-based chemoproteomic approach. To achieve this, 30 μM

SARS-CoV-2 M^{pro} was co-incubated with LJ (final concentration, 200 μg/mL) at 37 °C for 3 hours. The adduct was transferred to 10 kDa centrifugal filter tubes to remove unbound molecules, followed by washing with water. The sample was denatured in 100 μL of buffer containing 8 M urea for 1 hour and treated with dithiothreitol and iodoacetamide to reduce and alkylate cysteine residues, respectively. The protein was digested with chymotrypsin and trypsin in 100 μL of 50 mM NH_4HCO_3 at 37 °C overnight with an enzyme-to-substrate ratio of 1:50 (w/w). And further desalted using a Solid Phase Extraction Cartridge (MonoSpin C18, GL Sciences). The eluents were resolved in 40 μL of 0.1% formic acid after being vacuum-dried for analysis.

2.5 Inactivation kinetic analyses

The inactivation kinetic analysis of gallic acid and quercetin towards SARS-CoV-2 M^{pro} was performed in black 96-well plates. The enzyme was preincubated with inhibitors at varying concentrations and durations, and then the reaction was started with a constant substrate concentration. The natural logarithm of the residual activity of SARS-CoV-2 M^{pro} was plotted against the preincubation time using Graphpad Prism 7.0. Detailed information was as described in the reported literature[24,27].

2.6. Surface plasmon resonance assay

The interactions between SARS-CoV-2 M^{pro} and gallic acid and quercetin for were studied by SPR include immobilizing SARS-CoV-2 M^{pro} on a CM5 sensor chip surface and then flowing a solution containing the compound over the surface. The sensor surface was activated with a 7 min injection of the mixture of 50 mM N-hydroxysuccinimide (NHS) and 200 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). 10 mM sodium acetate was used as a running buffer. Compounds was diluted to different concentrations and injected over the surfaces at a flow rate of 10 μL/min. The contact time was 90 s, followed by a 180 s dissociation period. All SPR experiments were conducted on Biacore T200 instruments (Cytiva, Sweden). The specific experimental protocol and conditions were performed as described in previous study[28,29].

2.7 Covalent docking simulations

The MOE (Molecular Operating Environment 2019.01, Chemical Computing Group Inc., Montreal, Canada) was used to perform covalent docking simulations to analyze the potential binding modes of gallic acid and quercetin to M^{pro}[30]. First, the crystal structure of SARS-CoV-2 M^{pro} (PDB Code: 7NBY) was prepared using the QuickPrep module, which involved adding hydrogen atoms, removing water and hetero molecules, and energy minimization. Gallic acid and quercetin were generated as ligands, with Cys85 and Cys128 selected as reactive sites for gallic acid and Cys22 for quercetin, as indicated in Table 1. Afterwards, MarvinSketch was used to define the covalent reaction formula for cysteine residuals and compounds. The protein structure was set as rigid receptor, and binding scores were evaluated using GBVI/WSA dG. The poses with the lowest S-score were chosen for the ligand-M^{pro}interaction analysis.

2.8 Data analysis

The MS RAW files were converted to mgf format by MSconvert and output by pLabel v2.4.1. GraphPad Prism 7.0 was used to calculate the IC_{50} and K_I values.

Results

3.1 Screening the antiviral herbs with strong anti-SARS-CoV-2 M^{pro}effect

First of all, the anti-SARS-CoV-2 M^{pro} potentials of 60 antiviral herbs were assessed, while the residual activity of the protease was measured by a FRET-based assay. As shown in**Fig.2** , LJ exhibited potent inhibitory effects on M^{pro} at a final concentration of 100 μg/mL, resulting in a residual activity of 6.55%. Subsequent assays revealed that LJ could block the activity of SARS-CoV-2 M^{pro} in a time- and dose- dependent manner. The IC_{50} value for preincubation of 63 minutes was determined as 37.82±1.038 μg/mL**(Fig. 2).** Moreover, we used DTT sensitivity analysis to verify the inactivation mechanism of M^{pro} by *Lonicera japonica* . In the absence of DTT, the residual activity of 100 ug/mL LJ on M^{pro} was close to 0. In contrast, the addition of 1 mM DTT resulted in almost no inhibition of M^{pro}**(Fig. S2)** . These results suggested that

LJ could potently and time-dependently inhibit M^{pro} mainly through the interactions with cysteines of this viral enzyme.

3.2 Global chemical analysis of LJ

The chemical components of herbal medicines are extremely complex, with a significant proportion being trace or minor constituents[31]. To comprehensively identify the chemical components in LJ, we employed UHPLC-Q-Exactive Orbitrap HRMS for the analysis of this herbal extract. The structures of the components were determined by comparing their LC-MS characteristics,*i.e.* retention time, exact molecular weight, and tandem mass spectrometry fragmentation pattern, with reference standards, compounds reported in the literature, as well as those registered in PubChem or other databases. Under the optimized chromatography and mass spectrometry conditions, a total of 50 components in all were identified, including organic acids, flavonoids, catechins and terpenoids (**Fig. S3, S5-S54** and **Table S1**). Notably, some flavonoids (e.g., quercetin and luteolin) and catechins (e.g., gallic acid and caffeic acid) in LJ contain one or more catechol groups in their structures, which are susceptible to oxidation and form*o* -quinone. These *o* -quinones have the potential to form covalent bonds with the cysteines of M^{pro}[17,32,33].

3.3 Cysteine modification profiling of SARS-CoV-2 M^{pro} by LJ

After elucidating the natural compounds in LJ extract, we proceeded to the next step aiming at identifying those capable of covalently modifying M^{pro} and to determine the attachment site. This was achieved using a chemoproteomic approach based on bottom-up mass spectrometry. The LJ extract was served as a natural compound library to screen inhibitors that acting as covalent binders. Recombinant M^{pro} was directly incubated with LJ extract under near-physiological conditions, and subsequently, covalent protein-inhibitor conjugates were digested and evaluated using nanoflow LC-MS/MS.

To get a higher peptide coverage, we performed the method of filter aided sample preparation (FASP)[34,35]. The results demonstrated that the FASP method's peptide coverage could reach 88.56%, and more satisfactorily, the coverage of cysteine-containing peptides was up to 100%. The FASP method involves preparing a denatured enzyme-inhibitor mixture and then performing enzymatic digestion on the membranes of 10 kDa ultrafiltration tubes. It should be emphasized that both the catalytic domain cysteines and the cysteines essential for the formation of the dimeric form were discovered.

Given that each modification imparts specific fixed mass shifts to the peptide precursor ion and fragment ions, the mass increase corresponding to compounds adduction were considered as variable modifications of cysteine. The localization of chemical modification within the peptide sequence was determined by MS/MS fragment ion matching. Taking quercetin as an example, a mass shift of 300.02700 Da attributed to the Michael adduct of quercetin's *o* -quinone form was defined as a variable modification of cysteine during the database searching step. The MS/MS spectra of the modified peptides were further manually checked and verified. **Table 1** listed the 22 components in LJ that could covalently bind on M^{pro} and their binding sites. The MS^2 spectra of all modified peptides of M^{pro} were demonstrated in **Fig. S55-S70** . The catalytic site of SARS-CoV-2 M^{pro} is characterized by a cysteine-histidine catalytic dyad (Cys145 and His41)[34]. In addition to Cys145, the cysteine residues near the catalytic site (including Cys22, Cys44, and Cys85) may also play crucial roles in enzymatic catalysis or stabilizing this viral enzyme[17,36]. Peptide-level analysis reveals that quercetin and caffeic acid can covalently bind to these cysteine residues. The enzyme is active only as a dimer. Apigenin and quercetin were found to covalently bind to the cysteine residue (Cys156) at the dimerization interface, which may result in loss of enzyme function[37]. Modifying the remaining cysteine residues (such as Cys117 and Cys160) on SARS-CoV-2 M^{pro}, however, may have a minor impact on the enzyme's activity.

3.4 Anti-SARS-CoV-2 M^{pro} effects of the newly identified covalent binders

Next, the anti-M^{pro} effects of these newly identified covalent binders from LJ were validated by using the standards or the purified compounds. Herein, a total of 20 naturally occurring M^{pro} binders isolated from LJ were collected and their anti-M^{pro} effects were tested one by one. As demonstrated in **Fig. 4** , among

all tested natural covalent binders, nine compounds exhibited relatively strong inhibition to the proteolytic activity of M^{pro} in both time- and dose- dependent manners. Notably, gallic acid and quercetin exhibited the highest anti-M^{pro} potency with an IC_{50}value of 6.18 μM and 9.44 μM after preincubation for 63 min. The IC_{50} values for the other compounds and the positive inhibitor (myricetin) are listed in **Table 2** . The structures of all the above constituents are shown in **Fig. S4** .

3.5 Inactivation kinetics of gallic acid and quercetin against SARS-CoV-2 M^{pro}

Next, the inactivation kinetics of gallic acid and quercetin were studied *via* performing a suit of SARS-CoV-2 M^{pro} inhibition assays. The residual activity of gallic acid and quercetin were measured by varying inhibitor concentrations at various time intervals. The obtained data was analyzed to determine the rate and mechanism of inactivation. As illustrated in**Fig. 5** , gallic acid and quercetin could inactivate the activity of SARS-CoV-2 M^{pro} in dose- and time- dependent manners. The K_I value of gallic acid was determined to be 5.63 μM, and the K_{inact} value was calculated to be 0.05 min^{-1}. In contrast, the inactivation of M^{pro} by quercetin was relatively weak with theK_I value of 75.98 μM and theK_{inact} value of 0.05 min^{-1}.

3.6 Binding kinetics of gallic acid and quercetin with SARS-CoV-2 M^{pro}

The binding affinity of gallic acid and quercetin to M^{pro} was further investigated using SPR technique, which allowed the analysis of their interactions. As shown in**Fig. S72** , the observed association and dissociation phases of the gallic acid and quercetin exhibit clear biphasic behavior, which aligns with the anticipated two-step binding interaction of an irreversible inhibitor. Wherein, gallic acid and quercetin were both bound to M^{pro} in a dose-dependent manner and exhibited moderate affinity with K_D values of 0.321 μM and 0.308 μM, respectively (**Table S3**). Notably, the sensorgram plots suggested that gallic acid binds and dissociates more slowly from M^{pro} than quercetin, which might be caused by the multiple binding sites of gallic acid on M^{pro}[38].

3.7 Covalent docking

The binding modes of gallic acid covalently binding to SARS-CoV-2 M^{pro} at either Cys85 or Cys128 of were depicted in**Fig. 6** and **Fig. S73** . At the Cys85 site, gallic acid interacts with Asp187 and Arg40 through electrostatic interactions (π-Cation or π-Anion) and hydrogen bonds. At Cys128 site, hydrophobic interactions (π-Sigma, Alkyl and π-Alkyl) are responsible for fixing gallic acid to protein. The binding modes of quercetin covalently binding to SARS-CoV-2 M^{pro} were also analyzed. While quercetin forms covalent bonds with Cys22, it also forms hydrophobic interactions (π-Sulfur, π-Cation) and hydrogen bond with Lys61. In brief, covalent docking simulation revealed that gallic acid and quercetin inhibited SARS-CoV-2 M^{pro} through interacting with several key non-cysteine residues and covalently modifying some cysteine residues.

Discussion

M^{pro} plays a crucial role in the replication process of multiple β-coronaviruses including SARS-CoV-2 virus. M^{pro} are a class of highly conserved cysteine hydrolases distributed in coronaviruses, no similar mammalian genes are estimated to encode M^{pro} like proteases in healthy humans[39]. These characteristics prompted the researchers to discover and develop more efficacious covalent inhibitors of M^{pro} for completely blocking its catalytic activity. To this end, great efforts have been made to efficiently identify the covalent inhibitors targeting this protease utilizing various approaches. Given that M^{pro} is a cysteine-rich protease, it offers the potential to identify or design small molecules that can covalently bind to its key cysteine residues through a mechanism of nucleophilic addition, thereby inactivating the enzyme[40,41]. In the past few years, researchers have demonstrated that many natural ingredients found in medicinal and edible herbal products can covalently bind the key cysteines of SARS-CoV-2 M^{pro}. Not only do these ingredients possess strong ability to inactivate M^{pro}, but they also exhibit good safety. Inspired by these findings, we are committed to discover more covalent inhibitors of SARS-CoV-2 M^{pro} in herbal products. However, discovering covalent inhibitors for M^{pro} in clinically used herbs poses a significant challenge in antiviral pharmacology research. This is due to the highly complex chemical composition of these products, with many components existing

in extremely small amounts (less than 0.1% of the total weight). Efficient and accurate identification of such inhibitors remains a crucial task.

To address this issue, this study presents a practical platform for the rapid discovery of new SARS-CoV-2 M^{pro} covalent inhibitors from herbal medicines. The process involves screening antiviral herbs with fluorescent labeling techniques, analyzing the herbal medicine composition using HRMS, and characterizing modified peptides with mass spectrometry-based chemoproteomic method to identify compounds with potential covalent inhibitory activity. The comprehensive strategy proposed in this study for the rapid discovery of covalent inhibitors of SARS-CoV-2 M^{pro} from herbs has a few points that are worth noting. In the screening process, the time-dependent inhibition of M^{pro} by an herbal extract indicates the presence of covalent inhibitors of M^{pro}. Despite the complex and diverse nature of chemical components in herbal medicines poses a challenge in fully characterize them, the use of high-resolution mass spectrometry enables the assignment of most chemical structures by matching the LC-MS/MS features with natural compounds in the database or reference standards. Additionally, a high coverage of peptide sequence is critical for identifying all constituents that can covalently bind to the target protein. Identifying isomers individually is also necessary because their presence in the extract is inevitable. This can be done by separately incubating isomers or compounds of similar molecular weights with the target protein. Based on this strategy, we successfully found Lonicera japonica as having significant time-dependent inhibitory potential against SARS-CoV-2 M^{pro} among 60 herbs. Covalent inhibitors of M^{pro} were also uncovered from this herbal medicine.

Herbal medicines offer alternative therapies to address the multiple symptoms and complications associated with COVID-19 management[42,43]. It has been widely employed in the treatment of respiratory infections, and certain herbal components have gained approval as marketed drugs, over-the-counter nutritional supplements, or food additives[44]. Generally, the long-term use of herbal medicines and the availability of marketed herbal products have demonstrated satisfactory safety profiles, making them suitable for extended prophylactic use. Although the emergency phase of the pandemic may have subsided with the emergence of the less pathogenic omicron variant, the incidence of infection remains high among individuals who have received vaccines or have experienced natural infection[45,46]. In the post-pandemic period, the utilization of herbal medicines and their bioactive fractions holds great potential for both preventive measures and supportive treatment in relation to COVID-19[47]. As an extensively used herbal medicine in clinical settings, LJ possesses a wide range of health-promoting effects, such as heat-clearing and detoxifying, anti-inflammatory effects, activating meridians, broad-spectrum anti-bacterial and anti-viral effects[48-50]. These characteristics make LJ a commonly employed herb for treating respiratory infections, fever, sore throat, and other inflammatory conditions. Considering LJ's significant ability to inhibit M^{pro} and its anti-inflammatory effects, it is expected that LJ may exert multifaceted effects in terms of reducing the duration of COVID-19 symptoms, suppressing uncontrolled inflammation (such as cytokine release syndrome), and alleviating long COVID symptoms.

Conclusion

In summary, an integrative approach was constructed to efficiently discover SARS-CoV-2 M^{pro} covalent inhibitors from anti-viral herbs, including target-based high-throughput inhibition assay and mass spectrometry-based chemoproteomic approaches. By employing this multifaceted analytical approach, we successfully uncovered the covalent inhibitors of SARS-CoV-2 $M^{pro}$from a clinically used anti-viral herb *Lonicera japonica* that was found with significant time-dependent inhibition against SARS-CoV-2 M^{pro}. Our findings revealed that 22 constituents in LJ extract could covalently modify the cysteine residues of SARS-CoV-2 M^{pro}. Among these, gallic acid, quercetin and cynaroside could dose- and time- dependently inhibited SARS-CoV-2 M^{pro}, with the IC_{50} values of less than 20 μM. The inhibition mechanisms and binding modes between of two potent inhibitors (gallic acid and quercetin) were comprehensively characterized. Overall, we present the screening, discovering, biochemical and biophysical validation of covalent inhibitors targeting M^{pro} from antiviral herbal medicines. Beyond providing potential lead compounds for future development of novel anti-SARS-CoV-2 agents, this integrated approach is expected to facilitate the efficient and confident identification of naturally occurring anti-viral constituents derived from medicinal plants.

Declaration of Competing Interest

No competing interests.

CRediT authorship contribution statement

Ya-Ni Zhang : Methodology, Data Curation, Writing- Original Draft. **Guang-Hao Zhu** : Software, Visualization. **Wei Liu** : Methodology, Data Curation. **Yuan Xiong** : Methodology, Data curation. **Qing Hu** : Methodology. **Gui-Hua Jia** : Methodology. **Xiao-Yu Zhuang** : Writing- Reviewing and Editing.**Wei-Dong Zhang** : Project administration. **Guang-Bo Ge** : Supervision, Funding acquisition, Project administration, Writing, Reviewing and Editing.

Acknowledgments

This study was funded by National Key Research and Development Program of China (2022YFC3502000, 2021YFE0200900), the NSF of China (82273897, 81922070, 81973286, 82104281, 82173798), Shanghai Science and Technology Innovation Action Plans (21S21900600, 20S21901500, 20S21900900) supported by Shanghai Science and Technology Committee, the Innovation Team and Talents Cultivation Program of National Administration of Traditional Chinese Medicine (ZYYCXTDD-202004), the Three-year Action Plan for Shanghai TCM Development and Inheritance Program (ZY (2021-2023)-0401), Shanghai Municipal Health Commission's TCM research project (2022CX005), and Shanghai Municipal Science and Technology Major Project (ZD2021CY001).

Reference

1. Yan F, Gao F. An overview of potential inhibitors targeting non-structural proteins 3 (PLpro and Mac1) and 5 (3CLpro/Mpro) of SARS-CoV-2. *Computational and structural biotechnology journal.*2021;19:4868-4883.

2. Lee YR, Chang CM, Yeh YC, et al. Honeysuckle Aqueous Extracts Induced let-7a Suppress EV71 Replication and Pathogenesis In Vitro and In Vivo and Is Predicted to Inhibit SARS-CoV-2. *Viruses.* 2021;13(2).

3. Moradi M-T, Karimi A, Rafieian-Kopaei M, Rabiei-Faradonbeh M, Momtaz H. Pomegranate peel extract inhibits internalization and replication of the influenza virus: An in vitro study. *Avicenna Journal of Phytomedicine.* 2020;10(2):143.

4. Rajbhandari M, Wegner U, Schoepke T, Lindequist U, Mentel R. Inhibitory effect of Bergenia ligulata on influenza virus A. *Die Pharmazie-An International Journal of Pharmaceutical Sciences.*2003;58(4):268-271.

5. Guan W, Li J, Chen Q, et al. Pterodontic acid isolated from Laggera pterodonta inhibits viral replication and inflammation induced by influenza a virus. *Molecules.* 2017;22(10):1738.

6. Zheng X, Fu Y, Shi S-S, et al. Effect of Forsythiaside a on the RLRs signaling pathway in the lungs of mice infected with the influenza a virus FM1 strain. *Molecules.* 2019;24(23):4219.

7. Liang X, Huang Y, Pan X, et al. Erucic acid from Isatis indigotica Fort. suppresses influenza A virus replication and inflammation in vitro and in vivo through modulation of NF-κB and p38 MAPK pathway.*Journal of pharmaceutical analysis.* 2020;10(2):130-146.

8. Li T, Peng T. Traditional Chinese herbal medicine as a source of molecules with antiviral activity. *Antiviral research.*2013;97(1):1-9.

9. Abiri R, Abdul-Hamid H, Sytar O, et al. A brief overview of potential treatments for viral diseases using natural plant compounds: the case of SARS-Cov. *Molecules.* 2021;26(13):3868.

10. Sasidharan S, Chen Y, Saravanan D, Sundram K, Latha LY. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African journal of traditional, complementary and alternative medicines.* 2011;8(1).

11. Lv Z, Cano KE, Jia L, Drag M, Huang TT, Olsen SK. Targeting SARS-CoV-2 Proteases for COVID-19 Antiviral Development. *Front Chem.* 2021;9:819165.

12. Drayman N, DeMarco JK, Jones KA, et al. Masitinib is a broad coronavirus 3CL inhibitor that blocks replication of SARS-CoV-2.*Science.* 2021;373(6557):931-936.

13. Yang M, Lin L, Scartelli C, et al. Inhibition of Sars-Cov-2 Viral Replication and In Vivo Thrombus Formation By a Novel Plant Flavonoid.*Blood.* 2021;138:3144.

14. Hu Q, Xiong Y, Zhu GH, et al. The SARS-CoV-2 main protease (Mpro): Structure, function, and emerging therapies for COVID-19.*MedComm.* 2022;3(3):e151.

15. Kneller DW, Phillips G, O'Neill HM, et al. Room-temperature X-ray crystallography reveals the oxidation and reactivity of cysteine residues in SARS-CoV-2 3CL Mpro: insights into enzyme mechanism and drug design. *IUCrJ.* 2020;7(6):1028-1035.

16. Su H, Yao S, Zhao W, et al. Identification of pyrogallol as a warhead in design of covalent inhibitors for the SARS-CoV-2 3CL protease. *Nature communications.* 2021;12(1):3623.

17. Xiong Y, Zhu G-H, Zhang Y-N, et al. Flavonoids in Ampelopsis grossedentata as covalent inhibitors of SARS-CoV-2 3CLpro: Inhibition potentials, covalent binding sites and inhibitory mechanisms.*International Journal of Biological Macromolecules.*2021;187:976-987.

18. Davis DA, Bulut H, Shrestha P, et al. Regulation of the dimerization and activity of SARS-CoV-2 main protease through reversible glutathionylation of cysteine 300. *Mbio.* 2021;12(4):e02094-02021.

19. Tao X, Zhang L, Du L, et al. Allosteric inhibition of SARS-CoV-2 3CL protease by colloidal bismuth subcitrate. *Chemical science.*2021;12(42):14098-14102.

20. Chen Z, Cui Q, Cooper L, et al. Ginkgolic acid and anacardic acid are specific covalent inhibitors of SARS-CoV-2 cysteine proteases.*Cell Biosci.* 2021;11(1):45.

21. Xiong M, Nie T, Shao Q, Li M, Su H, Xu Y. In silico screening-based discovery of novel covalent inhibitors of the SARS-CoV-2 3CL protease.*European Journal of Medicinal Chemistry.* 2022;231:114130.

22. Zhong B, Peng W, Du S, et al. Oridonin inhibits SARS-CoV-2 by targeting its 3C-Like protease. *Small science.* 2022;2(6):2100124.

23. Khan A, Heng W, Imran K, et al. Discovery of Isojacareubin as a covalent inhibitor of SARS-CoV-2 main protease using structural and experimental approaches. *J Med Virol.* 2023;95(2):e28542.

24. Zhang YN, Zhu GH, Liu W, et al. Discovery and characterization of the covalent SARS-CoV-2 3CL(pro) inhibitors from Ginkgo biloba extract via integrating chemoproteomic and biochemical approaches.*Phytomedicine.* 2023;114:154796.

25. Alhadrami HA, Hassan AM, Chinnappan R, et al. Peptide substrate screening for the diagnosis of SARS-CoV-2 using fluorescence resonance energy transfer (FRET) assay. *Microchimica Acta.* 2021;188:1-10.

26. Wei L, Huang J, Zhang F, et al. Comprehensive profiling and characterization of the absorbed components and metabolites in mice serum and tissues following oral administration of Qing-Fei-Pai-Du decoction by UHPLC-Q-Exactive-Orbitrap HRMS. *Chinese Journal of Natural Medicines.* 2021;19(4):305-320.

27. Tu D-Z, Mao X, Zhang F, et al. Reversible and irreversible inhibition of cytochrome P450 enzymes by methylophiopogonanone A.*Drug Metabolism and Disposition.* 2021;49(6):459-469.

28. Chen Z, Du R, Cooper L, et al. Sulforaphane is a reversible covalent inhibitor of 3-chymotrypsin-like protease of SARS-CoV-2. *J Med Virol.* 2023;95(3):e28609.

29. Xiong J, Xiang Y, Huang Z, et al. Structure-Based Virtual Screening and Identification of Potential Inhibitors of SARS-CoV-2 S-RBD and ACE2 Interaction. *Front Chem.* 2021;9:740702.

30. Paul AS, Islam R, Parves MR, et al. Cysteine focused covalent inhibitors against the main protease of SARS-CoV-2. *Journal of Biomolecular Structure and Dynamics.* 2022;40(4):1639-1658.

31. Xia X-F, Xia G-Y, Wu Y-Z, et al. Trace therapeutic substances of traditional Chinese medicine: great resources of innovative drugs derived from traditional Chinese medicine. *Zhongguo Zhong yao za zhi= Zhongguo Zhongyao Zazhi= China Journal of Chinese Materia Medica.*2022;47(7):1705-1729.

32. Awad HM, Boersma MG, Boeren S, Van Bladeren PJ, Vervoort J, Rietjens IM. Quenching of quercetin quinone/quinone methides by different thiolate scavengers: stability and reversibility of conjugate formation.*Chemical research in toxicology.* 2003;16(7):822-831.

33. Baron G, Borella S, Della Vedova L, et al. An integrated metabolomic and proteomic approach for the identification of covalent inhibitors of the main protease (Mpro) of SARS-CoV-2 from crude natural extracts.*Talanta.* 2023;252:123824.

34. Ferreira JC, Fadl S, Villanueva AJ, Rabeh WM. Catalytic dyad residues His41 and Cys145 impact the catalytic activity and overall conformational fold of the main SARS-CoV-2 protease 3-chymotrypsin-like protease. *Frontiers in Chemistry.* 2021;9:692168.

35. Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nature methods.*2009;6(5):359-362.

36. Ravanfar R, Sheng Y, Shahgholi M, et al. Surface cysteines could protect the SARS-CoV-2 main protease from oxidative damage. *J Inorg Biochem.* 2022;234:111886.

37. Kneller DW, Phillips G, O'Neill HM, et al. Room-temperature X-ray crystallography reveals the oxidation and reactivity of cysteine residues in SARS-CoV-2 3CL M(pro): insights into enzyme mechanism and drug design. *IUCrJ.* 2020;7(Pt 6):1028-1035.

38. Bahun M, Jukic M, Oblak D, et al. Inhibition of the SARS-CoV-2 3CL(pro) main protease by plant polyphenols. *Food Chem.*2022;373(Pt B):131594.

39. Hu Q, Xiong Y, Zhu GH, et al. The SARS-CoV-2 main protease (M(pro)): Structure, function, and emerging therapies for COVID-19. *MedComm (2020).* 2022;3(3):e151.

40. Paasche A, Schiller M, Schirmeister T, Engels B. Mechanistic Study of the Reaction of Thiol-Containing Enzymes with α, β-Unsaturated Carbonyl Substrates by Computation and Chemoassays. *ChemMedChem: Chemistry Enabling Drug Discovery.* 2010;5(6):869-880.

41. Ramos-Guzmán CA, Ruiz-Pernia JJ, Tunon I. Inhibition mechanism of SARS-CoV-2 main protease with ketone-based inhibitors unveiled by multiscale simulations: insights for improved designs. *Angewandte Chemie International Edition.* 2021;60(49):25933-25941.

42. Chen Z, Lv Y, Xu H, Deng L. Herbal medicine, gut microbiota, and COVID-19. *Frontiers in Pharmacology.* 2021;12:646560.

43. Taylor-Swanson L, Altschuler D, Taromina K, et al. SEAttle-based Research of Chinese Herbs for COVID-19 Study: A Whole Health Perspective on Chinese Herbal Medicine for Symptoms that may be Related to COVID-19.*Global Advances in Health and Medicine.*2022;11:21649561211070483.

44. El Zakhem A, Chalhoub MA, Bassil M. The role of herbal and nutritional treatments in the fight against COVID-19 and other respiratory tract infections. *International Journal of Environmental Research and Public Health.* 2021;18(22):12001.

45. Hall V, Foulkes S, Insalata F, et al. Protection against SARS-CoV-2 after Covid-19 vaccination and previous infection. *New England Journal of Medicine.* 2022;386(13):1207-1220.

46. Wang X, Ma S, Zhao B, et al. Correlations between the viral loads and symptoms in the SARS-CoV-2-infected patients. *MedComm.*2023;4(4).

47. Zhu D, Su H, Ke C, et al. Efficient discovery of potential inhibitors for SARS-CoV-2 3C-like protease from herbal extracts using a native MS-based affinity-selection method. *Journal of Pharmaceutical and Biomedical Analysis.* 2022;209:114538.

48. Wang Y, Chen W, Zhong S, Zhang H, Xue M, Gu B. Effect of heat-clearing and detoxifying health function of lonicera japonica in rats based on metabonomics. *Zhong yao cai= Zhongyaocai= Journal of Chinese Medicinal Materials.* 2016;39(5):1129-1133.

49. Xin N, Li W, Li Y-J, Ma X-K, Fu Z-P, Li Y. Study of antivirus, antibacteria and immune functions of Gaoreqing freeze-dried powder.*J Med Plants Res.* 2011;5(22):5407-5412.

50. Chen W-C, Liou S-S, Tzeng T-F, Lee S-L, Liu I-M. Wound repair and anti-inflammatory potential of Lonicera japonica in excision wound-induced rats. *BMC Complementary and Alternative Medicine.*2012;12(1):1-9.

Hosted file

`Figures.docx` available at https://authorea.com/users/638144/articles/654053-discovery-of-the-covalent-sars-cov-2-m-pro-inhibitors-from-anti-viral-herbs-via-integrating-target-based-high-throughput-screening-and-chemoproteomic-approaches

Hosted file

`Tables.docx` available at https://authorea.com/users/638144/articles/654053-discovery-of-the-covalent-sars-cov-2-m-pro-inhibitors-from-anti-viral-herbs-via-integrating-target-based-high-throughput-screening-and-chemoproteomic-approaches