Anti-SARS-CoV-2 activities in vitro of Shuanghuanglian preparations and bioactive ingredients

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Human infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes coronavirus disease 2019 (COVID-19) and there is no cure currently. The 3CL protease (3CLpro) is a highly conserved protease which is indispensable for CoVs replication, and is a promising target for development of broad-spectrum antiviral drugs. In this study we investigated the anti-SARS-CoV-2 potential of Shuanghuanglian preparation, a Chinese traditional patent medicine with a long history for treating respiratory tract infection in China. We showed that either the oral liquid of Shuanghuanglian, the lyophilized powder of Shuanghuanglian for injection or their bioactive components dose-dependently inhibited SARS-CoV-2 3CLpro as well as the replication of SARS-CoV-2 in Vero E6 cells. Baicalin and baicalein, two ingredients of Shuanghuanglian, were characterized as the first noncovalent, nonpeptidomimetic inhibitors of SARS-CoV-2 3CLpro and exhibited potent antiviral activities in a cell-based system. Remarkably, the binding mode of baicalein with SARS-CoV-2 3CLpro determined by X-ray protein crystallography was distinctly different from those of known 3CLpro inhibitors. Baicalein was productively ensconced in the core of the substrate-binding pocket by interacting with two catalytic residues, the crucial S1/S2 subsites and the oxyanion loop, acting as a “shield” in front of the catalytic dyad to effectively prevent substrate access to the catalytic dyad within the active site. Overall, this study provides an example for exploring the in vitro potency of Chinese traditional patent medicines and effectively identifying bioactive ingredients toward a specific target, and gains evidence supporting the in vivo studies of Shuanghuanglian oral liquid as well as two natural products for COVID-19 treatment.

Keywords: SARS-CoV-2; 3CL protease; traditional Chinese medicines; Shuanghuanglian oral liquid; baicalin; baicalein

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INTRODUCTION
Coronaviruses (CoVs; subfamily Coronavirinae, family Coronaviridae, order Nidovirales) are a group of highly diverse, enveloped, positive-sense, single-stranded RNA viruses and are prevalent pathogens that are responsible for a wide range of diseases in animals as well as humans [1, 2]. Human CoV strains, including 229E, NL63, OC43, and HKU1, lead to mild to moderate upper respiratory tract infections such as colds and pneumonia, whereas human infections with zoonotic CoVs, such as severe acute respiratory syndrome (SARS) in 2003 and Middle East respiratory syndrome (MERS) in 2012, caused severe respiratory diseases with high morbidity and mortality, posing a significant threat to public health across the world. The outbreak of coronavirus disease 2019 (COVID-19) is an ongoing pandemic that is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a new human coronavirus that appears to be more readily transmitted from human to human [3, 4]. It is 96% identical at the whole-genome level to a bat coronavirus and shares 79.6% sequence identity to SARS-CoV [5]. The ongoing SARS-CoV-2 pandemic has caused over 13 million infections and over 560,000 deaths worldwide, and the numbers are still increasing [6]. It marks the third introduction of a highly pathogenic CoV into the human population in the twenty-first century. Currently, no efficacious antiviral drugs and vaccines have been approved for the prophylaxis or treatment of the highly virulent SARS-CoV, MERS-CoV, or SARS-CoV-2 infections in humans.

CoVs share key genomic elements that provide promising therapeutic targets [1, 7]. A chymotrypsin-like cysteine protease called 3C-like protease (3CLpro), together with a papain-like protease (PLpro), is required to process polyproteins into mature nonstructural proteins such as RNA-dependent RNA polymerase (RdRp) and helicase, which are essential for viral transcription and...
replication. These polyproteins contain 3 and 11 cleavage sites for PLpro and 3CLpro, respectively, so 3CLpro is also referred to as the main protease. Moreover, the substrate specificity of 3CLpro is highly conserved among different CoVs and is similar to that of the main picornavirus 3C protease, making it an ideal target for the development of broad-spectrum antiviral drugs [7, 8]. Although several types of substrate-like peptidomimetic inhibitors of SARS-CoV 3CLpro, MERS-CoV 3CLpro, or SARS-CoV-2 3CLpro with a covalent mode of action have been reported, none has yet moved into clinical trials yet [1, 7, 9, 10]. Classical small molecules with more drug-like properties and binding modes with 3CLpros that are validated by complex structure determination have not yet emerged.

Traditional Chinese medicines (TCMs) have evolved over thousands of years and are invaluable sources for drug discovery and development. As a notable example, the discovery of artemisinin (Qinghaosu), which was originally isolated from the TCM Artemisia annua L. (Qinghao), was a milestone in the treatment of malaria. Shuanghuanglian preparation, a Chinese traditional patent medicine (also called proprietary Chinese medicine) used for the treatment of acute respiratory tract infections since 1973, is a classical purified herbal preparation extracted from three Chinese herbal medicines, namely, Lonicera japonica Thunb., Scutellaria baicalensis Georgi, and Forsythia suspensa (Thunb.) Vahl [11]. A variety of formulations of Shuanghuanglian have been developed, including oral liquid (over the counter (OTC) in China), capsule, powder injection, and spray, and adverse drug reactions such as hypersensitivity reactions were reported with the powder injection [12, 13]. According to the theory of TCM, Shuanghuanglian preparations have the efficacy of cold-pungent diaphoresis and clearing away heat and toxic material [14, 15]. Multiple pharmacological activities of Shuanghuanglian, including antibacterial, anti-inflammatory, antiviral, and immune enhancement have been reported [12]. In particular, Shuanghuanglian formulations, its three source herbs and several compounds isolated from these herbs have shown antiviral activities against HIV, adenovirus, influenza virus, and EV71, but the mechanisms of action of these substances remain largely unknown [16–20].

In the present study, we first performed enzymatic assays to rapidly test whether Shuanghuanglian preparations inhibit the proteolytic activities of SARS-CoV-2 3CLpro and PLpro. As Shuanghuanglian preparations showed significant inhibitory activities against 3CLpro, three index compounds mentioned in the Chinese Pharmacopoeia (chlorogenic acid, phillyrin, and baicalin) together with 25 other compounds from Shuanghuanglian preparations were further investigated. Nine compounds were identified as lower-micromolar-range inhibitors of 3CLpro. Among these, the binding of baicalein as well as baicalin with 3CLpro was comprehensively characterized, and a crystal structure of SARS-CoV-2 3CLpro in complex with baicalein, the first noncovalent, nonpeptidomimetic small-molecule inhibitor, was also determined. Finally, the antiviral activities of Shuanghuanglian oral liquids and compounds in a cell-based system were examined. In summary, this study comprehensively explored the in vitro antiviral activities of the Shuanghuanglian preparation and its components at the molecular and cellular levels by using a network of phytochemical, biochemical, biophysical, and pharmacological methods.

MATERIALS AND METHODS

General chemistry
Shuanghuanglian oral liquids (Sanjing: 18051923; Tailong: 190808012; Xiren: 20190923) were purchased from Yifeng Pharmacy (Shanghai, China). Shuanghuanglian lyophilized powder (1910407) was provided by Harbin Pharmaceutical Group No.2 Traditional Chinese Medicine Factory (Harbin, China). Compounds were from an in-house natural product library. All compounds were analyzed by HPLC-MS (Waters 2695 instrument with a 2998 PDA detector coupled with a Waters Acuity Elsd and a Waters 3100 SQDMS detector using a Waters Sunfire RP-C18 column (4.6 x 150 mm, 5 μm) with a 1.0 mL/min flow rate). The identification of the constituents in Shuanghuanglian oral liquids was performed on a Waters i-Class UPLC-SYNAPT G2-Si HDMS according to the methods from literatures [21, 22].

Quantitative analysis of chlorogenic acid, phillyrin, baicalin, and baicalein in Shuanghuanglian preparations

Quantitative analysis of chlorogenic acid, phillyrin, baicalin, and baicalein in four Shuanghuanglian preparations was performed on a Waters Acuity UPLC-Acquity PAD. Components were separated on a Waters BEH RP-C18 column (2.1 x 100 mm, 1.7 μm) at 30 °C with a flow rate of 0.4 mL/min and an injection volume of 2.0 μL. Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. A nonlinear gradient elution was used (see Supplementary Information). The detection wavelength for these four compounds was set at 277.4 nm. The linear ranges of chlorogenic acid, phillyrin, baicalin, and baicalein were 4.0–150.0, 4.0–150.0, 5.0–250.0, and 3–120 μg/mL ($R^2 = 0.9999$), respectively. For each sample, at least three independent experiments were performed for the quantitative analysis of the four compounds.

Protein expression and purification

The cDNA of full-length SARS-CoV-2 3CLpro (GenBank: MN908947.3) or SARS-CoV 3CLpro (GenBank: AAP13442.1) was cloned into the PGE6p-1 vector. To obtain SARS-CoV-2 3CLpro or SARS-CoV 3CLpro with authentic N and C terminals, four amino acids (AVLQ) were inserted between the GST tag and the full-length SARS-CoV-2 3CLpro or SARS-CoV 3CLpro, while eight amino acids (GPHHHHHH) were added to the C-terminus of SARS-CoV-2 3CLpro. The plasmid was then transformed into BL21 (DE3) cells for protein expression. The N-terminal GST tag and four amino acids (AVLQ) were self-cleavable. The expressed protein with an authentic N-terminus was purified by a Ni-NTA column (GE Healthcare) and transformed into the cleavage buffer (150 mM NaCl, 25 mM Tris, pH 7.5) containing human rhinovirus 3C protease to remove the additional residues. The resulting protein sample was further passed through size-exclusion chromatography (Superdex200, GE Healthcare). The eluted protein samples were stored in a solution (10 mM Tris, pH 7.5) for the enzymatic inhibition assay, native-state mass spectrometry and protein crystallization.

To obtain SARS-CoV-2 3CLpro with the GST tag, the cDNA of full-length SARS-CoV-2 3CLpro was inserted after the PreScission protease digestion site of the PGE6p-1 vector. The plasmid was transformed into BL21 (DE3) cells for protein expression. The expressed protein was purified by a GST column (GE Healthcare) followed by size-exclusion chromatography (Superdex200, GE Healthcare). The resulting protein was stored in a solution (25 mM Tris, pH 7.3) for isothermal titration calorimetry measurements.

The cDNA of full-length SARS-CoV-2 PLpro (GenBank: MN908947.3) was cloned into the pET-22b vector. A cleavage site for the PreScission protease (LEVLFQGP) and 6His-tag were added to the C-terminus. The plasmid was then transformed into BL21 (DE3) cells for protein expression. The expressed protein was purified by a Ni-NTA column (GE Healthcare) and cleaved by the PreScission protease to remove the His-tag. The resulting protein sample was further passed through size-exclusion chromatography (Superdex200, GE Healthcare). The eluted protein samples were stored in a solution (50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM DTT) for the enzymatic inhibition assay.

The inhibition assay of proteases

A fluorescence resonance energy transfer (FRET) protease assay was applied to measure the inhibitory activity of compounds
against SARS-CoV-2 3CLpro and SARS-CoV 3CLpro. The fluorogenic substrate MCA-AVLQSGFR-Lys(Dnp)-Lys-NH₂ was synthesized by GenScript (Nanjing, China). The FRET-based protease assay was performed as follows. The recombinant SARS-CoV-2 3CLpro (30 nM final concentration) or SARS-CoV 3CLpro (100 nM final concentration) was mixed with serial dilutions of each compound, oral liquid or the dissolved lyophilized powder in 80 μL assay buffer (50 mM Tris–HCl, pH 7.3, 1 mM EDTA) and incubated for 10 min. The reaction was initiated by adding 40 μL fluorogenic substrate at a final concentration of 20 μM. After that, the fluorescence signal at 320 nm (excitation)/405 nm (emission) measured immediately every 35 s for 3.5 min with a Bio-Tek Synergy4 plate reader. The initial velocities of reactions with compounds added at various concentrations compared to the reaction added with DMSO were calculated and used to generate inhibition profiles.

The inhibition of SARS-CoV-2 PLpro by compounds as well as Shuanghuanglian preparations was measured similar to that described previously [23] with a fluorogenic peptide (RLRGG-AMC) synthesized by GenScript (Nanjing, China). The reactions were performed in a total volume of 120 μL. First, 50 nM PLpro was incubated with the indicated concentrations of tested compounds or Shuanghuanglian preparations in the condition of 50 mM HEPES, pH 7.5, 0.1 mg/mL BSA, 5 mM DTT for 10 min. The reactions were initiated by the addition of 10 μM fluorogenic peptide. After that, the fluorescence signal at 360 nm (excitation)/460 nm (emission) was measured immediately every 1 min for 5 min with a Bio-Tek Synergy4 plate reader. The initial velocities of reactions with compounds or Shuanghuanglian added at various concentrations compared to the reaction added with DMSO were calculated and used to generate inhibition profiles.

For each compound or Shuanghuanglian preparation, at least three independent experiments were performed for the determination of IC₅₀ values. The IC₅₀ values were expressed as the mean ± SD and determined via nonlinear regression analysis using GraphPad Prism software 8.0 (GraphPad Software, Inc., San Diego, CA, USA).

Isothermal titration calorimetry (ITC) measurements
All measurements were performed by an iTC200 calorimeter (General Electric Co.) in ITC buffer (25 mM Tris, pH 7.3) while stirring at 800 rpm. The stock solution of compounds and the SARS-CoV-2 3CLpro protein with a GST tag were diluted with the ITC buffer to a compound concentration of 50–100 μM and a protein concentration of 0.6–1.5 mM before titrations. The final concentration of DMSO in the reaction buffer was less than 2% by volume. Compounds were titrated by the protein solution. All titrations were performed using an initial injection of 0.4 μL followed by 19 identical injections of 2 μL with a duration of 4 s per injection and a spacing of 120 s between injections. The last five data points were averaged and subtracted from each titration to account for the heat of dilution. Additional background experiments where the protein solution was titrated into the buffer without the compound revealed no significant shift in the baseline during the course of the measurements. Each measurement was repeated three times.

Native-state mass spectrometry studies
All mass spectrometry (MS) measurements were performed with a scimaX magnetic resonance mass spectrometry (MRMS) system (Bruker Daltonik GmbH, Bremen, Germany). The sample solutions were infused into the system with a syringe (50 μL) at a flow rate of 120 μL/h using electrospray ionization in a positive ion mode. Data were acquired in the mass-to-charge (m/z) range of 400–6000 with a transient length of 3.92 s in quadrupolar detection, resulting in a resolving power of 54.0 ppp at m/z 3240. The source and ion transfer parameters were optimized as follows: skimmer 1 100 V, funnel 1 180 V, funnel RF amplitude 200 V, collision cell frequency 2.0 MHz, ion transfer frequency 2 MHz, time of flight to analyzer cell 2.0 ms with the RF amplitude of 350 V, and source temperature 200 °C. To optimize protein performance, the mass range around the charge state 18 was isolated with the quadrupole using a quadrupole mass window of 200 Da with a center mass of the quadrupole of m/z 3750; the magnitude was set to 4 M; 200 single scans were added. For ligand-binding screening and dissociation constant (Kd) determination, the magnitude was set to 128 K, and 1000 single scans were added.

The protein sample of SARS-CoV-2 3CLpro was buffer-exchanged with 10 mM ammonium acetate buffer (pH 6.8). For optimization of the MS conditions, the protein was diluted to 2 μM. For ligand-binding screening studies and Kd determination, samples were prepared with the protein at 1 μM and the ligand at a series of concentrations (50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 μM for baicalin and 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08 μM for baicalein). The incubation time was 1 h at 37 °C. Three independent experiments were performed for the determination of the Kd.

Mass spectra were deconvoluted with SNAP2 in DataAnalysis 5.2 (Bruker Daltonics, Bremen, Germany). Charge states 18+ and 19+ were used to calculate the free protein and protein–ligand complex intensities. The relative abundances of the protein–ligand complex to total protein in the mass spectra were calculated as reported previously [24].

Protein crystallization and structure determination
The purified SARS-CoV-2 3CLpro protein was concentrated to 7 mg/mL for crystallization. The protein was incubated 1 h with 10 mM bicine before crystallization condition screening. Crystals of the complex were obtained at 20 °C by mixing equal volumes of protein-baicalin and a reservoir (16% PEG6000, 100 mM MES, pH 5.8, 3% DMSO) with a hanging-drop vapor diffusion method. Crystals were flash-frozen in liquid nitrogen in the presence of the reservoir solution supplemented with 20% glycerol. X-ray diffraction data were collected at beamline BL18U1 at the Shanghai Synchrotron Radiation Facility [25]. The data were processed with HKL3000 software packages [26]. The complex structure was solved by molecular replacement using the program PHASER [27] with a search model of PDB code 6L7U. The model was built using Coot [28] and refined with a simulated annealing protocol implemented in the program PHENIX [29]. The refined structure was deposited to the Protein Data Bank with the accession code listed in Supplementary Table S1. The complete statistics as well as the quality of the solved structure are also shown in Supplementary Table S1.

The cell-based antiviral activity assay
The Vero E6 cell line was obtained from American Type Culture Collection (ATCC, Manassas, USA) and maintained in minimum Eagle’s medium (MEM; Gibco Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen, UK) in a humid incubator with 5% CO₂ at 37 °C. The cytotoxicity of the three Shuanghuanglian oral liquids and the tested compounds to the Vero E6 cells was determined by CCK8 assays (Beyotime, China). A clinical isolate SARS-CoV-2 [5] was propagated in Vero E6 cells, and the viral titer was determined as described previously [30]. All infection experiments were performed at biosafety level-3 (BSL-3).

Preseeded Vero E6 cells (5 × 10⁵ cells/well) were incubated with different concentrations of oral liquids or compounds for 1 h, and the virus was subsequently added (a multiplicity of infection of 0.01) to infect the cells for 2 h. After that, the virus–compound mixture was removed, and the cells were further cultured with a fresh compound-containing medium. At 48 h post infection, the cell supernatant was collected, and the viral RNA in the supernatant was submitted to quantitative real-time RT-PCR (qRT-PCR) analysis as described previously [30]. DMSO was used in the controls. At least three independent experiments were carried out for each oral liquid, baicalin and baicalein. The half-

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maximal effective concentration (EC₅₀) values are expressed as the mean ± SD from independent experiments and were determined via nonlinear regression analysis using GraphPad Prism software 8.0 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS
Inhibition of SARS-CoV-2 3CLpro and PLpro by Shuanghuanglian preparations
The pivotal role of the 3CL protease (3CLpro) in processing polyproteins into individual functional proteins for viral replication and the highly conserved substrate specificity of the enzyme among CoVs make it a promising target for screening inhibitors. A FRET protease assay was applied to measure the proteolytic activity of the recombinant SARS-CoV-2 3CLpro on a fluorogenic substrate. The details of the assay are described above. This FRET-based protease assay was utilized to measure the inhibitory activities of Shuanghuanglian preparations as well as compounds against SARS-CoV-2 3CLpro. To quickly find out whether Shuanghuanglian preparations and their components have inhibitory activities against the proteases, we added the diluted Shuanghuanglian oral liquid directly into the enzymatic reaction without visible precipitation or aggregation. To our surprise, the oral liquid showed dose-dependent inhibition of SARS-CoV-2 3CLpro, and the resulting IC₅₀ values were 0.090, 0.064, and 0.076 µg/mL for three Shuanghuanglian oral liquids produced by three different pharmaceutical companies (Table 1 and Fig. 1a–c). Moreover, we dissolved the lyophilized powder of Shuanghuanglian oral liquids produced by three different pharmaceutical companies (Table 1 and Fig. 1a–c). Taking these data into account, the calculated concentrations of baicalin in the reaction system when the four Shuanghuanglian samples reached 50% inhibition of the enzyme’s activity ranged from 1.83 to 6.14 µM, which were close to the IC₅₀ of pure baicalin (6.41 µM). These

Table 1. Inhibition of SARS-CoV-2 3CLpro by Shuanghuanglian preparations as well as their ingredients, and the determined content of chlorogenic acid, phillyrin, baicalin, and baicalein in Shuanghuanglian preparations.

| Entities               | IC₅₀     | Chlorogenic acid | Phillyrin | Baicalin | Baicalein |
|------------------------|---------|------------------|----------|----------|-----------|
| Oral liquid 1           | 0.090 ± 0.004 µg/mL | 0.72 ± 0.01 mg/mL | 0.29 ± 0.01 mg/mL | 12.72 ± 0.30 mg/mL | 0.12 ± 0.00 mg/mL |
| Oral liquid 2           | 0.064 ± 0.011 µg/mL | 0.78 ± 0.01 mg/mL | 0.32 ± 0.01 mg/mL | 12.78 ± 0.18 mg/mL | 0.06 ± 0.00 mg/mL |
| Oral liquid 3           | 0.076 ± 0.007 µg/mL | 0.78 ± 0.01 mg/mL | 0.28 ± 0.01 mg/mL | 17.52 ± 0.17 mg/mL | 0.22 ± 0.00 mg/mL |
| Lyophilized powdera    | 0.010 ± 0.001 mg/mL | 19.36 ± 0.35 mg/g | 2.92 ± 0.05 mg/g | 274.08 ± 2.82 mg/g | 1.87 ± 0.04 mg/g |
| Chlorogenic acid        | 39.48 ± 5.51 µM |                  |           |          |           |
| Phillyrin               | 7.8% inhibition @ 10 µM |            |          |          |           |
| Baicalin                | 6.41 ± 0.95 µM |                  |          |          |           |
| Baicalein               | 0.94 ± 0.20 µM |                  |          |          |           |

aShuanghuanglian oral liquids and the lyophilized powder for injection were produced by three different pharmaceutical companies.

Subsequently, a fluorogenic peptide (RLRGG-AMC) was used to determine the inhibitory activities of four Shuanghuanglian preparations against SARS-CoV-2 PLpro which is also a crucial protease in processing polyproteins into individual functional proteins for viral replication. All three oral liquids as well as the lyophilized powder of Shuanghuanglian again exhibited dose-dependent inhibition of SARS-CoV-2 PLpro, but with the much higher IC₅₀ values of 3.65 µg/mL, 4.44 µg/mL, 4.19 µg/mL, and 0.14 mg/mL, respectively, compared with their IC₅₀ values toward 3CLpro (Supplementary Fig. S1). These findings suggested that Shuanghuanglian preparations could inhibit the hydrolytic activity of both SARS-CoV-2 3CLpro and PLpro, and their potency toward 3CLpro is much more significant than toward PLpro.

Inhibition of SARS-CoV-2 3CLpro by baicalin and baicalein
According to the Chinese Pharmacopoeia (2015), chlorogenic acid from L. japonica Thunb., phillyrin from F. suspense (Thunb.) Vahl., and baicalin from S. baicalensis Georgi (Supplementary Fig. S2), are the index components of Shuanghuanglian oral liquids, with concentrations of over 0.6, 0.3, and 10 mg/mL, respectively. These three compounds were thus individually tested by the FRET-based protease assay. Baicalin and chlorogenic acid showed an IC₅₀ of 6.41 and 39.48 µM against 3CLpro, respectively, while phillyrin hardly had inhibitory activity at a concentration of 10 µM (Table 1). Furthermore, the contents of chlorogenic acid, phillyrin, and baicalin in the four tested Shuanghuanglian samples were determined by the ultra-high-performance liquid chromatography (UPLC) (Supplementary Fig. S3), and the results are shown in Table 1. Baicalin concentrations of 12.72 mg/mL, 12.78 mg/mL, 17.52 mg/mL, and 274.08 mg/g were included in the three oral liquids and the lyophilized powder, respectively. Taking these data and the IC₅₀ values of the four Shuanghuanglian samples into account, the calculated concentrations of baicalin in the reaction system when the four Shuanghuanglian samples reached 50% inhibition of the enzyme’s activity ranged from 1.83 to 6.14 µM, which were close to the IC₅₀ of pure baicalin (6.41 µM). These

Fig. 1 Inhibition of SARS-CoV-2 3CLpro by Shuanghuanglian. a Representative inhibition curves for oral liquid 1, b oral liquid 2, c oral liquid 3, and d the lyophilized powder for injection of Shuanghuanglian against SARS-CoV-2 3CLpro. All data are shown as mean ± SD.
data suggest that the inhibition of SARS-CoV-2 3CLpro by Shuanghuanglian preparations is mainly due to their abundant component, baicalin, although other ingredients may also possess inhibitory activities against the protease. Furthermore, we determined the inhibition of 3CLpro by baicalein, an aglycone of baicalin, and the resulting IC50 was 0.94 μM (Table 1 and Supplementary Fig. S2). There was a small amount of baicalein in Shuanghuanglian preparations (Table 1). Thus, baicalin and baicalein are two novel nonpeptidomimetic inhibitors of SARS-CoV-2 3CLpro with single-digit micromolar potency. In contrast, the inhibition of SARS-CoV-2 PLpro by these two compounds at a concentration of 50 μM is <50%.

Protein–ligand binding supported by ITC and ESI-MS
To validate the binding of baicalin and baicalein with SARS-CoV-2 3CLpro and exclude the possibility of the pan-assay interference compounds [31], their binding affinities with the protease were measured by ITC, widely known as an invaluable tool used to determine the thermodynamic parameters of protein–ligand interactions, such as the $K_d$ (Fig. 2a, b and Table 2). The resulting $K_d$ of baicalin and baicalein binding with SARS-CoV-2 3CLpro was 11.50 and 4.03 μM, respectively. These had good correlations with the IC50 values mentioned above, demonstrating that specific binding of the compounds with the enzyme is responsible for their bioactivities. Baicalein showed a high binding efficiency to

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Fig. 2 Binding of baicalin and baicalein with SARS-CoV-2 3CLpro characterized by ITC and ESI-MS. a Representative thermodynamic profiles of baicalin and (b) baicalein binding with SARS-CoV-2 3CLpro in solution, resulting from ITC measurements. c Native-state electrospray ionization mass spectrometry spectra of SARS-CoV-2 3CLpro with baicalin and (d) baicalein. e Binding affinities of baicalin and (f) baicalein with SARS-CoV-2 3CLpro determined by native-state mass spectrometry. Fraction of ligand bound protein ([PL]/[P]t) obtained under optimized conditions plotted against total ligand concentrations. All data are shown as mean ± SD.
SARS-CoV-2 3CLpro, with a calculated ligand efficiency of 0.37 kcal/mol. Moreover, the ITC profiles in combination with their chemical structures suggest that baicalin and baicalein act as noncovalent inhibitors of SARS-CoV-2 3CLpro.

Native-state electrospray ionization mass spectrometry (ESI-MS) has been used extensively to directly observe native-state proteins and protein complexes, allowing the direct detection of protein–ligand noncovalent complexes with $K_d$ values as weak as 1 mM [32]. A $\Delta m/z$ between the [protein + ligand] $m/z$ and the [unbound protein] $m/z$ identifies a ligand as a binder with the correct molecular weight, while the ratio of the intensity of the [protein + ligand] peaks relative to the [unbound protein] peaks provides a qualitative indication of the ligand-binding affinity. Herein, ESI-MS analysis using high-resolution MRMS was carried out to detect the binding of baicalin and baicalein with SARS-CoV-2 3CLpro. To optimize the free protease performance, the mass range around charge state 18 was isolated with a center mass of the quadrupole of $m/z$ 3750 (Supplementary Fig. S4). For the ligand-binding screening studies, two charge states (18+ and 19+) were used to calculate the free protein and protein–ligand complex intensities. The representative spectra of samples containing SARS-CoV-2 3CLpro (1 µM) and baicalin (3.13 µM), or baicalein (0.31 µM) acquired under both optimized and screening conditions are shown in Fig. 2c, d, demonstrating specific binding of baicalin or baicalein with the protease. Moreover, the plot of the fraction of the bound protease versus the total concentration of the compound showed that the $K_d$ values of baicalin and baicalein were 12.73 and 1.40 µM, respectively (Fig. 2e, f), in keeping with the results from the ITC measurements.

Crystal structure of SARS-CoV-2 3CLpro in complex with baicalin. The mode of action of baicalin and the structural determinants associated with its binding with SARS-CoV-2 3CLpro were further explored using X-ray protein crystallography. The crystal structure of SARS-CoV-2 3CLpro in complex with baicalein was determined at a resolution of 2.2 Å (Fig. 3 and Supplementary Table S1). The protease had a catalytic Cys145-His41 dyad and an extended binding site, features shared by SARS-CoV 3CLpro and SARS-CoV-2 3CLpro, with a calculated ligand efficiency of 0.37 kcal/mol. Moreover, the ITC profiles in combination with their chemical structures suggest that baicalin and baicalein act as noncovalent inhibitors of SARS-CoV-2 3CLpro.

> **Table 2.** Binding affinities of baicalin and baicalein with SARS-CoV-2 3CLpro determined by ITC and MRMS, and the calculated ligand efficiency (LE) of two compounds.

| Compounds   | $K_d$ ITC (µM) | $\Delta G$ ITC (kJ/mol) | LE ITC (kcal/mol) | $K_d$ MRMS (µM) | $IC_{50}$ (µM) |
|-------------|----------------|-------------------------|-------------------|-----------------|----------------|
| Baicalin    | 11.50 ± 1.89   | −28.20 ± 0.42           | 0.21              | 12.73 ± 0.81    | 6.41 ± 0.95    |
| Baicalein   | 4.03 ± 0.37    | −30.78 ± 0.24           | 0.37              | 1.40 ± 0.10     | 0.94 ± 0.20    |

Baicalin and baicalein are monosaccharides that are naturally present in the roots of Scutellaria baicalensis and are characterized by a furanone moiety. The furanone moiety in baicalin and baicalein is critical for their antiviral activity. The furanone moiety acts as a hydrogen bond acceptor and a hydrogen bond donor, which is essential for the binding of baicalin and baicalein to the protease.

In conclusion, baicalin and baicalein are effective inhibitors of SARS-CoV-2 3CLpro, with the former being a more potent inhibitor. The crystal structures of the complex of baicalin or baicalein with SARS-CoV-2 3CLpro provide insights into the binding mode and the structural determinants of the inhibition. These findings have implications for the development of novel antiviral therapies targeting 3CLpro.
Fig. 4 Unique binding mode of baicalein with 3CLpro. a The structure of SARS-CoV 3CLpro in complex with baicalein was generated by superimposing the structure of SARS-CoV-2 3CLpro/baicalein (PDB code 6M2N) with that of SARS-CoV 3CLpro/TG-0204998 (PDB code 2ZU4). SARS-CoV 3CLpro is shown in yellow cartoon and baicalein is shown as green spheres. Residues differing in two proteases are shown as cyan sticks. b A survey of the binding modes of known inhibitors as well as baicalein with SARS-CoV-3CLpro are shown by superimposing all the crystal structures of inhibitor-bound SARS-CoV 3CLpro (PDB codes: 2ASI, 2ASK, 2ALV, 2AMD, 2AMQ, 2G7B, 2GK4, 2OP9, 2YNB, 2ZU4, 2ZUS, 3ATW, 3AVZ, 3AW0, 3SN8, 3SNA, 3SNB, 3SNC, 3SND, 3SNE, 3SZN, 3V3M, 4MD5, 4TWW, 4TW4, 4WY3, 5CSN, and 5CSO). Baicalein is shown as green spheres, and other inhibitors and the two catalytic residues are shown as sticks.

Unique binding mode of baicalein with SARS-CoV-2 3CLpro

The amino acid sequence of SARS-CoV-2 3CLpro has 96% sequence identity to SARS-CoV 3CLpro. As a result, the inhibition assay showed that baicalein inhibited SARS-CoV 3CLpro with an IC50 of 1.18 ± 0.37 µM, similar to its IC50 toward SARS-CoV-2 3CLpro. Twelve residues differed between the two proteases, and none of them participated in the direct contact with baicalein. The high level of sequence identity between the two proteases allows one to assume that baicalein will bind to SARS-CoV 3CLpro in the same way as it does to SARS-CoV-2 3CLpro. Thus, a three-dimensional model of SARS-CoV 3CLpro in complex with baicalein was constructed by superimposing the crystal structure of SARS-CoV-2 3CLpro/baicalein with that of SARS-CoV 3CLpro/TG-0204998 (PDB code 2ZU4) (Fig. 4a). Subsequently, all of the crystal structures of the inhibitor-bound SARS-CoV 3CLpro were collected for a comparison analysis (Fig. 4b). This revealed that the binding mode of baicalein with SARS-CoV 3CLpro was distinctively different from those of known inhibitors. Assuming the peptidomimetic inhibitors were delineated as "swords" to resemble the binding of substrates, baicalein worked as a "shield" in front of two catalytic dyads to effectively prevent substrate access to the catalytic dyad within the active site (Fig. 4b). Such a unique binding mode in combination with its high ligand-binding efficiency and small molecular weight makes baicalein a valuable lead for further development.

Inhibition of SARS-CoV-2 3CLpro by other compounds in Shuanghuanglian preparations

Inspired by the good activities of baicalein and baicalin against SARS-CoV-2 3CLpro, we carried out a phytochemical analysis of Shuanghuanglian oral liquids. A total of 28 compounds, including baicalein and baicalin, were rapidly identified from the Shuanghuanglian oral liquid using UPLC-QTOF. The profiles of all 28 compounds in the Shuanghuanglian oral liquid on UPLC are shown in Supplementary Fig. S5. In addition to those of baicalein, baicalin, chlorogenic acid, and phillyrin, the inhibitory activities of the remaining 24 compounds against SARS-CoV-2 3CLpro were determined (Table 3). To our surprise, seven compounds besides baicalein and baicalin showed good inhibition of 3CLpro: Forsythoside B, scutellarein, forsythoside A, forsythoside I, isoforsythoside, forsythoside E, and forsythoside H had IC50 values of 2.88, 3.02, 3.18, 5.47, 5.85, 6.68, and 10.17 µM, respectively. Scutellarein is from S. baicalensis, but the other six compounds are ingredients of F. suspensa. These results provide a good basis to explain the potent inhibition of SARS-CoV-2 3CLpro by Shuanghuanglian oral liquids, although these compounds have very low concentrations in the oral liquids compared with baicalin. Nevertheless, the inhibition of SARS-CoV-2 Plpro by all 28 compounds is generally weak, which is consistent with the low potency of Shuanghuanglian preparations toward PLpro (Table 3).

Antiviral activities of Shuanghuanglian and its ingredients against SARS-CoV-2 in cells

We further evaluated the antiviral efficacy of Shuanghuanglian preparations and compounds against a clinical isolate of SARS-CoV-2 [5] in Vero E6 cells. The cytotoxicity of the three Shuanghuanglian oral liquids in Vero E6 cells was first determined by the CCK8 assay, and the resulting half-cytotoxic concentration (CC50) was over 12.5 µL/mL (Fig. 5a–c). Subsequently, the Vero E6 cells were infected with SARS-CoV-2 at a multiplicity of infection of 0.01 in the presence of different concentrations of the oral liquids. The antiviral efficacies were evaluated by quantifying the viral copy numbers in the cell supernatant via qRT-PCR. As shown in Fig. 5a–c, the three oral liquids all showed dose-dependent inhibition of SARS-CoV-2 replication, and the resulting half-maximal effective concentrations (EC50) were 1.20, 1.07, and 0.93 µL/mL, respectively. Like their IC50 values toward 3CLpro (Table 1), the EC50 values of the three oral liquids were also close to each other.

Analogously, the antiviral activities of the compounds identified in Shuanghuanglian oral liquids were determined. Most of the compounds at a concentration of 30 µM showed weak or no antiviral activity (Supplementary Fig. S6). However, both baicalin and baicalein showed dose-dependent inhibition of SARS-CoV-2 replication, and the resulting EC50 values were 27.87 and 2.94 µM, respectively (Fig. 5d, e). The resulting CC50 values of baicalin and baicalein in Vero E6 cells were over 200 µM, demonstrating the very low cytotoxicity of the two compounds (Fig. 5d, e). The corresponding selectivity index (SI = CC50/EC50) values were >7 and >68 for baicalin and baicalein, respectively. Therefore, the cell-based antiviral activity of baicalein was superior to that of most of the reported compounds and was close to those of chloroquine (EC50: 2.71 µM; SI = 100.81) and hydroxychloroquine (EC50: 4.51 µM; SI = 55.32) [30]. In addition, wogonin, which is an inhibitor of neither 3CLpro nor PLpro, also showed good activity against the replication of SARS-CoV-2 in Vero E6 cells (EC50: 6.88 µM, Supplementary Fig. S7). Taken together, these findings suggest that the good antiviral activities of Shuanghuanglian oral liquids in Vero E6 cells are mainly due to the antiviral activities of baicalin, considering its high concentration in the oral liquid, while baicalein and wogonin, as trace components of the oral liquids, also exhibit the potent antiviral activities.

DISCUSSION

Emerging CoVs such as SARS-CoV, MERS-CoV, and SARS-CoV-2 cause globally prevalent and severe diseases in humans, and there is still no cure for these diseases. In view of the long history of Shuanghuanglian preparations in treating respiratory tract infections and the urgent need for drugs against COVID-19, we investigated the anti-SARS-CoV-2 potential of Shuanghuanglian preparations in vitro. Both the oral liquids (OTC) and the lyophilized powder for injection of Shuanghuanglian demonstrate dose-dependent inhibition of SARS-CoV-2 3CLpro and PLpro, which are promising therapeutic targets for anti-CoV agents. In particular, the inhibitory activities of Shuanghuanglian preparations against 3CLpro are significant, as the relevant IC50 values of three Shuanghuanglian oral liquids produced by three different pharmaceutical companies are 0.090, 0.064, and 0.076 µL/mL.
respectively, and the IC50 of the lyophilized powder is 0.010 mg/mL. Remarkably, all three oral liquids also show dose-dependent inhibition of SARS-CoV-2 replication in Vero E6 cells, with EC50 values of 1.20, 1.07, and 0.93 μL/mL, respectively. Both the IC50 and EC50 values of the three oral liquids are close to each other, and the EC50 values are approximately tenfold the IC50 values, a common dose response at the molecular and cellular levels. We can thus conjecture that the inhibition of 3CLpro by baicalin, the major ingredient, accounts for most of the anti-SARS-CoV-2 activities of the four Shuanghuilian samples, although trace components such as baicalein and wogonin also contribute. These results together provide a good basis for further studies of Shuanghuilian oral liquids for COVID-19 treatment.

Encouragingly, the first family case of COVID-19 confirmed in Wuhan and treated with a combination of the Western medicine and Shuanghuilian oral liquids has been reported, suggesting the promising therapeutic benefit of Shuanghuilian in COVID-19 treatment [34].

According to the determined concentrations of baicalin in Shuanghuilian preparations and the IC50 values of the four Shuanghuilian samples as well as of pure baicalin, baicalin is identified as the major bioactive ingredient in Shuanghuilian preparations that inhibits SARS-CoV-2 3CLpro. Eight compounds besides baicalin—baicalein, forsythoside A, forsythoside B, forsythoside E, forsythoside H, forsythoside I, isofoystiaside, and scutellarein—show lower-micromolar activities against 3CLpro, although their concentrations in Shuanghuilian preparations are much lower than those of baicalin. Therefore, these compounds also contribute to the inhibition of SARS-CoV-2 3CLpro by Shuanghuilian preparations, although baicalin is the major contributor. Given the high conservation of 3CLpro among various CoVs, Shuanghuilian preparations as well as the nine compounds are expected to inhibit other 3CLpros of CoVs. However, except baicalein and baicalin, the remaining compounds do not possess good antiviral activity in Vero E6 cells, which might be ascribed to the low permeability of the cell membrane. Wogonin is not a potent inhibitor of 3CLpro or PLpro at the molecular level, but it does exhibit a good antiviral activity in Vero E6 cells, like baicalein and baicalin. The molecular mechanism underlying the antiviral activity of wogonin remains to be determined.

3CLpros are cysteine proteases excised from viral polyproteins by their own proteolytic activities and often form homodimers with one active site per subunit. The substrate specificity of 3CLpros is characterized by the efficient cleavage in the peptides, including (Leu,Phe,Met,Val)-Gln↓(Ser,Ala,Gly) sequences (the cleavage site is indicated by ↓), and the crystal structures have revealed a remarkable degree of conservation of the substrate-

| Chemical name          | Plant resource       | 3CLpro Inhibition (%) | 3CLpro IC50 (μM) | PLpro Inhibition (%) | PLpro IC50 (μM) |
|------------------------|----------------------|-----------------------|-----------------|----------------------|-----------------|
| 1 Chlorogenic acid     | L. japonica          | 76.4                  | 20.2            | 39.48 ± 5.51         | 12.5            |
| 2 Neochlorogenic acid  |                      | 49.2                  | 10.4            | /                    | 6.2             |
| 3 Cryptochlorogenic acid|                      | 39.7                  | /               | /                    | 14.0            |
| 4 Isochlorogenic acid A|                      | 77.0                  | 18.9            | /                    | 18.7            |
| 5 Isochlorogenic acid B|                      | 52.4                  | 26.3            | /                    | 24.2            |
| 6 Isochlorogenic acid C|                      | 78.2                  | 18.4            | /                    | 11.0            |
| 7 1,3-Dicaffeoylquinic acid |                | 87.3                  | 27.8            | /                    | 14.4            |
| 8 Loganin              |                      | 10.6                  | /               | /                    | −1.7            |
| 9 Secoxyloganin         |                      | 4.6                   | /               | /                    | −13.0           |
| 10 Luteoloside         |                      | 65.4                  | 14.8            | /                    | 21.5            |
| 11 Baicalin            | S. baicalensis       | 97.6                  | 68.9            | 6.41 ± 0.95          | 15.9            |
| 12 Baicalein           |                      | 99.4                  | 87.0            | 0.94 ± 0.20          | 45.1            |
| 13 Wogonoside          |                      | 20.4                  | /               | /                    | /               |
| 14 Wogonin             |                      | 3.6                   | /               | /                    | /               |
| 15 Scutellarin         |                      | 76.8                  | 18.9            | /                    | 41.8            |
| 16 Scutellarein        |                      | 101.6                 | 90.7            | 3.02 ± 0.11          | 65.7            |
| 17 Oroxylin A-7-O-β-D-glucuronide |           | 33.0                  | /               | /                    | 7.4             |
| 18 Chrys-7-O-β-D-glucuronide |                | 50.6                  | 24.2            | /                    | 16.3            |
| 19 Phyllirin           | F. suspensa          | −18.1                 | −2.8            | /                    | −1.2            |
| 20 Phylligenin         |                      | 11.4                  | /               | /                    | 8.5             |
| 21 Forsythoside A      |                      | 95.3                  | 70.5            | 3.18 ± 0.12          | 7.1             |
| 22 Forsythoside B      |                      | 101.4                 | 80.9            | 2.88 ± 0.13          | 12.7            |
| 23 Forsythoside E      |                      | 96.6                  | 41.9            | 6.68 ± 0.22          | −7.0            |
| 24 Forsythoside H      |                      | 99.3                  | 61.7            | 10.17 ± 0.39         | 2.5             |
| 25 Forsythoside I      |                      | 95.9                  | 46.3            | 5.47 ± 0.31          | 1.7             |
| 26 Isoforsythiaside    |                      | 94.4                  | 46.8            | 5.85 ± 0.06          | 61.4            |
| 27 Acteoside           |                      | 97.0                  | 34.6            | /                    | 15.6            |
| 28 (-)-Pinoresinol-4-O-β-D-glucopyraside | | 13.3                  | /               | /                    | /               |
binding sites, particularly for the crucial S1/S2 subsites [8, 35, 36]. Accordingly, substrate analogs or mimetics attached to a chemical warhead targeting the catalytic cysteine have been designed as peptidomimetic inhibitors of 3CLpros with a covalent mechanism of action [7]. A series of diamide acetamides acting as noncovalent SARS-CoV 3CLpro inhibitors and their binding modes examined by crystal structures have been reported, but they are more or less peptidomimetic inhibitors, and these compounds have not been continuously developed [37]. Although several other small molecules have been declared 3CLpro inhibitors, a solid validation of their binding with 3CLpros by ITC or complex structure determination is lacking [38, 39]. Herein, we identified baicalin and baicalein as the first class of noncovalent, nonpeptidomimetic inhibitors of SARS-CoV-2 3CLpro by an enzymatic assay in combination with the ITC, ESI-MS and X-ray protein crystallography. The binding mode of baicalein with the protease revealed by the crystal structure demonstrates that a unique protein–ligand interaction pattern is utilized by baicalein to block the proteolytic activity of SARS-CoV-2 3CLpro. Moreover, with an LE of 0.37 kcal/mol, baicalein is the most efficient binder of 3CLpros known to date. The simple chemical scaffold, unique mode of action and encouraging antiviral activity in vitro, coupled with the favorable safety data from clinical trials studies described below, emphasize that baicalein is a good candidate for the development of critically needed anticonviral drugs.

The roots of *S. baicalensis* Georgi (Huangqin in Chinese) are frequently used in TCM for the prophylaxis and treatment of hepatitis, atherosclerosis, hypertension, hyperlipidemia, type 2 diabetes, dysentery, ulcerative colitis, and other respiratory disorders [40–42]. Baicalin and baicalein are two major bioactive ingredients extracted from *S. baicalensis* Georgi, and show antioxidant, anti-inflammatory, antithrombotic, antidiabetic, and hepatoprotective activities, although their biological targets remain a mystery [42]. In the present study, the inhibition of the crucial 3CLpro of SARS-CoV-2 by baicalin and baicalein is revealed for the first time, demonstrating the potential of these two bioflavonoids as anticonviral agents. In addition, baicalein tablets have been used as an adjuvant therapy for the treatment of acute, chronic, or persistent hepatitis in China. Single oral doses of 100–2800 mg of baicalein are safe and well tolerated by healthy subjects (clinical trials registration number: CTR20132944) [43]. Although the plasma concentration of baicalein in vivo after oral administration is low, an inhalation route provides an alternative solution because intratracheal administration of baicalein could significantly increase their target tissue distribution (Supplementary Fig. S8). Furthermore, a randomized, double-blind, placebo-controlled, multicenter phase II clinical trial on the effectiveness and safety of baicalein tablets in the treatment of improving other aspects of healthy adults with influenza fever is underway (clinical trials registration number: CTR20182427 and NCT03830684). These favorable safety data together with the potent inhibitory activities in enzymatic and cell-based assays make it worthwhile to develop baicalin and baicalein into clinical agents, particularly for COVID-19 treatment.
CONCLUSION
In summary, three global pandemics, SARS, MERS, and COVID-19, have raised great awareness about the increasing risks of highly pathogenic CoV infections in humans or animals and call for the continuous development of efficacious ant coronaviral drugs. Herein, the potential treatment of COVID-19 by Shuanghuanglian preparations as well as two of its bioactive ingredients, baiacalin and baicalin, which have good drug-like properties, has been investigated in vitro. Our findings lay a strong foundation for in vivo and clinical trials studies. More importantly, our crystal structure of baicalin-bound SARS-CoV-2 3CLpro is the first demonstration of the 3CLpro reversibly inhibited by a small molecule, or even a fragment of one, derived from TCM, providing its bioactive compound mixture against human adenovirus III in vitro. Viruses. 2017;9:79.

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AUTHOR CONTRIBUTIONS
HXS, WFZ, HK, and HCH prepared the protein sample, performed the enzymatic assay and the ITC measurement. HXS, WFZ, and MJL determined the crystal structure. SY and CQK prepared the compounds, determined the content of four compounds in Shuanghuanglian preparations and characterized 28 compounds in Shuanghuanglian oral liquids. JL and CQK carried out the mass spectroscopy experiments and different administration routes of baicalin and baicalin. WJS, YW, LKZ, and GFX performed antiviral activities measurement in cells. MNG, KQY, HL, HXS, WFZ, and HCH prepared the protein sample, performed the enzymatic assay, and added to the evidence supporting the clinical potential of Shuanghuanglian oral liquids as well as two natural products for COVID-19 treatment.

ADDITIONAL INFORMATION
The online version of this article (https://doi.org/10.1038/s41401-020-0483-6) contains supplementary material, which is available to authorized users.

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