Small Molecules Blocking the Entry of Severe Acute Respiratory Syndrome Coronavirus into Host Cells

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Severe acute respiratory syndrome coronavirus (SARS-CoV) is the pathogen of SARS, which caused a global panic in 2003. We describe here the screening of Chinese herbal medicine-based, novel small molecules that bind avidly with the surface spike protein of SARS-CoV and thus can interfere with the entry of the virus to its host cells. We achieved this by using a two-step screening method consisting of frontal affinity chromatography-mass spectrometry coupled with a viral infection assay based on a human immunodeficiency virus (HIV)-luc/SARS pseudotyped virus. Two small molecules, tetra-O-galloyl-β-D-glucose (TGG) and luteolin, were identified, whose anti-SARS-CoV activities were confirmed by using a wild-type SARS-CoV infection system. TGG exhibits prominent anti-SARS-CoV activity with a 50% effective concentration of 4.5 μM and a selective index of 240.0. The two-step screening method described here yielded several small molecules that can be used for developing new classes of anti-SARS-CoV drugs and is potentially useful for the high-throughput screening of drugs inhibiting the entry of HIV, hepatitis C virus, and other insidious viruses into their host cells.
We used the FAC/MS approach to screen small molecule libraries consisting of extracts from 121 Chinese herbs, including _Prunella vulgaris_ and _Saussurea lappa_ Clarks, that exhibited antiviral activities against HIV-1 (7), RSV (30), and hepatitis B virus (27). We identified two small molecules that bind avidly to the SARS S2 protein and can interfere with the entry of SARS-CoV into Vero E6 cells, with potent antiviral activities against wild-type SARS-CoV with EC_{50} values of 4.5 and 10.6 µM.

**MATERIALS AND METHODS**

**Preparation for the polymeric carrier.** We mixed 4.7 mmol of functional monomer MAA (Acros, Geel, Belgium) and 24 mmol of cross-linker trimethylolpropane trimethacrylate (Sigma-Aldrich, Munich, Germany) and then added with 32 mg of initiator AIBN (Geel). The mixture was degassed and then placed in a 60°C water bath for 24 h. Finally, the mixture was frozen in N_{2}. The rigid polymers were ground in a mortar and passed through a 30-µm-pore-size sieve. The fine particles were removed by decanting them in acetone. The remaining were vacuum dried.

**Expression, purification, and activity detection of GST-S2 protein.** The full-length cDNA of the SARS-CoV S gene (strain BJ01, GenBank accession no. AY278488) was a gift provided by Shengli Bi at Institute of Virology, China CDC. We used it as a template to amplify the gene for S2 protein and cloned the PCR products into pGEM-T vector (Promega) and sequenced it. The desired fragments were then subcloned into pGEX-T1 vector (Amersham Biosciences). After we screened for the positive clones, the recombinant plasmids were transfected into _Escherichia coli_ JM109 (DE3)-competent cells. The gluthathione S-transferase (GST)-S2 fusion fragment was expressed and then purified from the inclusion body. The purified GST-S2 was then refolded with the Protein Refolding kit (Novagen).

The activity of the expressed GST-S2 protein was tested with convalescent SARS patient sera (provided by Beijing Ditan Hospital) by enzyme-linked immunosorbent assay (ELISA). Briefly, we precoated 96-well plates (Maxisorp; Nunc) with 1 µg of GST-S2 protein/well in 50 mM carbonate buffer (pH 9.6) for 2 h at 37°C and blocked them with 3% bovine serum albumin in carbonate buffer. We added 1,800-diluted human sera in phosphate-buffered saline with 0.05% Tween 20 (PBS-T; pH 7.4) to the wells, followed by incubation for 2 h at 37°C. The secondary antibodies conjugated with horseradish peroxidase were diluted in 3% bovine serum albumin in carbonate buffer. The mixture was then incubated with 1:100-diluted horseradish peroxidase and worked for 30 min. The mixture was then incubated with 1:10,000 in PBS-T with 1% bovine serum albumin. The optical densities at 450 nm were determined with an ELISA plate reader (Bio-Rad model 550).

**Immobilization of GST-S2 protein on the carrier.** First, 3.9 ml of a 1.12-mg/ml GST-S2 protein solution (0.05 M NaHCO$_3$ [pH 8.5]) was dialyzed in 200 ml of 0.01 M NaHCO$_3$ buffer (pH 8.0) at 4°C for 5 h, and then 14.8 mg of EDCI (Sigma-Aldrich, Germany) was added and used into the activation reagent. Then, 1.137 g of polymeric carrier that had been infiltrated in 3 ml of 0.01 M NaHCO$_3$ (pH 8.0) buffer was added, and the mixture was shaken at 20°C for 4 h. The affinity columns (PEEK tubing, 0.75 by 50 mm) were packed with the GST-S2 protein linked covalently to the polymeric carrier by using a self-pack device (PerSeptive Biosystems). There were in all about 20 µl of wet polymeric particles that had been linked with GST-S2 protein and filled in the column. The chromatographic columns were then equilibrated with 2 mM NH$_4$Ac (pH 6.7) solution and kept at 4°C.

**Preparation of the extract samples.** Next, 121 Chinese herbs were extracted by macerating them with 85% ethanol at room temperature for 2 weeks (16, 17, 52). The solvent was evaporated in a vacuum, and then the extract sample was redissolved in dimethyl sulfoxide to a final concentration of 10 mg/ml. Thereafter, individual extracts were pooled in groups of five, and each pool was diluted to a concentration of 50 µg/ml (total concentration) with 2 mM NH$_4$Ac (pH 6.7) before they were loaded onto the chromatographic column.

**Screening the crude extracts by using a GST-S2 protein column (FAC/MS).** The frontal affinity chromatographic experiments were carried out at room temperature as previously described (6, 41, 42). Briefly, the immobilized S2 proteins on the polymeric material were then packed wetly in the affinity columns (PEEK tubing, 0.75 by 50 mm). The diluted extracts were then loaded onto the chromatographic column. The flow rate was 5 µl/min. An extract sample solution containing various kinds of Chinese herbs at a concentration of 10 µg/ml (in 2 mM NH$_4$Ac [pH 6.7]) was then mixed with methanol to allow the mixture to enter the detector of Mariner electrospray ionization time-of-flight mass spectrometer (PE Biosystems). For electrospray ionization, the mass spectrometer was operated in the negative mode under the following conditions: spray tip potential, 5,000 V; nozzle potential, 100 V; detector voltage, 2,250 V; nozzle temperature, 140°C; and quad temperature, 140°C. All of the other parameters were set at the default values.

**RESULTS**

**Screening of small molecules by FAC/MS.** For the FAC/MS approach, we generated a fusion protein (GST-S2) in which an S2 protein fragment corresponded to the sequence between Asn733 to Gln1190 of the SARS-CoV S protein and then used the fusion protein to screen the small molecules pooled from the extracts of more than 121 Chinese herbs. The GST-S2 fusion protein was expressed in _E. coli_, purified from the inclusion body, and refolded. The purity and activity of the purified GST-S2 were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and ELISA (Fig. 1a and b). The results showed that the renatured recombinant GST-S2 could specifically bind to the anti-S protein antibodies present in the sera of convalescent SARS patients.

We used the FAC/MS method to identify the small herbal molecules that had a relatively strong binding affinity to the GST-S2 protein. Extracts of 121 Chinese herbs were separately applied to the FAC column that was packed with purified GST-S2 protein (GST was used as control [data not shown]).

**HIV-luc/SARS pseudotyped virus entry inhibition assays.** To produce HIV-lucSARS pseudotyped virus, 10 µg of pNL4-3E-R-Luc (HIV-luc) and 1 µg of humanized SARS-CoV spike protein expression plasmids pCTsh were mixed and transfected into 293T cells with calcium phosphate as described previously (21, 55). The pseudotyped virus was harvested after 24 h of incubation, filtered through a 0.45-µm-pore-size Millipore membrane and normalized by p24 ELISA by using a Vironostika HIV-1 Antigen MicroELISA kit (Biomerieux bv, Boxtel, The Netherlands).

For the serum testing, the samples were serially twofold diluted from 1:100 and mixed with 5 µg of pseudotyped virus (p24). After incubation for 30 min at 37°C, the mixture was added onto the target cells. After overnight incubation, the medium was replaced, and incubation was carried out for an additional 36 h. The cells were then measured for luciferase activities by a Wallac Microbeta 1420 Counter (Perkin-Elmer, Inc.) by using the luciferase assay system (Promega, Inc.).

For the small-molecule testing, the supernatant containing 5 ng of pseudotyped virus (p24) was incubated with different concentrations of small molecules at 37°C for 30 min, and then the mixture was transferred into 96-well plates seeded with Vero E6 cells (3 × 10^3 cells/well), each concentration was repeated eight times. After overnight incubation, the medium was replaced, and the sample was incubated for an additional 36 h. The cells were then measured for luciferase activities as described above.

**Wild-type SARS-CoV infection inhibition assay.** SARS-CoV Wild-type virus BJ01 strain was a gift of the Beijing Genomics Institute. We coincubated 200 µl of the cell culture infective doses of wild-type SARS-CoV with 50-µl aliquots of small molecules at different concentrations at 37°C for 30 min. The mixture was then transferred into 96-well plates with eight wells for each dilution. After incubation for 60 h, an MTI-3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide; Sigma-Aldrich] assay was performed as described previously (19). Briefly, 10 µl of 5 mg of MTT/ml was added to each well, followed by incubation for a further 4 h. The medium in each well was then replaced with 100 µl of dimethyl sulfoxide, and then the media were left for 10 min at room temperature for color development before being read by Bio-Rad model 550 ELISA reader (at a test wavelength of 570 nm and a reference wavelength of 630 nm). The cytotoxicity of each small molecule was also determined by MTT assay without the addition of the virus.
The binding affinity of each main component of an extract to the GST-S2 protein was monitored by its elution front that could be deduced from its FAC/MS spectra. For example, among the frontal affinity chromatographic traces (for a typical ion chromatogram from the mass spectra, see Fig. 1c) of the 10 main components of *Galla chinensis*, the front retention time of component 10 (432.5 μL) was much higher than those of the other components (Table 1); correspondingly, this component also displayed the longest retention time (t_{1/2} = 85 min [Fig. 1d]). These results demonstrated that the tenth component exhibited the strongest binding affinity to the SARS S2 protein.

**Inhibition of entry of HIV-luc/SARS pseudotyped virus into host cells.** We then used HIV-luc/SARS pseudotyped virus to investigate the antiviral activity of the 130 small molecule candidates. To produce the HIV-luc/SARS pseudotyped virus, we cotransfected a humanized S protein expression plasmid pCDSh with pNL4-3E-R-Luc (HIV-luc), an HIV-1 vector containing luciferase gene as a reporter into 293T cells. The pseudotyped viruses were then collected and used to infect Vero E6 cells, which are permissive to infection by wild-type SARS-CoV. To evaluate the relevance of our pseudovirus assay, we first tested the inhibition ability of normal sera and the sera of SARS patients. This infection could be blocked by the sera of SARS patients and appeared to be SARS specific because the same sera did not neutralize the vesicular stomatitis virus (VSV) G glycoprotein pseudotyped virus (Fig. 2a).

To test the anti-HIV-luc/SARS activity, we added different concentrations of the small molecules to the infection mixture. Of the 130 small molecules, two were found to have potent antiviral activities against the HIV-luc/SARS pseudotyped virus, with EC_{50} values of 2.86 and 9.02 μM. Structural analysis revealed that the two small molecules were TGG and luteolin (Fig. 2b).

**Specificity of small molecules.** To investigate the specificity of the small molecules, we tested their antiviral activities against HIV-luc/VSV pseudotyped virus, another pseudotyped virus enveloped with the G protein of VSV. Instead of the S protein of SARS-CoV, infection of the HIV-luc/VSV pseudotyped virus was also determined by the luciferase activity in the infected cells. Both TGG and luteolin showed little anti-VSV activity at the same concentration levels that can effectively inhibit the entry of HIV-luc/SARS pseudotyped virus to its host cells (Fig. 2c). HIV-luc/SARS pseudotyped virus and HIV-luc/VSV pseudotyped virus share the same genome and the genome replication apparatus; the only difference between them is that one is coated with SARS-CoV S protein, whereas the other is coated with VSV G protein. These studies indicate that the two small molecules, especially TGG, were highly specific against SARS-CoV. Since we isolated TGG and luteolin through analysis of their binding to the S2 protein of SARS-CoV, these small molecules most likely work through their ability to block the entry of HIV-luc/SARS pseudotyped virus to its host cells.

**Inhibition of wild-type SARS-CoV infection.** To further confirm the antiviral activities of the small molecules identified above, we analyzed their inhibitory effects against infection by a wild-type SARS-CoV by using a MTT assay. In our experiments, we compared the antiviral activity of TGG and luteolin with the previously described glycyrrhizin and ribavirin. Our results (Table 2) showed that (i) TGG and luteolin could inhibit, in a dose-dependent fashion, SARS-CoV infection with EC_{50} values of 4.5 and 10.6 μM, respectively; (ii) the EC_{50} of glycyrrhizin in our experiments was >607.6 μM, which was

### Table 1. Frontal volumes of the 10 main components in *Galla chinensis* extract

<table>
<thead>
<tr>
<th>Component</th>
<th>Frontal vol (μL)</th>
<th>Component</th>
<th>Frontal vol (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>74</td>
<td>6</td>
<td>233.5</td>
</tr>
<tr>
<td>2</td>
<td>101.5</td>
<td>7</td>
<td>345.5</td>
</tr>
<tr>
<td>3</td>
<td>183</td>
<td>8</td>
<td>180</td>
</tr>
<tr>
<td>4</td>
<td>243</td>
<td>9</td>
<td>340.5</td>
</tr>
<tr>
<td>5</td>
<td>49</td>
<td>10</td>
<td>432.5</td>
</tr>
</tbody>
</table>
relatively consistent with a previous report (9); and (iii) ribavirin, as reported earlier, showed little, if any, anti-SARS-CoV effects (9).

Cytotoxicity and toxicity of the small molecules. To test the biosafety of these small molecules, we examined their cytotoxicity against the Vero E6 cells by using the MTT assay. The CC\textsubscript{50}s (i.e., the concentrations of drug that caused 50% cell death) of TGG and luteolin were 1.08 and 0.155 mM, respectively (Table 2). Therefore, the selective index (i.e., the ratio of CC\textsubscript{50} to EC\textsubscript{50}) values of TGG and luteolin were 240.0 and 14,62, respectively (Table 2). These results suggest that TGG and luteolin can be used at high concentrations to inhibit SARS-CoV without substantial cytotoxic effects. We then assessed the acute toxicity of the small molecules on the ICR mice by administrating TGG and luteolin intraperitoneally. The 50% lethal doses (i.e., the concentration of drug that causes 50% of animal death) of TGG and luteolin were \(\sim 456\) and 232.2 mg/kg, respectively, suggesting that these molecules can be used in mice at relatively high concentrations.

Antiviral activity of an analog of luteolin. Since quercetin, which is structurally related to luteolin, is an ingredient of antioxidant and antiallergy medicines that had been approved by the U.S. Food and Drug Administration (FDA; the national drug code numbers of the medicines are 65448-3085, 65448-3005), we sought to determine whether quercetin could also antagonize SARS-CoV entry. Assays with the HIV-luc/SARS pseudotyped virus showed that quercetin also had antiviral activity against HIV-luc/SARS, with an EC\textsubscript{50} of 83.4 \(\mu\)M (Fig. 3). The cytotoxicity of quercetin was very low, with a CC\textsubscript{50} of 3.32 mM (data not shown). As an FDA-approved drug ingredient, quercetin offers great promise as a potential drug in the clinical treatment of SARS.

**DISCUSSION**

Using a two-step screening method, we screened a library containing extracts of 121 Chinese herbs and identified two small molecules, TGG and luteolin, that displayed inhibitory activity for the entry process of SARS-CoV into host cells. The combined use of FAC/MS and an infection assay utilizing the HIV-luc/SARS pseudotyped virus offers several advantages. (i) FAC/MS has been developed recently for high-throughput screening of enzymatic ligands (6) and for determining the strength of ligand and en-

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TABLE 2. Inhibitory effects of TGG and luteolin against infection by wild-type SARS-CoV

<table>
<thead>
<tr>
<th>Small molecule</th>
<th>MW</th>
<th>EC\textsubscript{50} ((\mu)M)</th>
<th>SI</th>
<th>CC\textsubscript{50} (mM)</th>
<th>LD\textsubscript{50} (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGG</td>
<td>788.3</td>
<td>4.5 (1.96–5.8)</td>
<td>240</td>
<td>1.08</td>
<td>232.2</td>
</tr>
<tr>
<td>Luteolin</td>
<td>286.3</td>
<td>10.6 (9.2–12.2)</td>
<td>14.62</td>
<td>0.155</td>
<td>456</td>
</tr>
<tr>
<td>Glycyrrhizin</td>
<td>507.7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>244.2</td>
<td>No effect</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(a\) Experiments were repeated three times, and the data in the table show the average EC\textsubscript{50} values. MW, molecular weight; SI, selective index; ND, not determined.

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FIG. 2. The inhibitory activities of SARS patients’ sera and selected small molecules against the HIV-luc/SARS pseudotyped virus to enter Vero E6 cells. (a) Detection of inhibitory activities of sera of SARS patients. Note the ability of the SARS patient sera to block the infectivity of HIV-luc/SARS (SARS) versus the absence of such blocking activity in normal serum; note also the lack of neutralizing activity of the SARS sera against the pseudotyped virus bearing the G protein of VSV (VSV), which could infect the target cells at a similar level to that of HIV-luc/SARS. The serum dilution are indicated. (b) Structures and inhibitory activities of TGG and luteolin. Note the high inhibitory activity of TGG (EC\textsubscript{50} = 2.86 \(\mu\)M), which is consistent with its high affinity to the SARS S2 protein, as our data demonstrated (Fig. 1c and d and Table 1). (c) Specificities of TGG and luteolin inhibitory activities. HIV-luc/VSV (VSV) pseudotyped viruses were used as a control. Note the inhibition by TGG and luteolin of the entry of HIV-luc/SARS virus but not the HIV-luc/VSV pseudotyped virus. Values are averages of triplicate determinations. The bars indicate the standard deviations.

FIG. 3. Structure and inhibitory activities of quercetin against the entry of HIV-luc/SARS pseudotyped virus into Vero E6 cells. Similar results were obtained in three independent experiments.
zyme binding (dissociation constant) (53, 54). (ii) Pseudotyped viruses have proven to be a powerful method for studying the entry of viruses to their host cells. For example, studies utilizing HIV pseudotyped virus has led to the discovery of CCR5, the coreceptor of HIV-1 (10), and recently it was used to identify TRIM5α (a component of cytoplasmic bodies), which can inhibit HIV-1 infection in Old World monkeys (46). Pseudotyped hepatitis C viruses (HCVs) are being used to search for HCV receptors (2, 18). Pseudotyped viruses provide particularly useful assays for neutralizing antibodies (3), cell tropism (29, 51), and the identification of drugs that inhibit the entry of viruses into host cells (12, 38, 39, 44, 48). In the present study, we have utilized the SARS pseudotyped virus to screen for the small molecules that can antagonize SARS-CoV entry. We showed that FAC/MS in conjunction with the HIV-luc/SARS pseudotyped virus entry assay led to the rapid identification of two small inhibitory molecules. A similar strategy should be applicable for the search of drugs that antagonize other enveloped viruses such as HIV-1, HCV, and RSV.

There are two ways to obtain small molecules that can be used as virus entry inhibitors. These are, first, through chemical design and synthesis, such as SCH-C and TAK-779 for HIV-1 (1, 47) and as entry inhibitors for measles virus (34). The second is through isolation from natural products, e.g., different plant varieties. Chinese herbs are a great source of small molecules, leading to clinically used drugs; we identified here two small molecules from extracts of Chinese herbs, i.e., TGG and luteolin, that appear to be highly effective in inhibiting the entry of both wild-typed SARS-CoV and HIV-luc/SARS pseudotyped virus into Vero E6 cells (Fig. 2 and Table 2). Their anti-SARS-CoV potency is much greater than that of glycyrrhizin, a small molecule that has recently been reported to have anti-SARS-CoV activity (9). The detailed mechanism by which TGG and luteolin exert anti-SARS-CoV activity has not yet been established. The entry process of enveloped viruses usually involves three steps: attachment, receptor binding, and virus-cell fusion, which are mediated by viral envelope proteins. For SARS-CoV, it is presumed that its S protein participates in the viral entry process (24, 31, 40, 45) and that the transmembrane subunit of S protein, the S2 subunit, plays a crucial role in the virus-cell fusion process (24, 45). Our FAC/MS results identified TGG and luteolin as having the highest affinity, among all of the Chinese herbal components that we have studied thus far with the S2 protein. These data raise the possibility that TGG and luteolin may achieve their antiviral activity by interfering with the virus-cell fusion process. Additional studies are needed to test this idea.

TGG and luteolin offer excellent opportunities for further optimization and potential clinical use as anti-SARS drugs. TGG is a component of Galla chinensis, which has been used in traditional Chinese medicine for treating chronic coughing. Luteolin has been identified in extracts of many Chinese herbs such as Veronica lina riiifolia Pall by MS (28). We found this was a component of Rhodiola kirilowii, which has been used in Chinese medicine for treating hepatitis and tuberculosis (43). These two compounds and the luteolin-related, FDA-approved quecertain, have potential for use in the clinical treatment of SARS.

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