

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 μ M 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.

Severe acute respiratory syndrome (SARS) is a viral respiratory disease that spread to more than two-dozen countries in Asia, North America, South America, and Europe in the spring of 2003 (36, 49). Approximately 8,400 people worldwide suffered from SARS and more than 900 died according to a report of the World Health Organization executive board on 27 November 2003. A novel coronavirus, SARS-coronavirus (SARS-CoV), was identified as the pathogen that caused SARS (13, 26). SARS-CoV is highly infectious and is transmitted via the respiratory route (13, 15, 26, 36, 49). Although the first outbreak is over, it is possible that even a low-level epidemic or accidental infection, as recently happened in Singapore and Taiwan, could cause a sudden worldwide epidemic. At present, few drugs are available for treating SARS. Ribavirin was initially used, but it proved to be barely effective and had serious side effects (25, 50). Cinatl et al. reported recently that interferon exhibited anti-SARS-CoV potency (8) and that glycyrrhizin had an anti-SARS-CoV activity with a 50% effective concentration (EC_{50}) of 300 mg/liter (364.5 μ M) (9).

We have targeted our drug discovery to small molecules that can inhibit SARS-CoV from entering its host cells. Virus entry is an attractive target step for therapy because it can block the

propagation of virus at an early stage, thus minimizing the chance for the virus to evolve and acquire drug resistance. Inhibitors of the entry of several important viruses have been developed. For example, a new class of virus entry inhibitors has been developed for the human immunodeficiency virus type 1 (HIV-1) (14, 32), and one of these, T20 (enfuvirtide), is now in clinical usage (14, 20, 23, 32). For the respiratory syncytial virus (RSV), the small molecules RFI 641 (33, 37) and VP-14637 (11) have been developed to inhibit its entry process. For herpes simplex virus, entry inhibitors such as FGF4 signal peptide (4, 5) and *n*-docosanol (35) are under development.

We have developed a two-step screening method combining frontal affinity chromatography-mass spectrometry (FAC/MS) and pseudotyped virus infection assay to search for drugs that can interfere with the entry of SARS-CoV into host cells. FAC/MS allows for competitive assays of interacting molecules, such as enzymes and ligands, as well as for a quick analysis and direct presentation of the assay (6, 41, 42). Here, in the first step, we use the FAC/MS to perform high-throughput screening of small molecules that can bind with high affinity to the SARS-CoV S2 protein. The other rationale of our study is that some of these S2-binding molecules may interfere with the function of the S protein, thus preventing the SARS-CoV from entering its host cells. To test this, we developed an HIV-luc/SARS pseudotyped virus that can infect Vero E6 cells resulting in the cellular expression of luciferase that can be detected by luminometry. Thus, as a second step, we used HIV-luc/SARS pseudotyped virus to check the function of the small molecules obtained in the first step. This system is highly sensitive, rapid, and reproducible; the materials are safe to handle and can be used for high-throughput screening.

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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 EC₅₀ 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 N₂. 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 remainders 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-4T-1 vector (Amersham Biosciences). After we screened for the positive clones, the recombinant plasmids were transfected into *Escherichia coli* JM109 (DE3)-competent cells. The glutathione 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 1:2,000 to 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 NaHCO₃ [pH 8.5]) was dialyzed in 200 ml of 0.01 M NaHCO₃ buffer (pH 8.0) at 4°C for 5 h, and then 4.8 mg of EDCI (Sigma-Aldrich, Germany) was added into the solution as an activation reagent. Then, 1.137 g of polymeric carrier that had been infiltrated in 3 ml of 0.01 M NaHCO₃ (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 NH₄Ac (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 NH₄Ac (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 precisely 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 NH₄Ac [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.

The volume of the small molecules that bind to S2 protein (V), the volume of the nonbinding small molecules (V_0), and the concentration of the bound small molecules (C) were recorded. Using these data, we calculated the binding affinity of the small molecules (K_d) to S2 protein according to the intercept in the following liner equation (22):

$$\frac{1}{C(V-V_0)} = \frac{1}{L_t} + \frac{K_d}{L_t} \cdot \frac{1}{C}$$

HIV-luc/SARS pseudotyped virus entry inhibition assays. To produce HIV-luc/SARS pseudotyped virus, 10 μg of pNL4-3E-R-Luc (HIV-luc) and 10 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 ng 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 the sample was incubated 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 cocultured 200 50% tissue 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 to 96-well plates, with eight wells for each dilution. After incubation for 60 h, an MTT [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.

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]).

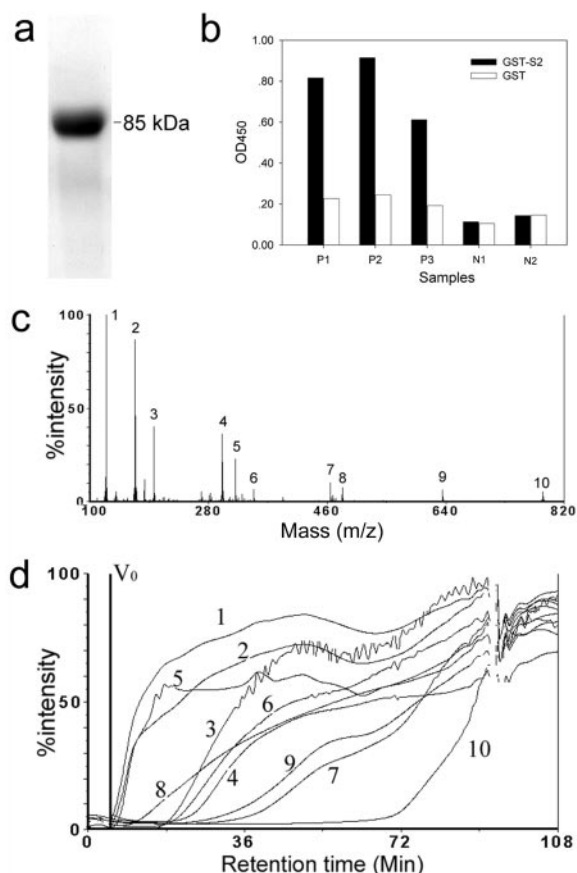


FIG. 1. Identification of *Galla chinensis* components that have a high affinity to the SARS S2 protein by MS coupled with frontal affinity chromatography. (a) Purity of the GST-S fusion protein as shown by SDS-15% PAGE. (b) The specific binding of the GST-S2 protein with sera of three convalescent SARS patients (P1, P2, and P3) as shown by ELISA. Two normal sera (N1 and N2) and GST were used as controls. (c) The mass spectra of the crude extract of *Galla chinensis* (the main components are numbered 1 to 10; also see Table 1). (d) Frontal affinity chromatographic traces (selected ion chromatogram from the mass spectra, Fig. 1c) of the 10 main components in *Galla chinensis*. V_0 indicates the volume of the nonbinding small molecules. Note the high retention time ($t_{1/2} = 85$ min) of component 10, indicating an exceptionally strong binding affinity to the SARS S2 protein.

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 frontal volume of the tenth component (432.5 μ l) 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 affinity to S2 protein. Structural analysis established that this component is tetra-*O*-galloyl- β -D-glucose (TGG). This method allowed us to identify \sim 130 small molecules with K_d values of <10 μ M for further analyses.

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 can-

didates. 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 μ M. 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 μ M, respectively; (ii) the EC_{50} of glycyrrhizin in our experiments was >607.6 μ M, which was

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

Component	Frontal vol (μ l)	Component	Frontal vol (μ l)
1	74	6	233.5
2	101.5	7	345.5
3	183	8	180
4	243	9	340.5
5	49	10	432.5

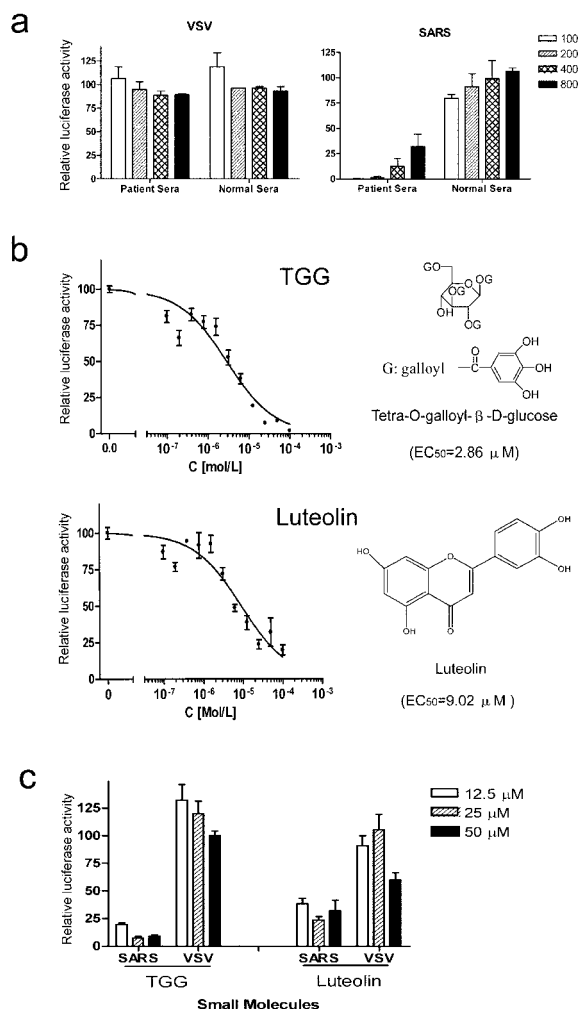


FIG. 2. The inhibitory activities of SARS patients' sera and selected small molecules against the HIV-luc/SARS pseudotyped virus to enter Vero E6 cells. (a) Detection of inhibitory activities of sera of SARS patients. Note the ability of the SARS patient sera to block the infectivity of HIV-luc/SARS (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 ($EC_{50} = 2.86 \mu\text{M}$), 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.

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_{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_{50} to EC_{50}) values of TGG and luteolin were 240.0 and

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

Small molecule	MW	EC_{50} (μM)	SI	CC_{50} (mM)	LD_{50} (mg/kg)
TGG	788.3	4.5 (1.96–5.8)	240	1.08	232.2
Luteolin	286.3	10.6 (9.2–12.2)	14.62	0.155	456
Glycyrrhizin	822.94	>607.6	ND	ND	ND
Ribavirin	244.2	No effect	ND	ND	ND

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

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 ~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_{50} of 83.4 μM (Fig. 3). The cytotoxicity of quercetin was very low, with a CC_{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 for the screening of antiviral drugs 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-

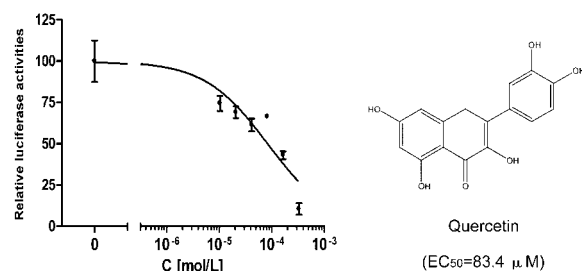


FIG. 3. Structure and inhibitory activities of quercetin against the entry of HIV-luc/SARS pseudotyped virus into Vero E6 cells. Similar results were obtained in three independent experiments.

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 TRIM5 α (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* that 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 riifolia* 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 quercetin, have potential for use in the clinical treatment of SARS.

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