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Identification of natural compounds with antiviral activities against SARS-associated coronavirus

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Abstract

More than 200 Chinese medicinal herb extracts were screened for antiviral activities against Severe Acute Respiratory Syndrome-associated coronavirus (SARS-CoV) using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay for virus-induced cytopathic effect (CPE). Four of these extracts showed moderate to potent antiviral activities against SARS-CoV with 50% effective concentration (EC_{50}) ranging from 2.4 ± 0.2 to 88.2 ± 7.7 μg/ml. Out of the four, *Lycoris radiata* was most potent. To identify the active component, *L. radiata* extract was subjected to further fractionation, purification, and CPE/MTS assays. This process led to the identification of a single substance lycorine as an anti-SARS-CoV component with an EC_{50} value of 15.7 ± 1.2 nM. This compound has a CC_{50} value of 14980.0 ± 912.0 nM in cytotoxicity assay and a selective index (SI) greater than 900. The results suggested that four herbal extracts and the compound lycorine are candidates for the development of new anti-SARS-CoV drugs in the treatment of SARS.
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Keywords: Severe Acute Respiratory Syndrome coronavirus (SARS-CoV); Drug screening; Natural products; Lycorine; *Lycoris radiata* herb

Severe Acute Respiratory Syndrome (SARS) is a respiratory illness caused by the Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) (Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003b; Poutanen et al., 2003). This febrile respiratory illness was initially described in early 2003 (Chan-Yeung and Yu, 2003; Donnelly et al., 2003; Lee et al., 2003; Peiris et al., 2003a; Tsang et al., 2003) and is life threatening and highly contagious. Currently, there are no approved or universally recommended therapies for SARS. Treatment for the disease is mainly supportive. Scientists worldwide have been vigorously trying to develop efficacious antiviral agents for the treatment of SARS in the event that SARS comes back in the future. Reports from several groups (Cinatl et al., 2003a,b; Scagnolari et al., 2004) have suggested that some reagents, such as interferon and glycyrrhizin, pose anti-SARS-CoV activity.

In China, traditional herbal medicine has been frequently used in conjunction with conventional medicine to treat SARS. There is evidence showing that the herbal medicine is effective (Lin et al., 2003; Xiao et al., 2003; Zhao et al., 2003; Zhong and Zeng, 2003). However, the mechanisms of this treatment have not been clearly understood. It has been shown that natural plants contain antiviral activities to other coronaviruses (McCutcheon et al., 1995) and the mechanism of action of these herbal products is mainly through inhibition

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of viral replication (Vlietinck and Vanden Berghe, 1991; Jassim and Naji, 2003).

In this study, we selected over 200 in-house-made extracts of medicinal herbs that have been historically used for the treatment of virus-induced infectious diseases in China and tested their antiviral activities against SARS-CoV using a high throughput screening approach. The screening was based on a MTS assay (Cory et al., 1991; Khabar et al., 1996). The active samples from screening were then subjected to structure activity relationship (SAR) study to identify a single active chemical substance. The results of these studies and the potential usage of identified lead compounds in the treatment of SARS-CoV-induced infectious diseases are presented here.

In searching for new reagents for anti-SARS-CoV, collected herbs were extracted by refluxing with 95% ethanol or chloroform for 3 h. The extracted solvents were filtered and lyophilized and then re-dissolved in dimethyl sulphoxide (DMSO) (Sigma) and stored in 96-well sample plates at −80 °C for assays and screening. Two strains of SARS-CoV (BJ001, BJ006) used for antiviral compound screening were obtained from the Laboratory of Virology at the Academy of Military Medical Sciences in Beijing, China. The viruses were propagated in Vero E6 cells at 37 °C in a humidified atmosphere of 5% CO_2. Vero E6 and HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) containing 5% heat-inactivated fetal bovine serum (FBS) (Hyclone) and sodium bicarbonate, 3.7 g/l; glucose, 4.5 g/l; and 15 mM HEPES buffer. The virus-induced cytopathic effect (CPE) was determined by MTS method and by visualization of cellular morphology change. The number of viable cells is correlated with absorbance at 490 nm in MTS assay. Approximately, 4×10^3 Vero E6 cells/well were seeded onto Corning 96-well tissue culture plates (Corning Incorporated) with final volume of 100 μl and cultured for 24 h. Ten microliters of compounds or plant extracts at a concentration of 100 μg/ml were added into each well in duplicates before inoculating with virus stock. Interferon alpha (Hualida Biotech Company), proven to show antiviral activities against SARS-CoV (Cinatl et al., 2003b; Scagnolari et al., 2004), was used as the positive control. The viral titers were assessed by cytopathic effect (CPE) determined visually under the light-phase microscope 2–4 days post-infection (PI). The concentration to achieve 90% of cell lysis was used in antiviral compound screening. The infected cells with or without compound were incubated at 37 °C in a 5% CO_2 atmosphere for 72 h. Then, 20 μl of MTS/phenazine methosulfate (PMS) (Promega) was added in each well. The cells were incubated for another 2 h in 37 °C. In the end, 50 μl 10% SDS was added to stop color reaction. The plates were measured at 490 nm using a VERSAMax microplate reader (Molecular Devices).

After primary screening, active compounds were cherry picked and a second round of test was performed for their antiviral effects. The pictures were taken to record cell morphology change caused by CPE and the inhibition effects of the compounds before MTS assay. As shown in Fig. 1, four of the extracts, *Lycoris radiata*, *Artemisia annua*, *Pyrrosia lingua*, and *Lindera aggregata* exhibited significant inhibition effects on virus-induced CPE when SARS-CoV strain BJ001 was used in screening. A dose dependency of antiviral activities was determined by serial dilutions of compounds. The percentage of CPE reduction was calculated by subtracting the mean value of virus-infected cell control (0%) from the measured absorbance, and resulting number was divided by the measured absorbance of uninfected cell control (100%). The mean values and the standard deviation (S.D.) were taken for result analysis. The inhibition effects of all four natural product samples showed dose-dependent patterns (Fig. 1). The EC_{50} values were determined as the concentration of the compounds needed to achieve the inhibition of SARS-CoV-induced CPE to 50% of control value (cells without viral infection) and data analysis for the assays was performed using Prism™ version 3 software (Graphpad Software, Inc.). The EC_{50} values of inhibition are 2.4 ± 0.2, 34.5 ± 2.6, 43.2 ± 14.1, and 88.2 ± 7.7 μg/ml, respectively, much lower than previously identified compounds (Cinatl et al., 2003a). To check whether there is any significant strain variation, we used SARS-CoV strain BJ-006 and tested the inhibition activity of active compounds. The results were quite similar for two viral strains (Table 1). Viability of Vero cells measured by MTS assay was consistent with what we observed visually under the microscope (photos not shown). The addition of active compounds significantly blocked viral infection or replication and kept cells in a viable state. Interferon alpha also showed limited inhibition effects on virus-induced CPE, either judged by visual observation or MTS assay. The results were consistent with previous reports from Cinatl and Scagnolari's groups. The inhibition for all four compounds to virus infection/replication was apparently more potent than that of interferon alpha judged by visual observation and color absorbance in MTS assay (Fig. 1).

The cytotoxicity test for active compounds was based on the cell viability after cells were treated with various concentrations of compounds, and was determined by MTS method. Vero E6 and HepG2 cells in 96-well microplates incubated with serial 10-fold dilutions of testing compounds in DMEM containing 5% FBS. Cells were allowed to grow for an additional 72 h before the measurement. The CC_{50} values were determined as the concentration of the compounds that reducing the cell viability to 50% of control (cells without addition of compound). For the four active compounds, *L. radiata*, *A. annua*, *P. lingua*, and *L. aggregata*, the CC_{50} values range from 886.6 ± 35.0 up to 2378.0 ± 87.3 μg/ml in assays using Vero cells (Table 1). The selective index (SI), which was determined as the ratio of CC_{50} versus EC_{50} for one of the potent active compound extracts, *L. radiata*, is more than 300. Three others also showed good SI values, with the exception of *L. aggregata*. In order to examine compound toxicity to different cell types, we tested all four extracts on both Vero E6 and human HepG2 cell lines. The CC_{50} values of *L. radiata*, *A. annua*, *P. lingua*, and *L. aggregata* were 690.5 ± 21.0,

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