1 Screening of Botanical Drugs against SARS-CoV-2 Entry

2 Junyuan Cao ^{a,b}, Yang Liu ^a, Minmin Zhou ^{a,b}, Siqi Dong ^{a,b}, Xiaoying Jia ^{a,b}, Xiaohao Lan ^{a,b},

3 Yueli Zhang ^{a,b}, Jiao Guo ^{a,b}, Gengfu Xiao ^{a,b}, Wei Wang ^{a,b} #

4 State Key Laboratory of Virology, Wuhan Institute of Virology, Center for Biosafety

5 Mega-Science, Chinese Academy of Sciences, Wuhan 430071, China ^a

6 University of the Chinese Academy of Sciences, Beijing 100049, China ^b

7

8 **Running title:** Botanical Drugs against SARS-CoV-2 Entry

9 **Key Words :** SARS-CoV-2, botanical drug, membrane fusion, entry inhibitor,

10 angeloylgomisin O, schisandrin B, procyanidin, oleanonic acid, combination therapy

11 **Word count:** abstract = 190, text = 2339

12 # Address correspondence to Wei Wang, wangwei@wh.iov.cn

13

14

15 **ABSTRACT**

16 An escalating pandemic caused by the novel severe acute respiratory syndrome coronavirus 2

17 (SARS-CoV-2) is impacting global health. Specific treatment options for diseases caused by

18 SARS-CoV-2 are largely lacking. Herein, we used a pseudotype virus (pv) bearing the

19 SARS-CoV-2 S glycoprotein to screen a botanical drug library to identify an agent against

20 SARS-CoV-2 entry. All the four hits, including angeloylgomisin O, schisandrin B,

21 procyanidin, and oleanonic acid, were identified for effective inhibition of SARS-CoV-2 S pv

22 entry in the micromolar range. A mechanistic study revealed that these four agents inhibit

23 SARS-CoV-2 S pv entry by blocking S-mediated membrane fusion. Furthermore,

angeloylgomisin O, schisandrin B, and oleanonic acid inhibited authentic SARS-CoV-2 with a high selective index (SI). We also showed that all the four hits could also inhibit the entry of pv of Middle East respiratory syndrome coronavirus (MERS-CoV) and newly emerged SARS-CoV-2 variants (D614G, K417N/E484K/N501Y/D614G). In drug combination studies performed in cellular antiviral assays, angeloylgomisin O and schisandrin B displayed synergistic effects in combination with remdesivir. These results indicated that angeloylgomisin O, schisandrin B, procyanidin, and oleanonic acid can inhibit SARS-CoV-2 and that they are potential therapeutic agents for COVID-19.

INTRODUCTION

COVID-19, caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), poses a severe threat to global public health as well as economic and social stability, requiring rapid development of treatment and preventive measures (1).

Coronavirus is an enveloped virus with four structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. SARS-CoV-2 shares a high degree of sequence identity with SARS-CoV that emerged previously and exploits the same human cell receptor, angiotensin-converting enzyme 2 (ACE2), for infection (2, 3). After the receptor binding domain (RBD) in the S1 subunit of the S protein on the virion binds to the ACE2 receptor on the target cell, the heptapeptide repeat 1 (HR1) and 2 (HR2) domains in the S2 subunit of the S protein interact with each other to form a six-helix bundle (6-HB) fusion nucleus. This interaction brings the virus membrane and cell membrane close together for fusion and infection (4, 5).

SARS-CoV-2 culture and assays need to be carried out in a biosafety level (BSL)-3 laboratory. The vesicular stomatitis virus (VSV) reverse genetics system can provide a safe, reliable, and stable platform to study SARS-CoV-2 S-glycoprotein inhibition by antibodies or small molecules with high assay throughput. Researchers have employed related viral systems that afford virus entry studies at BSL-2 and facilitate rapid inhibitor screening using fluorescence or luminescence-based reporters (6).

More than a year after its emergence, SARS-CoV-2 continues to cause a fervor with identification of new variants hailing from the United Kingdom, South Africa, Brazil, the United States, and India (7-9). The G614 variant was first detected in early 2020 and contains a D614G substitution in its S protein's RBD, which appears to increase viral transmissibility (10-12). As other new variants emerged over the past few months, the major focus of research has been on identifying further RBD substitutions and their impact on infection. Three other mutations in RBD, K417T/N, E484K, and N501Y, have been identified that affect host cell interactions and susceptibility to antibody-mediated neutralization (13-15).

Natural compounds have been universally used to prevent and cure various illnesses in many countries since ancient times. With the isolation and characterization of the structures of select natural products, these products have become a critical contributor to pharmaceuticals, and several critical pharmaceuticals have been developed that are modeled after natural products (16, 17).

This study focuses on screening drugs targeting the entry step of SARS-CoV-2 infection to block the early stages of viral infection and spread. Using SARS-CoV-2 S VSV-based-pseudovirus (SARS-CoV-2 S pv), we identified that angeloylgomisin O,

schisandrin B, procyanidin, and oleanonic acid could inhibit SARS-CoV-2 entry by inhibiting membrane fusion. Our findings may offer new therapeutic strategies for the treatment of COVID-19.

RESULTS

Construction of SARS-CoV-2 S pv (ct19)

To perform high-throughput screening under BSL-2 conditions, we constructed a SARS-CoV-2 S pv based on a VSV backbone. The cytoplasmic tail of the S glycoproteins SARS-CoV-2 is highly similar to that of SARS-CoV and carries signals for their retention in the endoplasmic reticulum (ER). Previous studies found that a 19 amino acid deletion in the cytoplasmic tail of SARS-CoV or SARS-CoV-2 S glycoprotein increased the infectivity of the single-cycle pseudotype virus (18-21).

We generated two recombinant plasmids expressing either the wild type (wt) S glycoprotein, pcDNA3.1-SARS-CoV-2 S, or the truncated S glycoprotein missing 19 amino acids from its C-terminus, pcDNA3.1-SARS-CoV-2 S (ct19), which were used to generate SARS-CoV-2 S pv (wt) or the shortened S glycoprotein, SARS-CoV-2 S pv (ct19), respectively.

As shown in Fig. 1A, the *Renilla* luciferase (Rluc) activity of the cells infected with SARS-CoV-2 S pv (ct19) was higher than that of cells infected with SARS-CoV-2 S pv (wt). Next, the VSV copy number was assayed in the different pv harvests, and a higher packaging efficiency was detected in the SARS-CoV-2 S pv (ct19) system than in SARS-CoV-2 S pv (wt) (Fig. 1A), reaching 1.15×10^9 copies/ml and 2.3×10^8 copies/ml, respectively.

Immunoblots confirmed the expression and accumulation of SARS-CoV-2 S protein in SARS-CoV-2 S pv (ct19) (Fig. 1B), enabling us to use SARS-CoV-2 S pv (ct19) for

high-throughput screening (HTS) of potential botanical drug candidates.

SARS-CoV-2 S pv (ct19) entry inhibitor screening

The HTS assay conditions were optimized to a seeding Caco-2 cell density of 2.4×10^4 cells and SARS-CoV-2 S pv (ct19) infective dose of MOI=5 per well in 96-well plates. Under these optimized conditions, the signal-to-basal (S/B) ratio, coefficient of variation, and Z' factor were 1286.09, 7.35%, and 0.705, respectively, making this assay promising for large-scale inhibitor screening. As shown in Fig. 2A, the HTS assay was performed by screening a library containing 1,037 botanical drugs. Compounds with inhibition of >80% and no apparent cytotoxicity at a concentration of 50 μ M were defined as prime candidates. Fifty-eight (5.59%) prime candidates (Fig. 2A and 2B) were selected. These prime candidates were then counter-screened to rule out the inhibition of VSV genome replication and RLuc activity. Four candidates (0.38%), angeloylgomisin O, schisandrin B, procyanidin, and oleanonic acid passed this secondary screen with mild inhibition against VSV (16.85%, 33.65%, 21.02%, and 32.41%, respectively) at 50 μ M (Fig. 2C).

Four hits inhibited SARS-CoV-2 S pv (ct19)

The dose-dependent inhibition of the four hits was further investigated using Caco-2 cells. As shown in Fig. 3, all the four hits suppressed SARS-CoV-2 S pv (ct19) infection in a dose-dependent manner. Angeloylgomisin O inhibited SARS-CoV-2 S pv (ct19) by 90% at 100 μ M and had little effect on VSV pv, demonstrating its specific inhibition of SARS-CoV-2 entry (Fig. 3A). Meanwhile, the cell viability was >80% when used at an extremely high

concentration (400 μ M). Schisandrin B inhibited SARS-CoV-2 S pv (ct19) by 90% at 100 μ M, whereas the cell viability was approximately 80% at 400 μ M (Fig. 3B). Procyanidin at 50 μ M and 100 μ M inhibited SARS-CoV-2 S pv (ct19) by approximately 80% and 95%, respectively. The cell viability was approximately 80% at 400 μ M (Fig. 3C). Oleanolic acid (50 μ M) inhibited SARS-CoV-2 S pv (ct19) by approximately 70% (Fig. 3D). While it is relatively toxic to Caco-2 cells with a 50% cytotoxic concentration (CC_{50}) of 53.88 μ M. To validate the antiviral effects, four hits were purchased from other commercial sources and tested. The antiviral effects were similar to those observed in our primary screening.

Four hits inhibited SARS-CoV-2 S (ct19)-mediated membrane fusion

To explore the antiviral mechanical properties of the four hits, the effects of the four hits on SARS-CoV-2 S-mediated membrane fusion were examined. Recent research has identified that SARS-CoV-2 induces syncytia formation in the lungs of patients with COVID-19 (22). It was reported that cells infected with SARS-CoV-2 exhibited a typical syncytium phenomenon (5). In this assay, fusion activity was conducted by co-transfecting SARS-CoV-2 S (ct19) and green fluorescent protein (GFP) into Vero E6 cells. After 24 h, the S protein of SARS-CoV-2 induced cell-cell fusion, resulting in the formation of syncytia (Fig. 4A). 25-hydrocholesterol (25HC) was used as the positive control (23, 24). As shown in Fig. 4A, 25 μ M of 25HC blocked syncytia formation. Likewise, all the four hits induced a dose-dependent reduction in syncytium size (Fig. 4B). Angeloylgomisin O blocked syncytia formation at a concentration of 6.25 μ M, while procyanidin, oleanonic acid, and schisandrin B required higher concentrations (12.5 μ M to 25 μ M) to achieve the inhibition (Fig. 4B). These results

suggested that angeloylgomisin O, schisandrin B, procyanidin, and oleanonic acid could inhibit SARS-CoV-2 entry by inhibiting membrane fusion.

Four botanical hits inhibited authentic SARS-CoV-2 infection

To evaluate the inhibition of four hits on authentic SARS-CoV-2, an immunofluorescence assay (IFA) was carried out using SARS-CoV-2 NP antibody. As shown in Fig. 5, all the four hits inhibited authentic SARS-CoV-2 in a dose-dependent manner. The IC_{50} values of angeloylgomisin O (3.727 μ M), schisandrin B (7.337 μ M), and oleanonic acid (1.414 μ M) were less than 10 μ M, While the IC_{50} value of procyanidin was 32.59 μ M. It seemed that angeloylgomisin O, schisandrin B, and oleanonic acid exerted stronger inhibition on the authentic SARS-CoV-2 than on the SARS-CoV-2 S pv (ct19).

The selective index (SI, CC_{50}/IC_{50}) was calculated using the authentic SARS-CoV-2 inhibition assay. The SI values of angeloylgomisin O and schisandrin were > 61.12 and > 27.25, respectively. Suggesting that both drugs are promising potential candidates against COVID-19.

Effects of the four hits against MERS-CoV-S pv and SARS-CoV-2 S variants pv

To verify the inhibitory effects of the four hits against other coronaviruses and the emergence of SARS-CoV-2 variants, we investigated the antiviral effect of the four hits on MERS-CoV S pv and SARS-CoV-2 S pv variants containing S protein mutations. As shown in Fig. 6A, all the four hits suppressed the entry of MERS-CoV S pv into Caco-2 cells in a dose-dependent manner. The IC_{50} values of angeloylgomisin O, schisandrin B, procyanidin, and oleanonic

acid against MERS-CoV S pv were 21.13 μ M, 18.99 μ M, 13.97 μ M, and 18.05 μ M, respectively. Indicating that their therapeutic potential against MERS-CoV. MERS-CoV employed the different receptor, dipeptidyl peptidase-4 (DPP4), from the SARS-CoV-2. It could be ruled out that four hits only interfere with ACE2 to block pseudovirus entry (25, 26). A SARS-CoV-2 S pv variant containing the S protein D614G substitution was constructed and used to evaluate its inhibitory effect. As shown in Fig. 6B, all the four hits suppressed the entry of SARS-CoV-2 S pv D614G into Caco-2 cells in a dose-dependent manner. The IC₅₀ values of angeloylgomisin O, schisandrin B, procyanidin, and oleanonic acid at which SARS-CoV-2 S pv D614G was inhibited were 16.35 μ M, 26.60 μ M, 33.36 μ M, and 25.21 μ M.

We introduced three additional amino acid substitutions, K417N, E484K, and N501Y, in the S D614G protein. These mutations have been observed in the South African variant, 501Y.V2, and play an essential role in immune escape (13-15). A substitution was constructed to evaluate the inhibitory effect. The results showed that all the four hits inhibited SARS-CoV-2 S pv K417N/E484K/N501Y/D614G in a dose-dependent manner (Fig. 6C). The IC₅₀ values of angeloylgomisin O, schisandrin B, procyanidin, and oleanonic acid against SARS-CoV-2 S pv K417N/E484K/N501Y/D614G were 23.76 μ M, 41.34 μ M, 26.62 μ M, and 35.18 μ M, respectively, indicating that the all the four hits may inhibit new variants such as 501Y.V2. Mutation of K417N/E484K/N501Y on the viral S proteins of 501.V2 variants renders a change in the RBD allowing additional interaction with human ACE2. All the four hits inhibited SARS-CoV-2 S variant with the similar effect. Further confirmed that four hits inhibited membrane fusion rather than interacted with RBD or ACE2 by inhibiting

SARS-CoV-2.

Combinatory treatments with the drug pairs remdesivir-angeloylgomisin O showed enhanced antiviral activity

As all the four hits could inhibit S-mediated membrane fusion, we assessed the efficacy of the combined treatment by using remdesivir, a SARS-CoV-2 viral RNA-dependent RNA polymerase inhibitor (27, 28), and four hits respectively. The degree of interaction was determined according to the bliss independence model using MacSynergy II software to analyze the results (29, 30). Volumes of statistically significant synergy were evaluated, and combination volumes (CV) of $> 25 \mu\text{M}^2\%$ were interpreted as evidence of synergy, and $> 100 \mu\text{M}^2\%$, as strong synergy (31).

Our results showed that strong synergy interactions occurred between remdesivir and angeloylgomisin O. Combination studies between remdesivir and angeloylgomisin O led to the CV of $158.27 \mu\text{M}^2\%$ (Fig. 7A). For combinations of remdesivir-angeloylgomisin O the maximal synergistic effect was seen at concentration of 0.032 to 0.8 μM remdesivir and 1.5625 to 6.25 μM angeloylgomisin O. Remdesivir and schisandrin B showed minor amount of synergy CV was $25.3 \mu\text{M}^2\%$ (Fig. 7A and 7C). Two-drug combinations of procyanidin-remdesivir and oleanonic acid-remdesivir had an additive antiviral inhibition profile (Fig. 7D and 7E).

DISCUSSION

In this study, we screened a botanical drug library containing 1,037 compounds and identified

four hits, including angeloylgomisin O, procyanidin, schisandrin B, and oleanonic acid, which blocked the entry of SARS-CoV-2 S (ct19) pv infection by inhibiting viral membrane fusion. Furthermore, the four hits inhibited the SARS-CoV-2 authentic virus at the micromolar level. The top two compounds angeloylgomisin O and schisandrin B inhibited authentic SARS-CoV-2 at a highly selective index (SI). All the four hits inhibited MERS-CoV S pv, SARS-CoV-2 S D614G pv, and SARS-CoV-2 S pv K417N/E484K/N501Y/D614G infection. Remdesivir-angeloylgomisin O drug combination showed stronger antiviral activities against SARS-CoV-2 than remdesivir monotherapy.

The top two hits, angeloylgomisin O and schisandrin B, were derived from *Schisandra chinensis*. Fruit from this plant and its extracts were used in traditional medicine in East Asia to treat liver disorders such as hepatitis. They also possess a broad spectrum of biological and pharmacological uses, including antiviral, anti-inflammatory, and antioxidative properties, without toxicity (32). Schisandrin B has been shown to inhibit cytochrome P450 3A (CYP3A) activity in rat liver and thus affects the metabolism of many drugs (33, 34). Furthermore, upon rat oral administration of *Schisandra chinensis* extract, schisandrin B has been reported to accumulate to a maximum plasma concentration (C_{max}) of 4.54 μ M (35). This value was similar to the IC_{50} value for schisandrin B in the present study, suggesting that this compound offers a biologically feasible treatment for SARS-CoV-2.

At the cellular level, CD4⁺T cells were rapidly activated to produce inflammatory cytokines after SARS-CoV-2 infection, which further induced CD14⁺ CD16⁺ monocyte activation with high levels of expression of interleukin 6 (IL-6). Thus, reduced IL-6 would potentially reduce immunopathology caused by SARS-CoV-2 (36, 37). Cai et al. demonstrated that

schisandrin B pretreatment mice at doses of 25, 50, and 75 mg/kg were shown to reduce lipopolysaccharide-induced acute lung injury with lowered the number of inflammatory cells and pro-inflammatory cytokines including tumor necrosis IL-6 in bronchoalveolar lavage fluid (38). This result suggested that schisandrin B could coordinate cytokine response to reduced host immune response, and schisandrin B might achieved a better therapeutic effect *in vivo*.

Procyanidin is a proanthocyanidin member that belongs to the class of flavonoids, the secondary metabolites of polyphenolic plants and fungi. Proanthocyanidins are also the most abundant polyphenolic compounds in lignin (39). Maroli et al. used molecular docking analyses to demonstrate that procyanidin can inhibit SARS-CoV-2 entry and replication (40). In this study, we confirmed that procyanidin inhibits SARS-CoV-2 infection in the entry step.

MATERIALS AND METHODS

Cells and viruses

HEK 293T, Vero E6, Caco-2, and BHK-21 cells were cultured in Dulbecco's modified Eagle medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). The SARS-CoV-2 strain used in this study is a clinical strain of SARS-CoV-2 (nCoV-2019BetaCoV/Wuhan/WIV04/2019). The pseudotype VSV bearing the S protein of SARS-CoV-2 S (GenBank QHD43416.1) and MERS-CoV S (GenBankNC_019843.3) were generated as previously reported (41, 42). Briefly, 293T cells transfected with pcDNA3.1-SARS-CoV-2 S (ct19), pcDNA3.1-SARS-CoV-2 S (wt), or pcDNA3.1-MERS-CoV S (ct16) for 48 h were infected with pseudotype VSV (described

below), in which the G gene was replaced with the luciferase gene, at an MOI of 0.1 for 2 h.

The culture supernatants were harvested 24 h later, centrifuged to remove cell debris, and

stored at -80°C . To generate VSVG pseudotype VSV, BHK-21 cells in 6-well plates were

infected with a recombinant vaccinia virus (vTF7-3) encoding T7 RNA polymerase at an

MOI of 5. After 45 min, the cells were transfected with 11 μg of mixed plasmids with a

5:3:5:8:1 ratio of pVSV Δ G-eGFP-GPC (pVSV Δ G-Rluc to generate pseudotype VSV),

pBS-N, pBS-P, pBS-G, and pBS-L. After 48 h, the supernatants were filtered to remove the

vaccinia virus and inoculated into BHK-21 cells that had been transfected with

pCAGGS-VSV G 24 h previously. The titer of the pseudotype virus was measured by

infecting BHK-21 cells previously transfected with pCAGGS-VSV G and determined by

plaque assay 24 hours post-infection (h.p.i). The titer of SARS-CoV-2 S pv(ct19) was 1.5×10^7

PFU/ml.

All point mutants (D614G, K417N/E484K/N501Y/D614G) were made from

pcDNA3.1-SARS-CoV-2 S (ct19) by using a fast mutagenesis system (Transgene Biotech),

and mutations were confirmed by sequencing (Sangon, Shanghai). We used the same method

to generate the SARS-CoV-2 S variants pv.

Viral copies assay

Equal volumes of SARS-CoV-2 S pv (ct19) and SARS-CoV-2 S pv (wt) were treated with

DNase and RNase. RNA from the viruses was extracted using Trizol (TaKaRa) and reverse

transcribed using the PrimeScriptTM RT reagent Kit (TaKaRa). Viral particles were quantified

via RT-qPCR using a specific primer pair to detect VSV Δ G-Rluc (primers

5'-GTAACGGACGAATGTCTCATAA-3' and 5'-TTTGACTCTCGCCTGATTGTAC -3').

Immunoblotting

Equal volumes of virus particles were precipitated with acetone and lysed using RIPA lysis buffer. Lysates were treated with loading buffer, subjected to SDS-PAGE, and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). SARS-CoV-2 S was detected using a rabbit anti-S2 subunit mouse monoclonal antibody (GeneTex, GTX632604;1:2000 dilution). Horseradish peroxidase-linked goat anti-mouse IgG antibody (Proteintech, CHI, USA, 1:5000).

HTS optimization and assaying of the botanical drug library

The appropriate cell density and the dose for SARS-CoV-2 S pv were selected by comparing the signal-to-basal ratio, the coefficients of variation, and Z' values under different conditions, as previously described. A library of 1,037 botanical compounds was purchased from Weikeqi Biotech (Sichuan, China). The compounds were collected and stored in 10 mM stock solutions in DMSO at -80 °C until use. The first round of HTS was carried out, and Caco-2 cells were seeded at a density of 2.4×10^4 cells per well in 96-well plates. After incubation overnight, cells were treated in duplicate with the compounds (50 μ M), and 1 h later, cells were infected with SARS-CoV-2 S pv (ct19) with the MOI=5, and the supernatant was removed 1 h.p.i. Camostat 100 μ M and 0.5% DMSO were used as positive and negative controls, respectively. After 24 h, luciferase activity was measured using the Rluc assay system (Promega, Madison, WI, USA). The primary compounds were then secondarily screened using VSV pv (MOI of 0.5) to rule out VSV genome replication inhibitors and Rluc activity.

Cell viability

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to evaluate the effect of the four hits on cell viability. Hits at the indicated concentrations were added to pre-seeded Caco-2 cells in 96-well plates. Twenty-four hours later, the cell viability was measured using the MTT kit.

Membrane fusion assay

Vero E6 cells were co-transfected with pcDNA3.1-SARS-CoV-2S (ct19) (0.25 µg) and pEGFP-N1 (0.25 µg) by using lipo2000 in 24-well plates. After transfection for 4 h, the medium was replaced with 2% DMEM containing different concentrations of the hits. After 24 h, syncytium formation was visualized using an M-shot image system (Micro-shot Technology, China)

Antiviral effect of four hits against SARS-CoV-2

Caco-2 cells were seeded at a density of 2.4×10^4 cells per well in 96-well plates. After overnight incubation, cell monolayers were treated in duplicate with the hits at a final concentration of 1 h and infected with SARS-CoV-2 at an MOI of 0.5. After an additional 24 h of incubation, the infection was stopped by rinsing each well, and the cells were fixed with 4% paraformaldehyde. Cells were permeabilized using PBS with 0.2% Triton X-100 for 15 min and blocked with 5% FBS (Gibco), followed by treatment with the primary antibody anti-SARS-CoV-2 NP (GeneTex GTX635678, USA) at a 1:500 dilution overnight at 4°C. After six rinses with PBS, the cells were stained with DyLight 488-labeled antibody against rabbit IgG (KPL, Gaithersburg, MD, USA). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA). Nine fields per well were imaged, and the percentages of infected and DAPI-positive cells were calculated

using Harmony 3.5 software.

Drug-drug interactions of remdesivir with hits

Caco-2 cells were seeded at a density of 2.4×10^4 cells per well in 96-well plates. After overnight incubation, different final concentrations of remdesivir were added to each row of the 96-well plate. Simultaneously, different final concentrations of angeloylgomisin O were added to each column of the plate. The cells were infected with SARS-CoV-2 at an MOI of 0.5. After an additional 24 h of incubation, the infection was stopped by rinsing each well, and the cells were fixed with 4% paraformaldehyde. The antiviral activities were determined using the IFA assay. To determine the drug-drug interactions, differential surface plots at the 95% confidence level (CI) were generated using MacSynergy II according to the Bliss independence model. The combinatorial effect was determined by subtracting the theoretical additive values from the experimental values (29, 30). The data sets were interpreted using the following scale: volumes of synergy or antagonism $> 100 \mu\text{M}^2\%$, strong synergy; 50–100 $\mu\text{M}^2\%$, moderate synergy; 25–50 $\mu\text{M}^2\%$, minor amount of synergy; 0–25 $\mu\text{M}^2\%$, additive (31).

ACKNOWLEDGMENT

We thank Tao Du and Jin Xiong from the BSL-3 Laboratory of the Wuhan Institute of Virology for their critical support. We thank the Center for Instrumental Analysis and Metrology, Core Facility, and Technical Support, Wuhan Institute of Virology, for providing technical assistance.

This work was supported by the National Key Research and Development Program of China (2018YFA0507204), the National Natural Sciences Foundation of China (31670165), Wuhan

333 National Biosafety Laboratory, Chinese Academy of Sciences Advanced Customer
334 Cultivation Project (2019ACCP-MS03), and the Open Research Fund Program of the State
335 Key Laboratory of Virology of China (2018IOV001).

336

337 REFERENCE

- 338 1. Hu B, Guo H, Zhou P, Shi ZL. 2021. Characteristics of SARS-CoV-2 and COVID-19.
339 Nat Rev Microbiol 19:141-154.
- 340 2. Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichsen S,
341 Schiergens TS, Herrler G, Wu NH, Nitsche A, Muller MA, Drosten C, Pohlmann S.
342 2020. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by
343 a Clinically Proven Protease Inhibitor. Cell 181:271-280 e8.
- 344 3. Cagliani R, Forni D, Clerici M, Sironi M. 2020. Computational Inference of Selection
345 Underlying the Evolution of the Novel Coronavirus, Severe Acute Respiratory
346 Syndrome Coronavirus 2. J Virol 94.
- 347 4. Bosch BJ, Martina BE, Van Der Zee R, Lepault J, Haijema BJ, Versluis C, Heck AJ,
348 De Groot R, Osterhaus AD, Rottier PJ. 2004. Severe acute respiratory syndrome
349 coronavirus (SARS-CoV) infection inhibition using spike protein heptad
350 repeat-derived peptides. Proc Natl Acad Sci U S A 101:8455-60.
- 351 5. Xia S, Liu M, Wang C, Xu W, Lan Q, Feng S, Qi F, Bao L, Du L, Liu S, Qin C, Sun F,
352 Shi Z, Zhu Y, Jiang S, Lu L. 2020. Inhibition of SARS-CoV-2 (previously
353 2019-nCoV) infection by a highly potent pan-coronavirus fusion inhibitor targeting
354 its spike protein that harbors a high capacity to mediate membrane fusion. Cell Res
355 30:343-355.
- 356 6. Nie J, Li Q, Wu J, Zhao C, Hao H, Liu H, Zhang L, Nie L, Qin H, Wang M, Lu Q, Li
357 X, Sun Q, Liu J, Fan C, Huang W, Xu M, Wang Y. 2020. Establishment and
358 validation of a pseudovirus neutralization assay for SARS-CoV-2. Emerg Microbes
359 Infect 9:680-686.
- 360 7. Plante JA, Mitchell BM, Plante KS, Debbink K, Weaver SC, Menachery VD. 2021.
361 The variant gambit: COVID-19's next move. Cell Host Microbe 29:508-515.
- 362 8. Tang JW, Tambyah PA, Hui DS. 2021. Emergence of a new SARS-CoV-2 variant in
363 the UK. J Infect 82:e27-e28.
- 364 9. Radhakrishnan C, Divakar MK, Jain A, Viswanathan P, Bhoyar RC, Jolly B, Imran M,
365 Sharma D, Rophina M, Ranjan G, Sehgal P, Jose BP, Raman RV, Kesavan TN,
366 George K, Mathew S, Poovullathil JK, Keeriyatt Govindan SK, Nair PR,
367 Vadakkandiyil S, Gladson V, Mohan M, Parambath FC, Mangla M, Shamnath A,
368 Sivasubbu S, Scaria V. 2021. Initial Insights Into the Genetic Epidemiology of
369 SARS-CoV-2 Isolates From Kerala Suggest Local Spread From Limited
370 Introductions. Frontiers in genetics 12:630542.
- 371 10. Plante JA, Liu Y, Liu J, Xia H, Johnson BA, Lokugamage KG, Zhang X, Muruato AE,

- 372 Zou J, Fontes-Garfias CR, Mirchandani D, Schariton D, Bilello JP, Ku Z, An Z,
373 Kalveram B, Freiberg AN, Menachery VD, Xie X, Plante KS, Weaver SC, Shi PY.
374 2021. Spike mutation D614G alters SARS-CoV-2 fitness. *Nature* 592:116-121.
- 375 11. Hou YJ, Chiba S, Halfmann P, Ehre C, Kuroda M, Dinno KH, 3rd, Leist SR, Schafer
376 A, Nakajima N, Takahashi K, Lee RE, Mascenik TM, Graham R, Edwards CE, Tse
377 LV, Okuda K, Markmann AJ, Bartelt L, de Silva A, Margolis DM, Boucher RC,
378 Randell SH, Suzuki T, Gralinski LE, Kawaoka Y, Baric RS. 2020. SARS-CoV-2
379 D614G variant exhibits efficient replication ex vivo and transmission in vivo. *Science*
380 370:1464-1468.
- 381 12. Korber B, Fischer WM, Gnanakaran S, Yoon H, Theiler J, Abfalterer W, Hengartner
382 N, Giorgi EE, Bhattacharya T, Foley B, Hastie KM, Parker MD, Partridge DG, Evans
383 CM, Freeman TM, de Silva TI, Sheffield C-GG, McDanal C, Perez LG, Tang H,
384 Moon-Walker A, Whelan SP, LaBranche CC, Saphire EO, Montefiori DC. 2020.
385 Tracking Changes in SARS-CoV-2 Spike: Evidence that D614G Increases Infectivity
386 of the COVID-19 Virus. *Cell* 182:812-827 e19.
- 387 13. Li Q, Nie J, Wu J, Zhang L, Ding R, Wang H, Zhang Y, Li T, Liu S, Zhang M, Zhao C,
388 Liu H, Nie L, Qin H, Wang M, Lu Q, Li X, Liu J, Liang H, Shi Y, Shen Y, Xie L,
389 Zhang L, Qu X, Xu W, Huang W, Wang Y. 2021. SARS-CoV-2 501Y.V2 variants lack
390 higher infectivity but do have immune escape. *Cell* 184:2362-2371 e9.
- 391 14. Hu J, Peng P, Wang K, Fang L, Luo FY, Jin AS, Liu BZ, Tang N, Huang AL. 2021.
392 Emerging SARS-CoV-2 variants reduce neutralization sensitivity to convalescent sera
393 and monoclonal antibodies. *Cell Mol Immunol* 18:1061-1063.
- 394 15. Hoffmann M, Arora P, Gross R, Seidel A, Hornich BF, Hahn AS, Kruger N, Graichen
395 L, Hofmann-Winkler H, Kempf A, Winkler MS, Schulz S, Jack HM, Jahrsdorfer B,
396 Schrezenmeier H, Muller M, Kleger A, Munch J, Pohlmann S. 2021. SARS-CoV-2
397 variants B.1.351 and P.1 escape from neutralizing antibodies. *Cell* 184:2384-2393
398 e12.
- 399 16. Cowan MM. 1999. Plant products as antimicrobial agents. *Clin Microbiol Rev*
400 12:564-82.
- 401 17. Dias DA, Urban S, Roessner U. 2012. A historical overview of natural products in
402 drug discovery. *Metabolites* 2:303-36.
- 403 18. Fukushi S, Watanabe R, Taguchi F. 2008. Pseudotyped vesicular stomatitis virus for
404 analysis of virus entry mediated by SARS coronavirus spike proteins. *Methods Mol*
405 *Biol* 454:331-8.
- 406 19. Schmidt F, Weisblum Y, Muecksch F, Hoffmann HH, Michailidis E, Lorenzi JCC,
407 Mendoza P, Rutkowska M, Bednarski E, Gaebler C, Agudelo M, Cho A, Wang Z,
408 Gazumyan A, Cipolla M, Caskey M, Robbani DF, Nussenzweig MC, Rice CM,
409 Hatzioannou T, Bieniasz PD. 2020. Measuring SARS-CoV-2 neutralizing antibody
410 activity using pseudotyped and chimeric viruses. *J Exp Med* 217.
- 411 20. Hu J, Gao Q, He C, Huang A, Tang N, Wang K. 2020. Development of cell-based
412 pseudovirus entry assay to identify potential viral entry inhibitors and neutralizing
413 antibodies against SARS-CoV-2. *Genes Dis* 7:551-557.
- 414 21. Case JB, Rothlauf PW, Chen RE, Liu Z, Zhao H, Kim AS, Bloyet LM, Zeng Q, Tahan
415 S, Droit L, Ilagan MXG, Tartell MA, Amarasinghe G, Henderson JP, Miersch S,

- 416 Ustav M, Sidhu S, Virgin HW, Wang D, Ding S, Corti D, Theel ES, Fremont DH,
417 Diamond MS, Whelan SPJ. 2020. Neutralizing Antibody and Soluble ACE2
418 Inhibition of a Replication-Competent VSV-SARS-CoV-2 and a Clinical Isolate of
419 SARS-CoV-2. *Cell Host Microbe* 28:475-485 e5.
- 420 22. Braga L, Ali H, Secco I, Chiavacci E, Neves G, Goldhill D, Penn R,
421 Jimenez-Guardeno JM, Ortega-Prieto AM, Bussani R, Cannata A, Rizzari G, Collesi
422 C, Schneider E, Arosio D, Shah AM, Barclay WS, Malim MH, Burrone J, Giacca M.
423 2021. Drugs that inhibit TMEM16 proteins block SARS-CoV-2 spike-induced
424 syncytia. *Nature* doi:10.1038/s41586-021-03491-6.
- 425 23. Wang S, Li W, Hui H, Tiwari SK, Zhang Q, Croker BA, Rawlings S, Smith D, Carlin
426 AF, Rana TM. 2020. Cholesterol 25-Hydroxylase inhibits SARS-CoV-2 and other
427 coronaviruses by depleting membrane cholesterol. *EMBO J* 39:e106057.
- 428 24. Zang R, Case JB, Yutuc E, Ma X, Shen S, Gomez Castro MF, Liu Z, Zeng Q, Zhao H,
429 Son J, Rothlauf PW, Kreutzberger AJB, Hou G, Zhang H, Bose S, Wang X, Vahey
430 MD, Mani K, Griffiths WJ, Kirchhausen T, Fremont DH, Guo H, Diwan A, Wang Y,
431 Diamond MS, Whelan SPJ, Ding S. 2020. Cholesterol 25-hydroxylase suppresses
432 SARS-CoV-2 replication by blocking membrane fusion. *Proc Natl Acad Sci U S A*
433 117:32105-32113.
- 434 25. Wang N, Shi X, Jiang L, Zhang S, Wang D, Tong P, Guo D, Fu L, Cui Y, Liu X,
435 Arledge KC, Chen Y-H, Zhang L, Wang X. 2013. Structure of MERS-CoV spike
436 receptor-binding domain complexed with human receptor DPP4. *Cell research*
437 23:986-993.
- 438 26. Lu G, Hu Y, Wang Q, Qi J, Gao F, Li Y, Zhang Y, Zhang W, Yuan Y, Bao J, Zhang B,
439 Shi Y, Yan J, Gao GF. 2013. Molecular basis of binding between novel human
440 coronavirus MERS-CoV and its receptor CD26. *Nature* 500:227-231.
- 441 27. Gordon CJ, Tchesnokov EP, Woolner E, Perry JK, Feng JY, Porter DP, Gotte M. 2020.
442 Remdesivir is a direct-acting antiviral that inhibits RNA-dependent RNA polymerase
443 from severe acute respiratory syndrome coronavirus 2 with high potency. *J Biol*
444 *Chem* 295:6785-6797.
- 445 28. Kokic G, Hillen HS, Tegunov D, Dienemann C, Seitz F, Schmitzova J, Farnung L,
446 Siewert A, Höbartner C, Cramer P. 2021. Mechanism of SARS-CoV-2 polymerase
447 stalling by remdesivir. *Nature communications* 12:279.
- 448 29. Prichard MN, Shipman C, Jr. 1990. A three-dimensional model to analyze drug-drug
449 interactions. *Antiviral Res* 14:181-205.
- 450 30. Prichard MN, Prichard LE, Baguley WA, Nassiri MR, Shipman C, Jr. 1991.
451 Three-dimensional analysis of the synergistic cytotoxicity of ganciclovir and
452 zidovudine. *Antimicrob Agents Chemother* 35:1060-5.
- 453 31. Fenwick C, Amad M, Bailey MD, Bethell R, Bos M, Bonneau P, Cordingley M,
454 Coulombe R, Duan J, Edwards P, Fader LD, Faucher AM, Garneau M, Jakalian A,
455 Kawai S, Lamorte L, LaPlante S, Luo L, Mason S, Poupart MA, Rioux N, Schroeder
456 P, Simoneau B, Tremblay S, Tsantrizos Y, Witvrouw M, Yoakim C. 2014. Preclinical
457 profile of BI 224436, a novel HIV-1 non-catalytic-site integrase inhibitor. *Antimicrob*
458 *Agents Chemother* 58:3233-44.
- 459 32. Azzam HS, Goertz C, Fritts M, Jonas WB. 2007. Natural products and chronic

- 460 hepatitis C virus. *Liver Int* 27:17-25.
- 461 33. Li W-L, Xin H-W, Yu A-R, Wu X-C. 2013. In vivo effect of Schisandrin B on
462 cytochrome P450 enzyme activity. *Phytomedicine : international journal of*
463 *phytotherapy and phytopharmacology* 20:760-765.
- 464 34. Li WL, Xin HW, Su MW, Xiong L. 2010. Inhibitory effects of schisandrin A and
465 schisandrin B on CYP3A activity. *Methods and findings in experimental and clinical*
466 *pharmacology* 32:163-169.
- 467 35. Xiao-tong W, Fan H, Xiao-ling S. 2017. Pharmacokinetics Study of Seven Lignans in
468 Alzheimer's Rats. *International Journal of Pharmacology* 14:68-75.
- 469 36. Li H, Zhou Y, Zhang M, Wang H, Zhao Q, Liu J. 2020. Updated Approaches against
470 SARS-CoV-2. *Antimicrob Agents Chemother* 64.
- 471 37. Zhou Y, Fu B, Zheng X, Wang D, Zhao C, qi Y, Sun R, Tian Z, Xu X, Wei H. 2020.
472 Aberrant pathogenic GM-CSF+ T cells and inflammatory CD14+CD16+monocytes
473 in severe pulmonary syndrome patients of a new coronavirus. *bioRxiv*
474 doi:10.1101/2020.02.12.945576:2020.02.12.945576.
- 475 38. Cai Z, Liu J, Bian H, Cai J, Zhu G. 2016. Suppression of P2X7/NF- κ B pathways by
476 Schisandrin B contributes to attenuation of lipopolysaccharide-induced inflammatory
477 responses in acute lung injury. *Archives of pharmacal research* 39:499-507.
- 478 39. Koyama K, Ikeda H, Poudel PR, Goto-Yamamoto N. 2012. Light quality affects
479 flavonoid biosynthesis in young berries of Cabernet Sauvignon grape.
480 *Phytochemistry* 78:54-64.
- 481 40. Maroli N, Bhasuran B, Natarajan J, Kolandaivel P. 2020. The potential role of
482 procyanidin as a therapeutic agent against SARS-CoV-2: a text mining, molecular
483 docking and molecular dynamics simulation approach. *J Biomol Struct Dyn*
484 doi:10.1080/07391102.2020.1823887:1-16.
- 485 41. Liu Y, Guo J, Cao J, Zhang G, Jia X, Wang P, Xiao G, Wang W. 2021. Screening of
486 Botanical Drugs against Lassa Virus Entry. *J Virol* doi:10.1128/JVI.02429-20.
- 487 42. Wang P, Liu Y, Zhang G, Wang S, Guo J, Cao J, Jia X, Zhang L, Xiao G, Wang W.
488 2018. Screening and Identification of Lassa Virus Entry Inhibitors from an
489 FDA-Approved Drug Library. *J Virol* 92.

490

491

492 **Figure legends**

493 Fig. 1. Construction of SARS-CoV-2 S pv (ct19). (A) The left-axis shows the RLU (relative
494 light unit) value detected 24 h after pseudovirus SARS-CoV-2 S pv (ct19) and SARS-CoV-2
495 S pv (wt) infection. The right y-axis shows virus copy number assays of both viral particles.
496 (B) Immunoblots verifying the incorporation of SARS-CoV-2 spike protein in the

pseudovirus. The cell control was the culture supernatant of 293T cell transfected with pcDNA3.1 48-h and infected with VSV pv.

Fig. 2. HTS for inhibitors of SARS-CoV-2 entry from a botanical drug library. (A) HTS assay flowchart. (B) HTS of a library of 1,037 natural extracts for primary candidates inhibiting SARS-CoV-2 S pv (ct19) infection. Each dot represents the percent inhibition achieved by each compound at a concentration of 50 μ M. (C) Inhibition of 58 candidates against SARS-CoV-2 S pv entry >80%. The second screen of the 58 selected compounds. VSV pv was used as a control to exclude compounds targeting the backbone. The hits depicted in red box showed mild inhibition of VSV pv infection and were designated hits.

Fig. 3. Dose-response curves and the structures of the four hits: angeloylgomisin O (A), schisandrin B (B), procyanidin (C), oleanonic acid (D) for inhibiting SARS-CoV-2 S pv (ct19) infection. (Right) The structure of each hit. Caco-2 cells were seeded at a density of 2.4×10^4 cells per well in 96-well plates. After overnight incubation, cell monolayers were treated in duplicate with the hits at a final concentration; 1 h later, cells were infected with different pv, and the supernatant was removed 1 h post infection. Cell viability was evaluated using MTT assay. Hits at the indicated concentrations was added to pre-seeded Caco-2 cells in 96-well plates. Twenty-four hours later, cell viability was measured. Data are represented as the mean $\pm$ standard deviation (SD) from three to four experiments.

Fig. 4. Four hits inhibited SARS-CoV-2 S (ct19)-mediated membrane fusion. (A)

SARS-CoV-2 S (ct19)-mediated Cell-cell fusion on Vero E6 cells. (Left) Vero E6 cells that were co-transfected with SARS-CoV-2 S (ct19) and GFP plasmid. (Middle) Cells were transfected with GFP. (Right) Cells were co-transfected with SARS-CoV-2 S (ct19), GFP and treated with 25 μ M 25HC. (B) Four hits inhibited SARS-CoV-2 S (ct19)-mediated membrane fusion in dose-dependent manner. Vero E6 cells were co-transfected with SARS-CoV-2 S (ct19) and GFP later treated with the four hits in different concentrations. Syncytium formation was visualized 24–36 h later using fluorescent microscopy. Images are representative fields from three independent experiments.

Fig. 5. The four hits inhibit authentic SARS-CoV-2 infection. IFA analysis and images of angeloylgomisin O (A and B), schisandrin B (C and D), procyanidin (E and F), and oleanonic acid (G and H) inhibition of SARS-CoV-2 infection in Caco-2 cells. Caco-2 cells were seeded at 96-well plates. After overnight incubation, cell monolayers were treated in duplicate with the hits at a final concentration of 1 h and infected with SARS-CoV-2 at an MOI of 0.5, followed by incubation for 24 h. IFA images showing the viral NP (green) and cell nuclei (blue) are displayed for Caco-2 cells. Cells were treated with different concentrations of angeloylgomisin O (B), schisandrin B (D), procyanidin (F), and oleanonic acid (H). (I) CC_{50} , IC_{50} , and SI values of four hits. Data were presented as means $\pm$ SD from three independent experiments.

Fig. 6. Antiviral activity of the four hits against MERS-CoV S pv (A), SARS-CoV-2 S pv D614G mutant (B), and SARS-CoV-2 S pvK417N/E484K/N501Y/D614G mutant (C). The

541 IC₅₀ of four hits for inhibition against the variants MERS-CoV-S pv, SARS-CoV-2 S pv
 542 D614G and SARS-CoV-2 S pv K417N/E484K/N501Y/D614G (D). Caco-2 cells were seeded
 543 at a density of 2.4×10^4 cells per well in 96-well plates. After overnight incubation, cells were
 544 treated in duplicate with the hits at a final concentration; 1 h later, cells were infected with
 545 MERS-CoV-S pv, SARS-CoV-2 S pv D614G, and SARS-CoV-2 S pv
 546 K417N/E484K/N501Y/D614G with the MOI=5. Data were presented as means±SD from
 547 three independent experiments.

548

549 Fig. 7. Combinatory treatments with the drug pairs remdesivir-angeloylgomisin O and
 550 remdesivir-schisandrin B show enhanced antiviral activity (A) Synergy volumes for pairwise
 551 combination studies performed between remdesivir and each hit. Three-dimensional plot of
 552 synergy and antagonism at 99% confidence for the pairwise combination of remdesivir with
 553 angeloylgomisin O (B), schisandrin B (C), procyanidin (D), oleanonic acid (E). Analysis was
 554 performed with MacSynergy II software.

A

B

A

B

C

A

angeloylgomisin O

C

schisandrin B

B

procyanidin

D

oleanonic acid

● SARS-CoV-2 S pv (ct19) □ Caco-2 cell viability □ VSV pv

A

SARS-CoV-2 S	+	-	+
25HC	-	-	25 μ M
pEGFP-N1	+	+	+

B

6.25 12.5 25 (μ M)

angeloylgomisin O

schisandrin B

procyanidin

oleanonic acid

	CC ₅₀	SARS-CoV-2 IC ₅₀	SI
angeloylgomisin O	>200 μ M	3.272 μ M	>61.12
schisandrin B	>200 μ M	7.337 μ M	>27.25
procyanidin	>200 μ M	32.95 μ M	>6.06
oleanonic acid	53.88 μ M	1.414 μ M	38.10

D

	IC_{50} (μM)		
	MERS-CoV S pv	SARS-CoV-2 S pv D614G	SARS-CoV-2 S pv K417N/E484K/N501Y/D614G
angeloylgomisin O	21.13	16.35	23.76
schisandrin B	18.99	26.60	41.34
procyanidin	13.97	33.36	26.62
oleanonic acid	18.05	25.21	35.18

A

hits combination with remdesivir	synergy volume (μM^2 %)	combination effect
angeloylgomisin O	158.27	strong synergy
schisandrin B	25.3	minor amount of synergy
procyanidin	0	additive
oleanonic acid	0	additive

B

C

D

E