Anti-SARS-CoV-2 Potential of Artemisinins In Vitro

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ABSTRACT: The discovery of novel drug candidates with anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) potential is critical for the control of the global COVID-19 pandemic. Artemisinin, an old antimalarial drug derived from Chinese herbs, has saved millions of lives. Artemisinins are a cluster of artemisinin-related drugs developed for the treatment of malaria and have been reported to have multiple pharmacological activities, including anticancer, antiviral, and immune modulation. Considering the reported broad-spectrum antiviral potential of artemisinins, researchers are interested in whether they could be used to combat COVID-19. We systematically evaluated the anti-SARS-CoV-2 activities of nine artemisinin-related compounds in vitro and carried out a time-of-drug-addition assay to explore their antiviral mode of action. Finally, a pharmacokinetic prediction model was established to predict the therapeutic potential of selected compounds against COVID-19. Arteannuin B showed the highest anti-SARS-CoV-2 potential with an EC50 of 10.28 ± 1.12 μM. Artesunate and dihydroartemisinin showed similar EC50 values of 12.98 ± 5.30 μM and 13.31 ± 1.24 μM, respectively, which could be clinically achieved in plasma after intravenous administration. Interestingly, although an EC50 of 23.17 ± 3.22 μM was not prominent among the tested compounds, lumefantrine showed therapeutic promise due to high plasma and lung drug concentrations after multiple dosing. Further mode of action analysis revealed that arteannuin B and lumefantrine acted at the post-entry step of SARS-CoV-2 infection. This research highlights the anti-SARS-CoV-2 potential of artemisinins and provides leading candidates for anti-SARS-CoV-2 drug research and development.

KEYWORDS: artemisinin, SARS-CoV-2, COVID-19, antiviral drug, drug repurposing

The COVID-19 pandemic caused by severe acute respiratory coronavirus 2 (SARS-CoV-2) has taken a heavy toll on public health and the global economy. As of July 18, 2020, 13.9 million confirmed cases including 593,087 deaths have been reported worldwide since the pathogen was first identified in January 2020.1,2 Unfortunately, there are currently no specific and effective antiviral drugs available to treat a large number of infected patients. Chloroquine, hydroxychloroquine, remdesivir, and lopinavir/ritonavir were highlighted as repurposed drugs to treat COVID-19. However, according to the COVID-19 Treatment Guidelines released by the NIH in April 21, 2020, there are insufficient clinical data to recommend either for or against the use of chloroquine, hydroxychloroquine, and remdesivir for the treatment of COVID-19, and the use of lopinavir/ritonavir or other HIV protease inhibitors was no longer recommended.3 Although a series of Food and Drug Administration (FDA)-approved drugs that are capable of inhibiting SARS-CoV-2 in vitro were reported, the discovery of more drug candidates with anti-SARS-CoV-2 potential is urgently needed to fuel antiviral drug research for COVID-19.

Received: July 21, 2020
Published: July 31, 2020
Previously, we reported that chloroquine, a decades-old antimalarial drug with immune-modulation activities, and its derivative hydroxychloroquine could efficiently inhibit SARS-CoV-2 in vitro. This raises an interesting question of whether other antimalarial drugs also have anti-SARS-CoV-2 potential. Artemisinins comprise another series of well-known antimalarials with immune-modulatory activities. Among the reported artemisinins, artemisinin, dihydroartemisinin, artemether-lumefantrine, artesunate, arteether, and artemisone are approved drugs derived from artemisinin. Arteannuin B and artemisinic acid are artemisinin derivatives reported to have therapeutic efficacy against malaria in vivo. Previous studies have reported the broad-spectrum antiviral potential of artemisinins. For example, artesunate effectively inhibits a wide range of DNA and RNA viruses, including human cytomegalovirus (HCMV), human herpes simplex virus (HSV), hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), and polyomavirus BK. Clinical trials focusing on the antiviral efficacy of artemisone suggested that it shows promise for the treatment of patients with HCMV and HSV-2 infection. Dihydroartemisinin also showed inhibitory effects on viruses such as HCMV and Zika virus. In addition, artemisone was proven to be a potent inhibitor of HCMV and had synergistic antiviral activity in combination with other approved and experimental anti-HCMV agents. Considering the broad-spectrum antiviral effects of artemisinins, it is necessary to systematically explore the anti-SARS-CoV-2 potential of artemisinins, which consists of multiple FDA-approved drugs and drug candidates at the late stage of pharmacological development, and predict their therapeutic efficacy based on a physiologically based pharmacokinetic (PBPK) modeling.

**RESULTS**

**Artemisinins Inhibit SARS-CoV-2 In Vitro.** In this study, nine artemisinins (Figure 1) were chosen to test their anti-SARS-CoV-2 potential using African green monkey kidney Vero E6 cells. Cytotoxicity assays were carried out before the antiviral assay to determine the cytotoxicity of the selected compounds, and viral RNA copies in the supernatants were determined by quantitative real-time PCR (qRT-PCR) to determine the antiviral effects of the compounds. The results showed that the half-cytotoxic concentrations (CC$_{50}$) of arteether, artemether, artemisone, artemisinin, and artemisone were greater than 200 μM. However, the half-maximal effective concentrations (EC$_{50}$) were 31.86 ± 4.72 μM, 73.80 ± 26.91 μM, >100 μM, 64.45 ± 2.58 μM, and 49.64 ± 1.85 μM, respectively for these compounds, indicating suboptimal selective indexes (SIs) (Figure 2). The EC$_{50}$ of dihydroartemisinin...
Artemisinins were 13.31 ± 1.24 μM and the SI was 2.38 ± 0.22. Notably, artesunate, which was reported to have broad-spectrum antiviral potential against multiple medical viruses, showed an ideal EC50 value of 12.98 ± 5.30 μM against SARS-CoV-2 virus, and its SI was 5.10 ± 2.08. For arteannuin B, the EC50 against SARS-CoV-2 was 10.28 ± 1.12 μM, and a CC50 of 71.13 ± 2.50 μM led to an optimal SI of 7.00 ± 0.76 among all artemisinins tested. Interestingly, for lumefantrine, another antimalarial drug that is structurally distinct from artemisinins and is a major component of the compound preparation “coartem”, the EC50 against SARS-CoV-2 was 23.17 ± 3.22 μM, and its SI was greater than 4.40 ± 0.61.

Artemisinins Reduce the Production of SARS-CoV-2 Protein.

To provide more direct evidence of the inhibitory effect of artemisinins, an immunofluorescence assay (IFA) was performed. SARS-CoV-2 nucleoprotein (NP) was stained with a specific antibody and detected with a secondary antibody with a fluorescence label. Inhibition of fluorescence was observed in a dose-dependent manner for several artemisinins, as shown in Figure 3. The expression of viral NP protein was completely inhibited when arteannuin B was added at 25 μM, and most viral NP protein was inhibited when artesunate, dihydroartemisinin, and lumefantrine were added at 25 μM, 25 μM, and 100 μM, respectively. The IFA results were consistent with the viral yield based on qRT-PCR analysis (Figure 4A).

Arteannuin B and Lumefantrine Block SARS-CoV-2 Infection at the Post-entry Level.

To explore the antiviral mechanism of the selected drugs, the time-of-drug-addition assays were performed for arteannuin B and lumefantrine, which were selected as representatives for different core structure types (Figure 1). Cells were treated with 25 μM arteannuin B or 100 μM lumefantrine at different steps of infection (full-time, entry, and post-entry), which was followed by qRT-PCR, IFA, and Western blot assays to determine the overall virus replication efficiency. For arteannuin B, addition of the compounds at the entry step failed to inhibit the extracellular viral RNA production and intracellular viral protein expression, but significant inhibition of viral RNA (Figure 4A) and viral protein (Figure 4B,C) was observed when the drug was added at the post-entry step. Similarly, lumefantrine showed inhibitory effects when added during the full-time infection process or post-entry stage, but not during virus entry (Figure 4A,D,E). These data revealed that arteannuin B and lumefantrine might function at a similar stage by interfering with the intracellular events of the SARS-CoV-2 infection cycle, which requires further investigation.

Physiologically Based Pharmacokinetic Modeling and In Vitro to In Vivo Extrapolation (IVIVE) for Lumefantrine.

The IVIVE could be estimated for most artemisinins due to the known pharmacokinetic profiles; however, there are limited data on the pharmacokinetics of lumefantrine. We thus carried out PBPK modeling and IVIVE for lumefantrine. Due to the low hepatic clearance and negligible renal excretion of lumefantrine, the prolonged half-life of up to 6 days in healthy volunteers led to a cumulative effect after multidose administration.25

https://dx.doi.org/10.1021/acsinfecdis.0c00522
ACS Infect. Dis. XXXX, XXX, XXX–XXX
after six oral doses of 480 mg over 3 days, the EC\textsubscript{50} of lumefantrine was reached both in plasma and in the lungs. These results suggest the potential of lumefantrine as a potential anti-SARS-CoV-2 agent.

**DISCUSSION**

During the fight against the COVID-19 pandemic, drug repurposing has been highlighted, as the known safety and pharmacokinetic profiles of repurposed drugs indicate that they are more likely to be applied in a timely manner compared to new drugs. Antimalarial drugs such as chloroquine, quinines, and artemisinins have long histories of clinical application and have been reported to have broad-spectrum antiviral potential in recent years. Chloroquine is effective against influenza virus, dengue virus, and SARS-CoV-2 in vitro and has recently been proven to be clinically effective against HCV.\textsuperscript{22} Quinines were reported to have antiviral effects against dengue virus and HSV-1.\textsuperscript{23,24} Artesunate is a structural derivative of artemisinin characterized by its broad-spectrum antiviral potential against DNA and RNA viruses.\textsuperscript{14} In this study, we systematically evaluated the antiviral potential of artemisinins against SARS-CoV-2 in vitro and discovered that artemisinins could inhibit SARS-CoV-2 replication in a dose-dependent manner. Arteannuin B is another artemisinin derivative that had an ideal EC\textsubscript{50} value, suggesting its anti-SARS-CoV-2 effect in vitro. Interestingly, we found that the antimalarial drug lumefantrine, which is structurally distant from artemisinins and is a major component of an approved drug coartem, could inhibit SARS-CoV-2 in vitro with an EC\textsubscript{50} of 23.17 ± 3.22 \( \mu \text{M} \).

For the emergency use of repurposed drugs, the pharmacokinetic profile is an important reference for estimating clinical efficacy. The C\textsubscript{max} of artesunate was 42 \( \mu \text{M} \) following a single intravenous injection dose of 120 mg, which is greater than the EC\textsubscript{50} of 13.31 ± 1.24 \( \mu \text{M} \) (the in vitro metabolite of artesunate was dihydroartemisinin) against SARS-CoV-2, indicating that artesunate is a potential countermeasure against COVID-19.\textsuperscript{25} Coartem is a pharmaceutical compound preparation composed of artemether–lumefantrine (20 mg artemether and 120 mg lumefantrine per tablet). The C\textsubscript{max} of artemether was found to be low (0.28 \( \mu \text{M} \)); however, the C\textsubscript{max} of lumefantrine was much higher. Moreover, the plasma half-life of lumefantrine was determined to be 119 h, and the long half-life caused drug accumulation, which might lead to enhanced plasma and tissue drug concentrations.\textsuperscript{26}

Figure 3. Immunofluorescence images of virus infection upon treatment with indicated antivirals. Virus infection and drug treatment were performed as mentioned previously herein. The nuclei (blue) were stained with Hoechst dye. The viral NP protein (green) was stained with rabbit serum against NP, followed by incubation with the secondary antibody, specifically Alexa 488-labeled goat anti-rabbit.
Indeed, based on the PBPK model of lumefantrine, the plasma and the lung concentrations could exceed 23.17 μM (12.26 μg/mL) after six oral doses of 480 mg over 3 days, which would exceed its EC₅₀ value against SARS-CoV-2. Arteannuin B showed anti-SARS-CoV-2 potential with an EC₅₀ of 10.28 ± 1.12 μM, and its unique core structure provides information for the future optimization of artemisinins as anti-SARS-CoV-2 agents.

Artemisinins, especially artesunate and its active metabolite dihydroartemisinin, have been shown to have antiviral potential in the present and previous studies. Accumulating studies have suggested that artesunate is likely to impair viral infection by modulating host cell metabolic pathways. In particular, the anti-HCMV efficacy of artesunate is associated with the PI3-K/Akt/p70S6K signaling pathway. Artesunate was also found to interact directly or indirectly with cellular DNA-binding factors such as NF-xB or Sp1, leading to the...
inhibition of viral replication.

For coartem and arteannuin B, although there are some studies on their antiviral efficacy, our research has demonstrated their promising therapeutic advantages for the treatment of SARS-CoV-2 infection in vitro. Notably, synergistic effects of artemisinins and conventional antiviral drugs were observed in antiviral research, including HCMV, HBV, and bovine viral diarrhea virus. Facing the global outbreak of SARS-CoV-2, the combination of artemisinins and other antiviral drugs with different mechanisms, such as remdesivir and favipiravir, might be a promising clinical option.

In summary, we systematically explored the antiviral activities of artemisinins against SARS-CoV-2 in vitro. Artesunate, arteannuin B, and lumefantrine showed promise as anti-SARS-CoV-2 agents in vitro. Combined with the safety and potential immunoregulatory activities of artemisinins, we believe that artemisinin might represent a potential medical countermeasure against COVID-19.

**METHODS**

**Cells and Virus.** Vero E6 cells (ATCC, no. 1586) were grown and maintained in minimum Eagle’s medium (Gibco Invitrogen) supplemented with 10% fetal bovine serum (Gibco Invitrogen) at 37 °C in 5% CO₂. The SARS-CoV-2 strain (nCoV-2019BetaCoV/Wuhan/WIV04/2019) was propagated, stored, and titrated as previously described. All studies on infectious viruses were performed in a biosafety level-3 (BLS-3) laboratory.

**Cytotoxicity and Antiviral Assays.** Cytotoxicity was evaluated in Vero E6 cells using a cell counting kit-8 (CCK8) (Beyotime, China) according to the manufacturer’s instructions. For the antiviral assay, 4.8 × 10⁶ Vero E6 cells were seeded onto 48-well cell-culture Petri dishes and grown overnight. After pretreatment with a gradient of diluted experimental compounds for 1 h at 37 °C, cells were infected with virus at an MOI of 0.01 for 1 h. After incubation, the inoculum was removed, cells were washed with PBS, and culture vessels were replenished with fresh drug-containing medium. At 24 h postinfection, total RNA was extracted from the supernatant and qRT-PCR was performed to quantify the virus yield as described previously.

**Immunofluorescence Assay.** The IFA was performed according to the previous method with modifications. Briefly, Vero E6 cells were inoculated in 48-well cell-culture Petri dishes and grown overnight. After pretreatment with a gradient of diluted experimental compounds for 1 h at 37 °C, cells were infected with virus at an MOI of 0.01 for 1 h. After incubation, the inoculum was removed, cells were washed with PBS, and culture vessels were replenished with fresh drug-containing medium. At 24 h postinfection, cells were washed with PBS, fixed with 4% (w/v) paraformaldehyde, and permeabilized with 0.2% (v/v, in PBS) triton X-100. After blocking with 5% (m/v, in PBS) bovine serum albumin at 37 °C for 1 h, the cells were further incubated with the primary antibody, rabbit serum against NP (anti-NP antibody, 1:1000), followed by incubation with the secondary antibody, Alexa 488-labeled goat anti-rabbit (1:500; Abcam). The nucleus was stained with Hoechst 33258 (Beyotime, China). Immunofluorescence images were obtained using a fluorescence microscope.

**Time-of-Drug-Addition Assay.** The time-of-drug-addition assay was performed according to a previous description. Briefly, Vero E6 cells were seeded at 1 × 10⁵ cells/well and incubated overnight. Twenty-five micromolar arteannuin B, 100 µM lumefantrine, or DMSO was added at the indicated time points. At 16 h postinfection, the viral NP protein in the infected cells was detected by IFA and Western blotting. IFA was performed as described previously herein. Rabbit serum against NP and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000; Proteintech, China) were used as primary and secondary antibodies, respectively, for Western blotting.

**Physiologically Based Pharmacokinetic Modeling and Simulations.** PBPK simulations were performed using the Simcyp simulator (version 18, release 2, Simcyp Limited, Sheffield, U.K.) run on a Lenovo computer platform with an Intel Core i5 processor. All simulations were carried out using
the virtual clinical trials composed of the prevalidated built-in “Healthy Volunteer” population groups. The parameters and methods of PBPK modeling and simulations are available in the Supporting Information.

Data and Statistical Analysis. The data and statistical analysis in this study compiled with the recommendations on experimental design and analysis in pharmacology. The data are presented as the mean ± SEM. Statistical analyses between two groups were performed using the unpaired Student’s t test. Differences among groups were assessed by one-way analysis of variance with the Bonferroni post hoc test. In all cases, a value of p < 0.05 was considered statistically significant.

Materials. Artemisinin (CAS No. 63968-64-9), artemether (CAS No. 71963-77-4), artesunate (CAS No. 88495-63-0), dihydroartemisinin (CAS No. 71939-50-9), artemisinic acid (CAS No. 80286-58-4), arteether (CAS No. 75887-54-6), and lumefantrine (CAS No. 82186-77-4) were purchased from Selleck. Arteannuin B (CAS No. 50906-56-4) and artemisone (CAS No. 255730-18-8) were purchased from MedChemExpress. All compounds were dissolved in DMSO for subsequent experiments.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00522.

Detailed methods and results of PBPK modeling (PDF)

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Author Contributions
R.C., H.H., and Y.L. contributed equally. W.Z., M.W., Z.H., and R.C. conceived the overall study and designed the experiments. H.H., Y.L., X.W., M.X., J.L., H.Z., Y.Y., L.Z., W.L., T.Z., D.X., X.G., Y.L., and J.Y. performed most of the biological and functional experiments and analyzed the data. R.C., M.W., and Z.H. wrote and edited the manuscript. All authors have made important comments regarding the manuscript.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was supported by grants from the National Science and Technology Major Projects (2018ZX09711003) and the National Key Research and Development Project (2020YFC0841700). We thank Jia Wu, Hao Tang, and Jun Liu from BSL-3 Laboratory from the Core Faculty of Wuhan Institute of Virology for their critical support.

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