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Abstract

Background: Medicines for the treatment of 2019-nCoV infections are urgently needed. However, drug screening using live 2019-nCoV requires high-level biosafety facilities, which imposes an obstacle for those institutions without such facilities or 2019-nCoV. This study aims to repurpose the clinically approved drugs for the treatment of coronavirus disease 2019 (COVID-19) in a 2019-nCoV-related coronavirus model.

Methods: A 2019-nCoV-related pangolin coronavirus GX_P2V/pangolin/2017/Guangxi was described. Whether GX_P2V uses angiotensin-converting enzyme 2 (ACE2) as the cell receptor was investigated by using small interfering RNA (siRNA)-mediated silencing of ACE2. The pangolin coronavirus model was used to identify drug candidates for treating 2019-nCoV infection. Two libraries of 2406 clinically approved drugs were screened for their ability to inhibit cytopathic effects on Vero E6 cells by GX_P2V infection. The anti-viral activities and anti-viral mechanisms of potential drugs were further investigated. Viral yields of RNAs and infectious particles were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) and plaque assay, respectively.

Results: The spike protein of coronavirus GX_P2V shares 92.2% amino acid identity with that of 2019-nCoV isolate Wuhan-hu-1, and uses ACE2 as the receptor for infection just like 2019-nCoV. Three drugs, including cepharanthine (CEP), selamectin, and mefloquine hydrochloride, exhibited complete inhibition of cytopathic effects in cell culture at 10 μmol/L. CEP demonstrated the most potent inhibition of GX_P2V infection, with a concentration for 50% of maximal effect (EC50) of 0.98 μmol/L. The viral RNA yield in cells treated with 10 μmol/L CEP was 15,393-fold lower than in cells without CEP treatment (1.48 ± 0.02) × 10⁻³ vs. 1.00 ± 0.12, t = 150.38, P < 0.001) at 72 h post-infection (p.i.). Plaque assays found no production of live viruses in media containing 10 μmol/L CEP at 48 h p.i. Furthermore, we found CEP had potent anti-viral activities against both viral entry (0.46 ± 0.12, vs. 1.00 ± 0.37, t = 2.42, P < 0.05) and viral replication ([6.18 ± 0.95] × 10⁻⁴ vs. 1.00 ± 0.43, t = 3.98, P < 0.05).

Conclusions: Our pangolin coronavirus GX_P2V is a workable model for 2019-nCoV research. CEP, selamectin, and mefloquine hydrochloride are potential drugs for treating 2019-nCoV infection. Our results strongly suggest that CEP is a wide-spectrum inhibitor of pan-beta-coronavirus, and further study of CEP for treatment of 2019-nCoV infection is warranted.

Keywords: Coronavirus disease 2019; 2019-Novel coronavirus; Cepharanthine; Selamectin; Mefloquine hydrochloride

Introduction

The coronavirus disease 2019 (COVID-19), which is caused by 2019 novel coronavirus (2019-nCoV), imposes a grand immediate challenge for global public health.[1,2] In the major affected area, the mainland of China, the death toll and the number of confirmed cases are still growing. There is an urgent need for effective vaccines and specific therapies for the prevention and treatment of 2019-nCoV infection.

A coronavirus closely related to 2019-nCoV was identified in a sample collected from Rhinolophus affinis bat in Yunnan in 2013, suggesting bats are likely the reservoirs of 2019-nCoV.[3] Recently, the searching of reservoirs or intermediate hosts of 2019-nCoV turned to pangolins. Xiao et al.[4] reported the isolation and characterization of a 2019-nCoV-like coronavirus from pangolins (Manis javanica). Similarly, in October 2019, a viral metagenomic study of pangolins identified severe acute respiratory syn-
drome-coronavirus (SARS-CoV)-related sequences, which can be re-identified as 2019-nCoV-related sequences. Moreover, we also reported the isolation and identification of 2019-nCoV-related coronaviruses (2019-nCoVr) in pangolins seized in anti-smuggling operations in southern China. Altogether, pangolins are likely a reservoir or an intermediate host of 2019-nCoV.

Due to its pathogenicity and transmissibility of 2019-nCoV, working with live 2019-nCoV requires high-level biocontainment facilities, which impedes the urgent need for drug screening. Without prior experience of therapy, the current treatment of 2019-nCoV infection is mainly empirical and symptomatic, and a limited number of therapeutics in ongoing clinical trial were adopted from previous research on SARS-CoV and Middle East respiratory syndrome-coronavirus (MERS-CoV), which are only remotely related with 2019-nCoV. With low or no pathogenicity in humans and close genetic relationship with 2019-nCoV, our pangolin coronavirus provides an ideal alternative model for 2019-nCoV research. Our reasoning of this isolate having low or no pathogenicity in humans was based on the fact that, back in 2017, no suspected infections were found in those having close contacts with pangolins; and our pangolin coronavirus isolate was routinely cultured in biosafety level 2 facilities. Here we present a screening of clinically approved drugs for anti-coronavirus activity in this 2019-nCoVr model, and identify the potent inhibitors for pangolin coronavirus infection.

Methods

Cell lines, coronavirus, and key reagents

Vero E6 cells (American Type Culture Collection, Manassas, VA, USA) were grown in high-glucose-containing Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum. 2019-nCoVr GX_P2V/pangolin/2017/Guangxi was isolated in Vero E6 cells from a dead smuggled pangolin in 2017, and its complete genome has been submitted to GenBank. A library of 2080 approved drugs (catalog No. L1000) and a library of 326 anti-virus compounds (catalog No. L1700) produced by TargetMol were purchased from Topscience (Shanghai, China). Oligonucleotides used in the study can be found in Supplementary Table 1, http://links.lww.com/CM9/A216.

Plaque assay for determining virus titer

Confluent monolayer Vero E6 cells were infected with serially ten-fold diluted 2019-nCoVr, at a range of 10^-1 to 10^-6. At 2 h post-infection (p.i.), the virus was removed and the cells were washed twice with phosphate-buffered saline. And then 3 mL 1% agarose overlay was added to each well to prevent cross-contamination. At 3 days p.i. or 5 days p.i., cells were fixed with 4% paraformaldehyde for 1 h at room temperature. Then the upper semi-solid agarose medium was removed. Fixed cells were stained with crystal violet for 10 min, and rinsed with water gently for several times. The number of plaques was counted and virus titers were calculated.

small interfering RNA (siRNA)-mediated silencing of angiotensin-converting enzyme 2 (ACE2)

Vero E6 cells were transfected with 0.8, 4, and 20 nmol/L angiotensin-converting enzyme 2-specific siRNApool (siACE2) or negative control siRNA (siNC) by using the RNAiMax transfection reagent (Invitrogen, Carlsbad, CA, USA) as previously described. At 48 h post-transfection (p.t.), cells were infected with cell culture-grown 2019-nCoVr at a multiplicity of infection (MOI) of approximately 10. At 24 h p.i., 2019-nCoVr-infected cells were lysed in lysis buffer, and the messenger RNA (mRNA) levels of ACE2, 2019-nCoVr and β-actin were determined by quantitative real-time polymerase chain reaction (qRT-PCR).

Drug screening for 2019-nCoV using approved drug library

Vero E6 cells were plated in 96 well plates at a density of 5000 cells/well. Cells were treated with 2019-nCoVr (MOI = 0.01) and different chemical drugs of the approved drug library with the final concentration of 10 μmol/L. At 2 h p.i., 2019-nCoVr and drugs were removed, and fresh culture medium containing 10 μmol/L drugs were added to each well. At 72 h p.i., the cytopathic effect (CPE) was observed using microscopy (Nikon, Cat:TS100 and TS2-S-5M, Tokyo, Japan). And the cells in the wells without obvious CPE were further analyzed.

Viral RNA extraction and real-time polymerase chain reaction (qRT-PCR)

Cell culture supernatants and Vero E6 cells were harvested for RNA extraction using the AxyPrep™ body fluid viral DNA/RNA Miniprep kit (Axygene, Cat No. AP-MN-BF-VNA-250, Hangzhou, Zhejiang, China) and AxyPrep™ multisource total RNA Miniprep kit (Axygene, Cat No. AP-MN-MS-RNA-250G) according to the manufacturer’s instructions. Reverse transcription was performed with a Hifair II 1st Strand cDNA synthesis kit with gDNA digester (Yeasen Biotech, Cat:111211ES60, Shanghai, China), and qRT-PCR was performed using QuantStudio 1 Real-Time PCR detection system (Applied Biosystems, Foster City, CA, USA) with Hieff qPCRSYBR Green Master Mix (Yeasen Biotech, Cat:11202ES08, Shanghai, China) or two-step Taqman probe assay. The sequence information of primers used is listed in Supplementary Table 1, http://links.lww.com/CM9/A216. And the PCR products were inserted into T vector (Ruibo Xingke Biotech, Beijing, China) to generate the standard plasmid after sequencing confirmation. The standard curve was generated by determination of copy numbers from serially dilutions (10^3 – 10^9 copies) of the plasmid. qRT-PCR amplification of SYBR Green method was performed as follows: 95°C for 5 min followed by 40 cycles consisting of 95°C for 10 s, 55°C for 20 s, and 72°C for 31 s. And the Taqman method was performed as follows: 50°C for 2 min, 95°C for 10 min followed by 40 cycles consisting of 95°C for 10 s, 60°C for 1 min.

Time-of-addition experiment of cepharanthine

The cepharanthine (CEP; 10 μmol/L) was used for the time of addition experiment. Vero E6 cells (5 x 10^4 cells/well)
were plated in 12 well plates and treated with CEP at different stages of virus infection. The “Full time” treatment, “Entry” treatment, and “Post-entry” treatment experiments were performed according to a previous study. And the cytotoxicity of CEP to Vero E6 cells was measured by cell titer blue according to the manufacturer’s protocol (Promega, Catalog Number: PR-G8081; Madison, WI, USA).

**Statistical analysis**

The data were analyzed using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA) and presented as the mean ± standard deviation (normal distribution of data was checked by Kolmogorov-Smirnov test). Comparisons between the two groups were analyzed using the Student’s t tests. A P value of <0.05 was considered statistically significant.

**Results**

**Pangolin coronavirus isolate GX_P2V is as an alternative model for 2019-nCoV research**

We reported the identification of 2019-nCoVr, which were composed of a lineage of 2019-nCoV and related viruses. Our GX_P2V isolate is hitherto one of the few 2019-nCoVr cultured from wildlife. Its spike protein shares 92.2% amino acid identity with the spike protein of 2019-nCoV isolate Wuhan-hu-1. Their receptor binding motifs of the 2019-nCoV-related viruses. It is noted, however, that having a possible receptor of ACE2 does not correlate to viral pathogenicity. In fact, no human infection relating to our pangolin CoV was identified or suspected, suggesting that CoV GX_P2V is nonpathogenic in humans. Altogether, the close relationship to 2019-nCoV, the shared receptor and non-pathogenicity, support that pangolin CoV GX_P2V can be used as an accessible *in vitro* model for developing therapies against 2019-nCoV.

**Three drugs are potent inhibitors of 2019-nCoVr infection**

In our 2019-nCoVr and Vero E6 cell model, we first screened a total of 2406 drugs and compounds for their inhibitory effects on viral infection-dependent CPE in 96 well plates [Figure 2A]. Each drug or compound was added to 10 μmol/L at the starting time point of infection and all drugs were tested in duplicate. At 72 h p.i., cells were observed under phase microscopy. Infected cells without any drug treatment showed typical CPE-cell rounding with no obvious lysis. Importantly, three drugs, CEP, selamectin, and mefloquine hydrochloride, exhibited complete inhibition of CPE in infected cells [Supplementary Figure 1, http://links.lww.com/CM9/A217]. The viral RNA level in the infected cells treated with 10 μmol/L CEP was 15,393-fold lower than that in infected cells without drug treatment ([6.48 ± 0.02] × 10⁻⁴ vs. 1.00 ± 0.12, t = 150.38, P < 0.001) [Figure 2B]. Viral RNA quantification indicated that viral replication in other two drug-treated cells was also dramatically inhibited [Supplementary Figure 2, http://links.lww.com/CM9/A218].

Among the three drug candidates, CEP is of particular attention due to its profound anti-viral activity and previous reports of its inhibitory effects on both SARS-CoV and HCoV-OC43.[12,13] It can inhibit 2019-nCoVr at a low concentration (concentration for 50% of maximal effect [EC₅₀] = 0.98 μmol/L; cytotoxicity concentration 50% [CC₅₀] = 39.30 μmol/L; selectivity index = 39.91) [Figure 3A]. We next investigated this drug’s anti-viral mechanism by conducting viral entry, post-entry, and full-time assays in 12 well plates [Figure 3B]. In the viral entry assay, cells were incubated with media containing both viruses and CEP during the first 2 h of infection, then were washed with phosphate-buffered saline and were supplemented with media containing no drugs. In the viral post-entry assay, cells were incubated in media containing no CEP during the first 2 h of infection, then were supplemented with media containing CEP. In the viral full-time assay, cells were constantly incubated in media containing CEP. Viral RNA yields were determined by qRT-PCR at 48 h p.i. Compared to normal infections without drug treatment, the viral RNA yields in the entry, post-entry and full-time assays were 2.17-fold (0.46 ± 0.12 vs. 1.00 ± 0.37, t = 2.42, P < 0.01), 1618-fold ([6.18 ± 0.95] × 10⁻⁴ vs. 1.00 ± 0.43, t = 3.98, P < 0.001), and 12,459-fold ([4.58 ± 1.27] × 10⁻⁵ vs. 1.00 ± 0.43, t = 4.03, P < 0.001) lower, respectively. Further plaque assays found no production of live viruses in the media containing 10 μmol/L CEP at 48 h p.i. [Figure 3C]. Thus, our data suggest that CEP can potently inhibit coronavirus infection at viral entry and post-entry.

**Discussion**

Here we first described a 2019-nCoVr model for research of 2019-nCoV. This model is suitable for work at biosafety level-2. We then identified three clinically approved drugs (CEP, selamectin, and mefloquine hydrochloride) that can inhibit a 2019-nCoVr infection, and suggested that these drugs be considered for further investigation in the treatment of 2019-nCoV infection.
Our finding of CEP as a potential drug for 2019-nCoV is especially instructive. This drug is an anti-inflammatory and anti-neoplastic alkaloid and is approved for leukopenia. It has multiple functions, such as inhibiting the efflux transporter ABCC10 of anti-tumor drugs,\cite{14} inhibiting the entry of human immunodeficiency virus type 1 (HIV-1) by reducing plasma membrane fluidity,\cite{15} and binding to central portion of heat shock protein 90.\cite{16} Importantly, as a naturally occurring plant alkaloid with more than 40 years of clinic use, CEP has low toxicity in animals and has no significant side effects in humans.\cite{17,18} Given the observed strong inhibition of virus replication and the drug’s established role of anti-inflammatory response, we think CEP is a promising candidate for the treatment of 2019-nCoV infection.

Nonetheless, our finding of CEP and mefloquine as anti-2019-nCoV agents was in agreement with previous studies in other coronaviruses of the genus Betacoronavirus. Two groups reported CEP as a drug candidate for SARS-CoV and HCoV-OC43, respectively.\cite{12,13} Mefloquine, which is approved for malaria, was found to have anti-viral activity against both MERS-CoV and SARS-CoV.\cite{19} Furthermore, we identified a previously
unknown anti-CoV compound, selamectin, which is marketed as a topical broad-spectrum parasiticide in cats and dogs to control fleas, heartworms, hookworms, roundworms, etc. The anti-viral mechanisms of these three drugs are unknown. We speculate that CEP and mefloquine are likely to target host cell pathways while selamectin might be a 2019-nCoVr-specific inhibitor.
The libraries of drugs used in this study contain 2406 compounds in total. Many of them have anti-viral activities against MERS-CoV and SARS-CoV. Clearly, our finding of only three inhibitors of 2019-nCoVr is not a comprehensive answer of all potential inhibitors in our libraries, as our goal is to find drugs that have the most potent anti-viral activities and we did the initial screening of virus inhibition by observing the existence of intact cell monolayers, not by quantitative methods.

In conclusion, this is the first report of a 2019-nCoVr model. We suggest the three drugs (CEP, selamectin, and melofloxacin hydrochloride) be considered for further investigation to treat the 2019-nCoV infection. Due to its amenable nature, our 2019-nCoVr model could play a more important role in the development of therapies and vaccines against 2019-nCoV. With high homology to 2019-nCoV, this 2019-nCoV isolate might be a potential live vaccine candidate. Cultured long before the outbreak of 2019-nCoV, our 2019-nCoVr isolate might play a significant role in the combat against COVID-19. Thus, our model in part reflects the importance of sustained coronavirus surveillance in wildlife.

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Conflicts of interest
None.

References