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Repurposing of clinically approved drugs for treatment of coronavirus disease 2019 in a 2019-novel coronavirus (2019-nCoV) related coronavirus model

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Abstract

Background: Medicines for the treatment of 2019-novel coronavirus (2019-nCoV) infections are urgently needed. However, drug screening using live 2019-nCoV requires high-level biosafety facilities, which imposes an obstacle for those without such facilities or 2019-novel coronavirus (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_P2X 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_P2X infection. The antiviral activities and antiviral 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-cepharanthine (CEP), selamectin and mefloquine hydrochloride exhibited complete inhibition of cytopathic effects in cell culture at 10 $\mu\text{mol/L}$. CEP demonstrated the most potent inhibition of GX_P2V infection, with a concentration for 50% of maximal effect [EC₅₀] of 0.98 $\mu\text{mol/L}$. The viral RNA yield in cells treated with 10 $\mu\text{mol/L}$ CEP was 15,393-fold lower than in cells without CEP treatment ($[6.48 \pm 0.02] \times 10^{-4}$ 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

$\mu\text{mol/L}$ CEP at 48 h p.i. Furthermore, we found CEP has potent antiviral activities against both viral entry (1.00 ± 0.37 vs. 0.46 ± 0.12 , $t=2.42$, $P<0.05$) and viral replication (1.00 ± 0.43 vs. $[6.18\pm 0.95]\times 10^{-4}$, $t=3.98$, $P<0.05$).

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-betacoronavirus, and clinical trial of CEP for treatment of 2019-nCoV infection is warranted.

Keywords: Coronavirus disease 2019; 2019-novel coronavirus (2019-nCoV); Cepharanthine; Selamectin; Mefloquine hydrochloride

Introduction

The coronavirus disease 2019 (COVID-19), which is caused by 2019 novel coronavirus (2019-nCoV) and was first detected in Wuhan, China in December 2019, imposes a grand immediate challenge for global public health.^[1,2] So far, COVID-19 has spread to many other countries with thousands of infected cases. 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 syndrome-coronavirus (SARS-CoV) related sequences,^[5] which can be re-identified as 2019-nCoV related sequences.^[6] Moreover, we also reported the isolation and identification of 2019-nCoV related coronaviruses in pangolins seized in anti-smuggling operations in southern China.^[7] Altogether, pangolins are likely a reservoir or an intermediate host of 2019-nCoV.^[5]

Due to its pathogenicity and transmissibility of 2019-nCoV, working with live 2019-nCoV requires high-level biocontainment facilities, which impedes the urgent needs 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 with 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 human 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-nCoV related coronavirus 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 (FBS). 2019-nCoV related coronavirus (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.^[7] 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 10-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 (PBS). And then 3 mL 1% agarose overlay was added to each well to prevent cross-contamination. At 3 d p.i. or 5 d 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.

siRNA-mediated silencing of ACE2

Vero E6 cells were transfected with 0.8, 4 and 20 nmol/L angiotensin-converting enzyme 2 (ACE2)-specific siRNApool (siACE2) or negative control siRNA (siNC) by using the RNAiMax transfection reagent (Invitrogen, Carlsbad, CA, USA) as previously described.^[8] 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-nCoVr using approved drug library

Vero E6 cells were plated in 96 well plates at a density of 2.5×10^4 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 μ mol/L. At 2 h p.i., 2019-nCoVr and drugs were removed, and fresh culture medium containing 10 μ mol/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-SM, Tokyo, Japan). And the cells in the wells without obvious CPE were further analyzed.

Viral RNA extraction and qRT-PCR

Cell culture supernatants and Vero E6 cells were harvested for RNA extraction using the AxyPrep™ Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, 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:11121ES60, 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 (CEP)

The CEP (10 μmol/L) was used for the time of addition experiment. Vero E6 cells (5×10^4

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.^[9] 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 \pm 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-nCoV related coronaviruses, which were composed of a lineage of 2019-nCoV and coronaviruses found in eight pangolin samples and three bat samples.^[7] Our GX_P2V isolate is hitherto one of the few 2019-nCoV related coronaviruses cultured from wildlife. Its spike protein shares 92.2% amino acid identity with the spike protein of 2019-nCoV isolate Wuhan-hu-1. Their major differences are in the S1 domain, especially the receptor binding domain (RBD). Alignment of the receptor binding motifs of the 2019-nCoV related lineage revealed a surprising diversity in the five critical residues for postulated binding between coronavirus RBD and human ACE2 protein [Figure 1A]. Compared to the five critical residues in SARS-CoV, two in GX_P2V and four in 2019-nCoV are replaced with different

residues.

Angiotensin converting enzyme II (ACE2) is the cell receptor for both SARS-CoV, bat SARS-like CoV, and 2019-nCoV.^[3,10,11] We next tested whether ACE2 is involved in pangolin CoV GX_P2V infection by using siRNA-mediated knockdown of ACE2 expression. In Vero E6 cells treated with 0.8 nmol/L, 4 nmol/L and 20 nmol/L concentrations of ACE2-targeting siRNAs, the yields of ACE2 mRNA and viral RNA are all significantly reduced, suggesting that ACE2 expression was knocked down by siRNA [Figure 1B] and ACE2 is also a receptor of 2019-nCoVr GX_P2V [Figure 1C]. It appears that ACE2 is a conserved receptor of both SARS-CoV and 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 infections relating to our pangolin CoV was identified or suspected, suggesting that CoV GX_P2V is nonpathogenic in human. Altogether, the close relationship to 2019-nCoV, the shared receptor and non-pathogenicity, support that pangolin CoV GX_P2X 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 μ mol/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 $\mu\text{mol/L}$ CEP was 15,393-fold lower than that in infected cells without drug treatment (Figure 2B, 1.00 ± 0.12 vs. $[6.48\pm 0.02]\times 10^{-4}$, $t=150.38$, $P<0.001$). Viral RNA quantification indicated that viral replication in other two drugs-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 antiviral 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 $[\text{EC}_{50}] = 0.98$ $\mu\text{mol/L}$; cytotoxicity concentration 50% $[\text{CC}_{50}] = 39.3$ $\mu\text{mol/L}$; selectivity index $[\text{SI}] = 39.91$) [Figure 3A]. We next investigated this drug's antiviral 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 PBS 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 2.18-fold (1.00 ± 0.37 vs. 0.46 ± 0.12 , $t=2.42$, $P<0.05$), 1618-fold (1.00 ± 0.43 vs. $[6.18\pm 0.95]\times 10^{-4}$, $t=3.98$, $P<0.05$) and 12,459-fold (1.00 ± 0.43 vs. $4.58\pm 1.27\times 10^{-5}$, $t=4.03$, $P<0.05$) lower, respectively. Further plaque assays found no production of live viruses in the media containing 10 $\mu\text{mol/L}$ 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-nCoV related coronavirus 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-nCoV related coronavirus infection, and suggest that these drugs be considered for clinical trials in 2019-nCoV patients.

Our finding of CEP as a potential drug for 2019-nCoV is especially instructive. This drug is an anti-inflammatory and antineoplastic alkaloid and is approved for leukopenia. It has multiple functions, such as inhibiting the efflux transporter ABCB1 of antitumor drugs,^[14] inhibiting the entry of human immunodeficiency virus type 1 (HIV-1) by reducing plasma membrane fluidity,^[15] and binding to central portion of Hsp90.^[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 human.^[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 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.^[12,13] Mefloquine, which

is approved for malaria, was found to have antiviral activity against both MERS-CoV and SARS-CoV.^[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 antiviral 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 antiviral activities against MERS-CoV and SARS-CoV.^[19] 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 antiviral 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-nCoV related coronavirus model. We suggest the three drugs (CEP, selamectin and mefloquine hydrochloride) be considered for trials in 2019-nCoV infection patients. 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-nCoVr isolate could 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.

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References

1. Gorbalenya AE. Severe acute respiratory syndrome-related coronavirus: The species and its viruses, a statement of the Coronavirus Study Group. *bioRxiv* 2020; 2020.2002.2007.937862. doi: 10.1101/2020.02.07.937862.
2. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. A new coronavirus associated with human respiratory disease in China. *Nature* 2020;doi: 10.1038/s41586-020-2008-3.
3. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 2020;doi: 10.1038/s41586-020-2012-7.
4. Xiao K, Zhai J, Feng Y, Zhou N, Zhang X, Zou J-J, et al. Isolation and Characterization of 2019-nCoV-like Coronavirus from Malayan Pangolins. *bioRxiv* 2020; 2020.2002.2017.951335. doi: 10.1101/2020.02.17.951335.
5. Liu P, Chen W, Chen JP. Viral Metagenomics Revealed Sendai Virus and Coronavirus Infection of Malayan Pangolins (*Manis javanica*). *Viruses* 2019;11:doi: 10.3390/v11110979.
6. Liu P, Jiang J-Z, Hua Y, Wang X, Hou F, Wan X-F, et al. Are pangolins the intermediate host of the 2019 novel coronavirus (2019-nCoV) ? *bioRxiv* 2020; 2020.2002.2018.954628. doi: 10.1101/2020.02.18.954628.
7. Lam TT-Y, Shum MH-H, Zhu H-C, Tong Y-G, Ni X-B, Liao Y-S, et al. Identification of 2019-nCoV related coronaviruses in Malayan pangolins in southern China. *bioRxiv* 2020; 2020.2002.2013.945485. doi: 10.1101/2020.02.13.945485.
8. Fan H, Qiao L, Kang KD, Fan J, Wei W, Luo G. Attachment and Postattachment Receptors Important for Hepatitis C Virus Infection and Cell-to-Cell Transmission. *J Virol*

2017;91:doi: 10.1128/JVI.00280-17.

9. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res* 2020;doi: 10.1038/s41422-020-0282-0.
10. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 2003;426:450-454. doi: 10.1038/nature02145.
11. Ge XY, Li JL, Yang XL, Chmura AA, Zhu G, Epstein JH, et al. Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature* 2013;503:535-538. doi: 10.1038/nature12711.
12. Zhang CH, Wang YF, Liu XJ, Lu JH, Qian CW, Wan ZY, et al. Antiviral activity of cepharanthine against severe acute respiratory syndrome coronavirus in vitro. *Chin Med J (Engl)* 2005;118:493-496.
13. Kim DE, Min JS, Jang MS, Lee JY, Shin YS, Song JH, et al. Natural Bis-Benzylisoquinoline Alkaloids-Tetrandrine, Fangchinoline, and Cepharanthine, Inhibit Human Coronavirus OC43 Infection of MRC-5 Human Lung Cells. *Biomolecules* 2019;9:doi: 10.3390/biom9110696.
14. Kathawala RJ, Wang YJ, Ashby CR Jr, Chen ZS. Recent advances regarding the role of ABC subfamily C member 10 (ABCC10) in the efflux of antitumor drugs. *Chin J Cancer* 2014;33:223-230. doi: 10.5732/cjc.013.10122.
15. Matsuda K, Hattori S, Komizu Y, Kariya R, Ueoka R, Okada S. Cepharanthine inhibited HIV-1 cell-cell transmission and cell-free infection via modification of cell membrane fluidity. *Bioorg Med Chem Lett* 2014;24:2115-2117. doi: 10.1016/j.bmcl.2014.03.041.

16. Haginaka J, Kitabatake T, Hirose I, Matsunaga H, Moaddel R. Interaction of cepharanthine with immobilized heat shock protein 90 α (Hsp90 α) and screening of Hsp90 α inhibitors. *Anal Biochem* 2013;434:202-206. doi: 10.1016/j.ab.2012.11.010.
17. Liu X. Studies on the immune evaluation of inactivated SARS-CoV experimental vaccine and the screening of antiviral drugs in vitro. Jinan: Jinan University; 2004.
18. Rogosnitzky M, Danks R. Therapeutic potential of the biscochlorine alkaloid, cepharanthine, for a range of clinical conditions. *Pharmacol Rep* 2011;63:337-347. doi: 10.1016/s1734-1140(11)70500-x.
19. Dyllal J, Coleman CM, Hart BJ, Venkataraman T, Holbrook MR, Kindrachuk J, et al. Repurposing of clinically developed drugs for treatment of Middle East respiratory syndrome coronavirus infection. *Antimicrob Agents Chemother* 2014;58:4885-4893. doi: 10.1128/AAC.03036-14.

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SARS-CoV BJ01	GCVLAWNTRNIDATSTGNYNYKYRYL <u>LRHGKLRPFERDISNVPFSPDG</u>
Wuhan HCoV-19	... I... SN. L. SKVG... L. LF. KSN. K. TEIQAGS
Guangdong Pangolin CoV	... I... SN. L. SKVG... L. LF. KSN. K. TEIQAGS
Guangxi Pangolin CoV	... I... SVKQ.. LTG. --.. L. LF. KS. .K. TEIQAGS
Yunan Bat CoV RaTG13	... I... SKH... KEG.. F.. L.. LF. KAN. K. TEIQAGS
Zhejiang Bat CoV ZXC21	... I... AKQ. T---. --H. F.. SH. ST.. K.... L. ----. DE-
Zhejiang Bat CoV ZC45	... I... AKQ. V---. --.. F.. SH. ST.. K.... L. ----. DE-

SARS-CoV BJ01	KPCT-PPA <u>L</u> NCYWPL <u>N</u> DYGFYTT <u>T</u> GIGY <u>Q</u> PYRVVLSFELLNAPATV
Wuhan HCoV-19	T.. NGVEGF... F.. QS... QP. N. V. H.
Guangdong Pangolin CoV	T.. NGVEGF... F.. QS... HP. N. V.
Guangxi Pangolin CoV	T.. NGQVG... Y.. ER... HP... VN... F..... G....
Yunan Bat CoV RaTG13	... NGQTG... Y.. YR... P. D. V. H.
Zhejiang Bat CoV ZXC21	----- . GVRT. ST. D. NPNVPLE. . AT.
Zhejiang Bat CoV ZC45	----- . GVRT. ST. D. NPNVPLE. . AT.

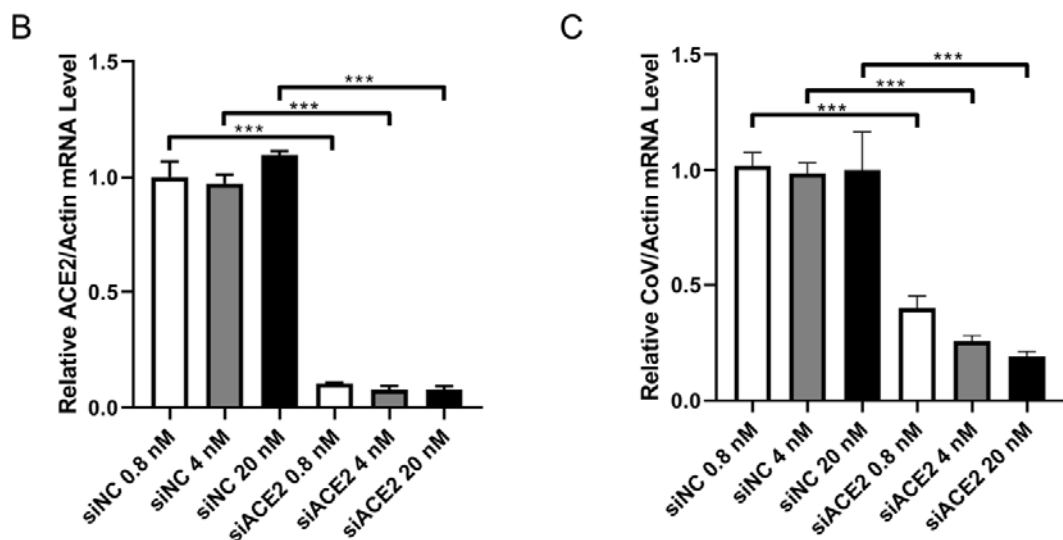


Figure 1: Pangolin coronavirus GX_P2V has two residue changes among the five key residues for receptor binding when compared to SARS-CoV, but likely still utilizes ACE2 as the cell receptor. (A) Alignment of receptor binding domains of 2019-nCoV related CoVs and SARS-CoV. Dots represent residues that are identical to those in SARS-CoV and hyphens represent gaps. Five key residues for binding between SARS-CoV RBD and ACE2 protein are indicated as

underlined capital letters. (B) The ACE2 expressions and (C) viral RNA yields were significantly reduced in ACE2-specific siRNA treated cells ($P < 0.01$). Vero cells were transfected with 0.8, 4 and 20 nmol/L ACE2-specific siRNAs or NSC siRNA by using the RNAiMax transfection reagent. At 48 h p.t., the cells were infected with pangolin CoV at a MOI of approximately 10. At 24 h p.i., CoV-infected cells were lysed in lysis buffer, and the RNA levels of ACE2, CoV and β -actin were determined by qRT-PCR. SARS-CoV: Severe acute respiratory syndrome-coronavirus; ACE2: Angiotensin-converting enzyme 2; 2019n-CoV: 2019 novel coronavirus; CoV: Coronavirus; RBD: Receptor binding domain; siRNAs: Small interfering RNA; NSC: non-specific control; p.t.: Post-transfection; MOI: Multiplicity of infection; p.i.: Post-infection; qRT-PCR: quantitative real time-polymerase chain reaction.

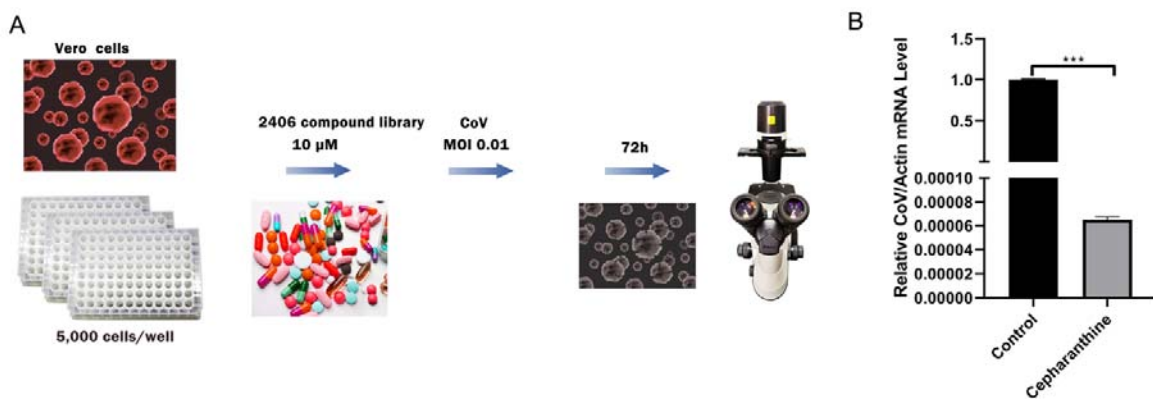


Figure 2: Screening clinically approved drugs for their antiviral activities against 2019-nCoV by observing CPE inhibition and relative quantification of viral RNA yields. (A) Vero E6 cells (2.5×10^4 cell/well) were infected with pangolin CoV (MOI=0.01) and were incubated in media containing different chemical drugs with a final concentration of 10 μ mol/L. At 72 h p.i, the CPE was observed by using phase microscopy. (B) Infected cells with cepharanthine treatment had no obvious CPE and were further analyzed by detecting viral RNA level. 2019n-CoV: 2019 novel coronavirus; CPE: cytopathic effect; CoV: Coronavirus; p.i.: Post-infection.

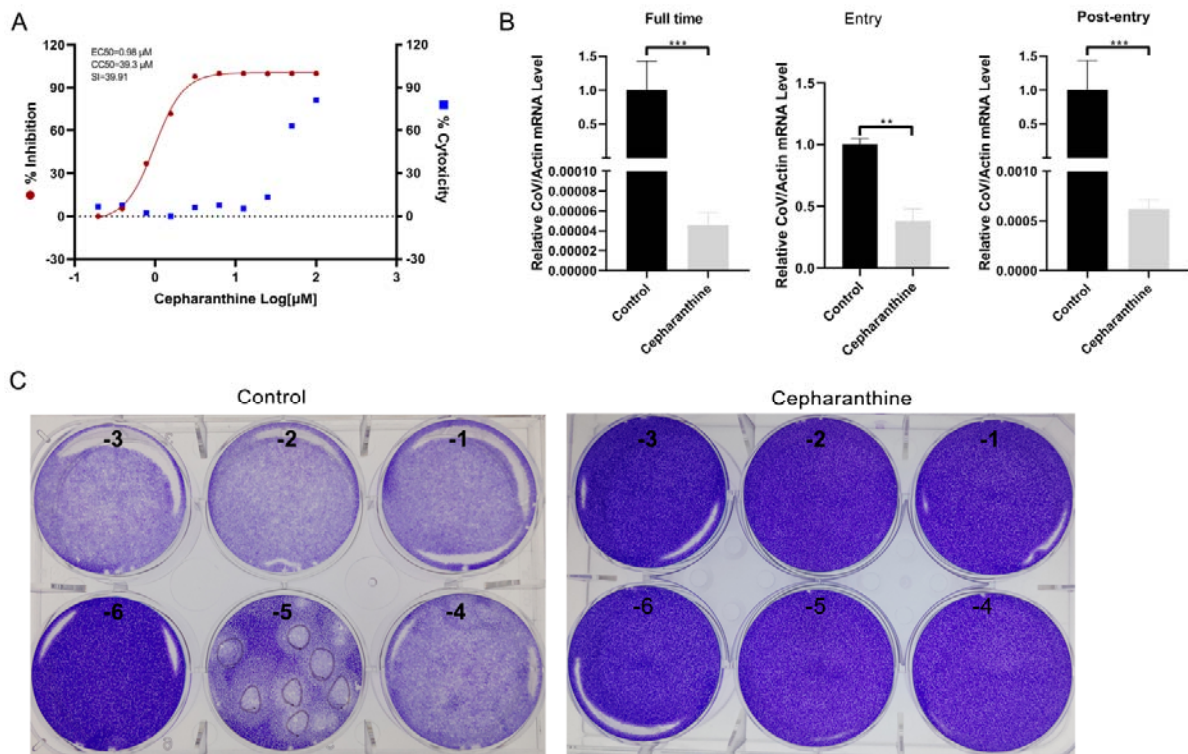


Figure 3: The antiviral activities of cepharanthine against CoV *in vitro*. (A) Vero E6 cells were infected with CoV at a MOI of 0.05 in the treatment of different doses of cepharanthine for 48 h. The viral yield in the cell was then quantified by qRT-PCR and normalized by β -actin level. Cytotoxicity of these drugs to Vero E6 cells was measured by CellTiter-Blue assay. The left and right Y-axis of the graphs represent mean percentage of inhibition of virus yield and cytotoxicity of cepharanthine, respectively. The experiments were done in triplicates. (B) Time-of-addition experiment of cepharanthine. The detailed steps for time-of-addition experiment of cepharanthine were described in the method section, and virus and β -actin mRNA level in the infected cell was quantified by qRT-PCR at 48 h p.i. (C) Cepharanthine inhibits infectious virus production in the supernatant. The virus titers of supernatant of control cells (left) and cepharanthine treated cells (right) in the “full time” experiment were determined by plaque assay. CoV: Coronavirus; MOI: Multiplicity of infection; qRT-PCR: quantitative real time-polymerase chain reaction; p.i.: Post-infection.