Supplementary information, Materials and Methods

Human serum samples

Human serum from healthy individuals and the COVID-19 patient were collected at TaiHe Hospital, Hubei University of Medicine. This study was performed according to the principles of the Declaration of Helsinki and approved by the TaiHe Hospital, Hubei University of Medicine, for the protection of human subjects. The study was approved by the ethics committee of the TaiHe Hospital (Reference Number 2020KS020) according to the medical research regulations of China.

Mice and ethics statement

hACE2-KI/NIFDC mice (expressing human ACE2 gene) were from National Institutes for Food and Drug Control (NIFDC).[1] BALB/c mice were purchased from the Vital River Laboratory. All animal experiments were approved by the Animal Experiment Committee of Laboratory Animal Center, Beijing Institute of Microbiology and Epidemiology (approval number: IACUC-DWZX-2020-002).

Cells, viruses and antiviral drugs

Human colorectal adenocarcinoma Caco-2 cell, African green monkey kidney Vero cell and human alveolar epithelial A549 cell were purchased from American Type Culture Collection (ATCC; Cat no. HTB-37, CCL-81 and CCL-185). Human live cancer Huh-7 cell was from NIFDC. All the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco Invitrogen) with 10 % fetal bovine serum (FBS; Gibco Invitrogen), 100 units/mL penicillin (Gibco Invitrogen) and 50 μg/mL streptomycin (Gibco Invitrogen) at 37 °C in a humidified atmosphere of 5 % CO_2.

SARS-CoV-2 strain BetaCoV/Beijing/IME-BJ01/2020 was originally isolated from a COVID-19 patient, and the full genome sequence is deposited in the Genome Warehouse in national Genomics Data Center, Beijing Institute of Genomic, CAS, with

the accession Nos. GWHACAX01000000. The SARS-CoV-2 mouse-adapted strain, MASCp6, has been described elsewhere,[2] and the full genome sequencing has been deposited in under the accession Nos. GWHACFH01000000. Viral titer was determined by plaque assay on Vero cells as previously described.[1] All experiments involving infectious SARS-CoV-2 were performed under biosafety level-3 (BLS-3) facilities at AMMS; VSV-based SARS-CoV-2 pseudovirus was from NIFDC.[3]

25-Hydroxycholesterol (25HC) using *in vitro* was purchased from Sigma-Aldrich (Sigma, Cat no. H1015); 25HC granules (containing 20% of 25HC) using in mice and rats was synthesized by Porton Biologics Ltd. and formulated by Bejing CoSci Me-Tech Co., Ltd.

Detection of SARS-CoV-2 induced genes

Human Caco-2 cells were infected with SARS-CoV-2 at 100 $TCID_{50}$ for 48 hours (hrs), total RNA from cells was extracted with TRIzol reagent (Thermo Fisher) according to the manufacturer's instructions, reverse transcription was performed with a PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Cat no. RR047A). SYRB Green qPCR mix (TransGen, Cat no. AQ131-04) was used to analyze the mRNA level of SARS-CoV-2 induced genes. Primers used to amplify SARS-CoV-2 induced genes were listed in Table S1. Primers of mGAPDH and mCH25H were used to test the mRNA level of CH25H in mice lung. Data analysis was calculated using the $2(-\Delta\Delta Ct)$ method.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of 25HC

To measure the concentration of 25HC in serum, blood was collected and centrifuged to obtain serum, which was inactivated at 56 °C for 1 hr. The inactivated serum samples were mixed with acetonitrile solution (Honeywell, AH015-4HC) of equal volume by vortex and sorted at -80 °C until analysis. The pretreated samples were centrifuged at 13000 rpm for 5 minutes (mins) at 4°C, 100 μL of the centrifuged supernatant was

transferred into a centrifuge tube. 50 μL of internal standard working solution (50 ng/mL of 25-Hydroxy Cholesterol-d6 solution; Toronto Research Chemicals, Cat no. 4-NWW-11-2) and 150 μL of acetonitrile solution were added into the tube, the mixture was mixed for 5 mins by vortex and centrifuged at 13000 rpm for 5 mins at 4°C. 200 μL of supernatant was added into a 96-well plate, which was added with 100 μL of 0.4% formic acid in acetonitrile, then the 96-well plate was treated by vortex for 5 mins. 5μL supernatant was measured by LC-MS/MS (LC 20ADXR-SCIEX API 4000). Data analysis was done using Analyst 1.6.3. LC-MS/MS experiment was performed by Union-Genius.

CH25H overexpression against SARS-CoV-2 infection

Vero cells were transfected with 800 ng CH25H expressing plasmid[4] or control plasmid in a 24-well plate for 12 hrs by Lipofectamine 3000 (Thermo Fisher), according to the manufacturer's instructions. Cells were infected with SARS-CoV-2 at 100 $TCID_{50}$ for 1hr and changed with fresh medium. Cell supernatant were used to test viral loads at 24 hour-post-infection (hpi) by quantitative reverse transcription PCR (RT-qPCR).

Measurement of viral RNA

Viral RNA from cell supernatant was extracted by using the QIAamp Viral RNA Mini Kit (Giagen, Cat no. 52904) and viral RNA from mice tissues was extracted by using TRIzol reagent according to the manufacturer's instructions, respectively. Viral RNA was performed by RT-qPCR using One Step PrimeScript RT-PCR Kit (Takara, Japan) with the following primers and probe: CoV-F3 (5'-TCCTGGTGATTCTTCTTCAGGT-3'); CoV-R3 (5'-TCTGAGAGAGGGTCAAGTGC-3'); and CoV-P3 (5'-FAM-AGCTGCAGCACCAGCTGTCCA-BHQ1-3').

Cytotoxicity assay

The cytotoxicity of 25HC on Vero cell was determined by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) cell proliferation assays (Promega, Cat no. G3582) as described previously.[5] Briefly, Vero cells were added with different doses of either compound or with EtOH in triplicate. After 4 days incubation at 37 °C, MTS assays were performed according to the manufacturer's protocols. After adjusting the absorbance for background (medium) and comparing to untreated controls (untreated cell medium), the cytotoxic concentration CC_{50} was calculated using a sigmoidal nonlinear regression function to fit the dose-response curve using the GraphPad Prism 7.01 software.

The in vitro antiviral activity

The *in vitro* antiviral efficacy of 25HC on Vero cell was determined by RT-qPCR assay. Briefly, Vero cells were pretreated with different doses of either compound or with EtOH as the control for 12 hrs and then infected with 20 $TCID_{50}$ of SARS-CoV-2 for 1 hr at 37 °C. The virus-drug mixture was removed and cells were further cultured with fresh drug-containing medium. At 48 hpi, the SARS-CoV-2 RNA copies in supernatant was quantified by RT-qPCR. The EC_{50} of 25HC was calculated according to the dose-response curves obtained and analyzed using the GraphPad Prism software.

Western blot analysis

Protein samples from SARS-CoV-2 infected Vero cells that received 25HC treatment were separated on 10% SDS-PAGE and then the gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). After being blocked at room temperature for 2 hrs, the blot was probed with mouse anti-coronavirus spike 2 antibody (MP, Cat no. 087204) and mouse anti-GAPDH antibody (CWBIO, Cat no. CW0100M) as primary antibody and the horseradish peroxidase (HRP)-conjugated Goat Anti-

Mouse IgG (ZSGB-Bio, Cat no. ZB-5305) as secondary antibody. Protein bands were detected by Immobilon Western Chemiluminescent HRP substrate (Sigma, Cat no. WBKLS0500).

SARS-CoV-2 pseudovirus neutralization assay

Pseudovirus neutralization assay for SARS-CoV-2 were basically performed as described before with some modifications.[3] Briefly, Huh-7 cells were seeded in a 96-well plate for 12 hrs, and then were treated with indicated doses of 25HC for 12 hrs. The cells were infected with SARS-CoV-2 pseudovirus at 2×10^4 $TCID_{50}$ for 24 hrs, and the inhibition rate is calculated by comparing the OD value to the negative and positive control wells.

Immunofluorescence staining

SARS-CoV-2 infected Vero cells that received 25HC treatment were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Then, the cells were incubated with the primary antibody (Sino Biological, Cat no. 40150-T62) for 1 hr at 37 °C, followed by incubation with the secondary antibody (Bioworld Technology , Cat no. BS10017) for 1 hr at 37 °C. The nuclei were stained with DAPI (Solarbio, Cat no. C0060) for 5 mins at room temperature. The images were taken by fluorescence microscopy.

Mouse challenge experiment

The *in vivo* therapeutic efficacy of 25HC was assessed by using a newly established mouse model based on a SARS-CoV-2 mouse adapted strain MASCp6.[2] Briefly, 6-8-week-old BALB/c mice were administrated intragastrically with 25HC (100mg/kg) or vehicle 24 hrs prior to infection intranasally with 2×10^4 $TCID_{50}$ of MASCp6. The administration was continued once daily until 3 dpi, when the lung and trachea tissues of mice were collected for viral RNA loads assay. The hACE2 mice were infected

intranasally (i.n.) with 5×10^5 $TCID_{50}$ of SARS-CoV-2.

Statistical analysis

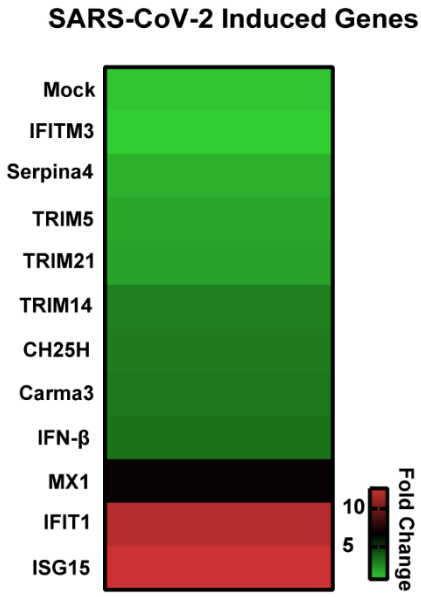
All data were analyzed using the GraphPad Prism 7.01 software. Statistical evaluation was performed by Student's unpaired t test. Data are presented as mean ± SEM.

REFERENCES

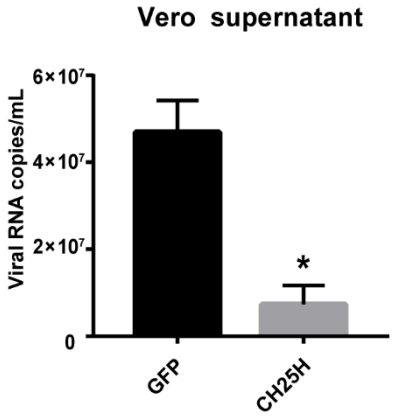
1. Sun, S. H. et al. *Cell Host Microbe* **28**, 124-133 e4 (2020).
2. Gu, H. et al. *Science* https://doi.org/10.1126/science.abc4730 (2020).
3. Nie, J. et al. *Emerg Microbes Infect* **9**, 680-686 (2020).
4. Li, C. et al. *Immunity* **46**, 446-456 (2017).
5. Jin, Z. et al. *Nature* **582**, 289-293 (2020).

Table S1. Primers used in this study

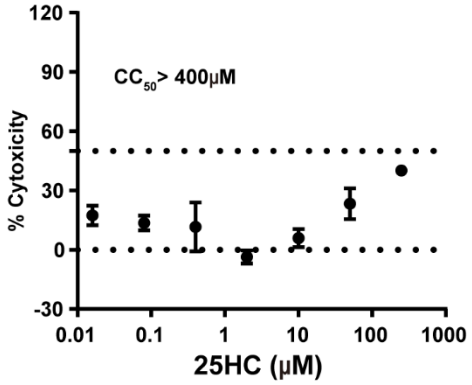
Gene	Forward primer	Reverse primer
Carma3	AGCTGGTGCAGAACGAGATCTAC	CCCTGACTTCCCGGACATT
CH25H	GCTGGCAACGCAGTATATGAG	CGAGCAGTGTGACGTTCATC
IFITM3	CATCCTCATGACCATTCTGC	TCAGTGATGCCTCCTGATCT
IFIT1	TGACTCTTTTGCCTCTTTCTTCTAA	TTCTTGGGGT GCTCTGTGG
IFN-β	ATGACCAACAAGTGTCTCCTCC	GGAATCCAAGCAAGTTGTAGCTC
ISG15	GAGAGGCAGCGAACTCATCT	CTTCAGCTCTGACACCGACA
MX1	GTTTCCGAAGTGGACATCGCA	CTGCACAGGTTGTTCTCAGC
Serpina4	CGAGC TGTCT GAGTC CGATG	GCCCA CAGTG TCGTA GAAGT
TRIM5	CTGGCATCCTGGGCTCTCAAAGT	CATACCCCCAGGATCCAAGCAGTT
TRIM14	TGAAGGGGAAATTCACTGAACTC	AGCCTCTGGACAGGATCGG
TRIM21	AGAGAGACTTCACCTGTTCTGT	TCAGTTCCCCTAATGCCACCT
mGAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
mCH25H	GCTGGCGACCCAATACATGAG	GAAGCACCGCGACGTTCAGC



Supplemental information, Fig. S1. SARS-CoV-2 induced genes mRNA expression. Human Caco-2 cells were infected with SARS-CoV-2 at 100 $TCID_{50}$ for 48 hrs, intended genes mRNA expression were measured by RT-qPCR.



Supplemental information, Fig. S2. Overexpression of CH25H inhibits SARS-CoV-2 replication. Vero cells transfected with plasmids expressing GFP and CH25H were infected with SARS-CoV-2 at 100 $TCID_{50}$. The viral RNA copies in supernatant were quantified by RT-qPCR.



Supplemental information, Fig. S3. The cytotoxicity of 25HC on Vero cells was determined by MTS assay. Vero cells were treated with indicated dose of 25HC or with EtOH for 96 hrs, the cells lysates were used to determine the cell toxicity of 25HC.