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Supporting Information for

2-Thiouridine is a broad-spectrum antiviral nucleoside analogue against positive-strand RNA viruses.

Kentaro Uemura, Haruaki Nobori, Akihiko Sato, Shinsuke Toba, Shinji Kusakabe, Michihito Sasaki, Koshiro Tabata, Keita Matsuno, Naoyoshi Maeda, Shiori Ito, Mayu Tanaka, Yuki Anraku, Shunsuke Kita, Mayumi Ishii, Kayoko Kanamitsu, Yasuko Orba, Yoshiharu Matsuura, William W. Hall, Hirofumi Sawa, Hiroshi Kida, Akira Matsuda, and Katsumi Maenaka

Katsumi Maenaka
Email: maenaka@pharm.hokudai.ac.jp

This PDF file includes:

Supporting text

Materials & Methods

In vitro growth kinetics of drug-resistant mutants

VeroE6 cells were seeded onto 24-well plates the previous day and were infected with rgDENV2 (rgDENV2-WT or rgDENV2-NS5-G605V) at an MOI of 0.01 for 1 h. After incubation, the unbound virus was removed, and new medium was added. At 24, 48, 72, and 96 hpi, total RNA was isolated with PureLink RNA Mini Kit. Viral RNA level was quantified by qRT-PCR analysis as described above with *ACTB* transcripts used as internal controls.

Mitochondrial protein synthesis assays

HepG2 cells were seeded onto 96-well plates the previous day and treated with the 3-fold serially diluted compound (n = 2). At 5 dpi, one set of plates was fixed with 4% paraformaldehyde (Nacalai tesque) and the intracellular levels of two mitochondrial proteins, the mitochondrial DNA-encoded cytochrome *c* oxidase I (COX-I) and nuclear DNA-encoded succinate dehydrogenase A (SDH-A) were determined using the MitoBiogenesis In-Cell Enzyme-linked immunosorbent assay (ELISA) Kit (Colorimetric, Abcam, ab110217) following the manufacturer's instructions. To monitor the cell viability as an ATP level, the second set of plates was analyzed by adding CellTiter-Glo 2.0 Reagent (Promega, G9242/3) and measuring luminescence on a GloMax Discover System (Promega). The IC_{50} value was defined in GraphPad Prism version 8.4.3 with a variable slope (four parameters).

Establishment of mouse-adapted DENV2 and SARS-CoV-2

AG129 mice (IFN-α/β and IFN-γ receptors deficient 129/Sv mice) were purchased from Marshall BioResources and bred in-house under the specific pathogen-free (SPF) conditions. To establish mouse-adapted DENV2, named DENV2 AG-P10 strain, virus passage in mice was carried out according to a previous report (1). Briefly, 7-weeks-old female AG129 mice were inoculated intraperitoneally with 100 µL of 4×10^5 PFU/mouse of DENV2 D2/hu/INDIA/09-74 strain. On day 3 after infection, the infected mice were euthanized under deep anesthesia by isoflurane inhalation, and serum was collected. Virus in the serum was amplified in C6/36 cells and then intraperitoneally injected into other AG129 mice. This adaptation process was performed a total of 10 times.

To establish mouse-adapted SARS-CoV-2, named SARS-CoV-2 MA-P10 strain, virus passage in mouse was carried out according to a previous report (2, 3). Briefly, SPF, 30–45-week-old female BALB/c mice (BALB/cAJcl, CLEA Japan) were inoculated intranasally with 50 µL of 1×10^5 $TCID_{50}$/mouse of SARS-CoV-2 WK-521 strain under anesthesia. On day 3 after infection, the infected mice were

euthanized, and whole lung tissues were harvested and homogenized in DMEM supplemented with 10% FBS and P/S with TissueRuptor (Qiagen). Virus in the supernatants of lung homogenates were intranasally injected into other BALB/c mice. This adaptation process was performed a total of 10 times.

Viral RNA was extracted and purified using QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Next-generation sequencing (NGS) was conducted on an iSeq 100 System (Illumina, Inc.) and the sequences were analyzed using CLC Genomics Workbench ver. 21.0.3 software (CLC bio, Qiagen).

In vitro ADME assay

Solubility assay: The Japanese Pharmacopeia (JP) 1st fluid (pH 1.2) or JP 2nd fluid (pH 6.8) for dissolution testing was used for solubility measurements. A test solution of test compound was prepared by diluting 10 mM DMSO stock solution 2 µL:165 µL in JP1st or 2nd fluid and mixed at 37˚C for 4 h by rotation at 1,000 rpm. After loading the mixed solution into 96-well MultiScreen Filter Plates (product number MSHVN4510, 0.45 µm hydrophilic PVDF membrane, Millipore), filtration was performed by centrifugation. The filtrates were mixed with acetonitrile and analyzed by HPLC-UV (254 nm). Solubility was calculated by comparing the peak area of the filtrate mixture with that of a 100 µM standard solution. When the peak area of the filtrate mixture was larger than the peak area of the standard solution, it was described as >100 µM.

PAMPA assay to determine the passive membrane diffusion rates: A Corning Gentest Pre-coated PAMPA Plate System was used in the PAMPA permeability test. The acceptor plate was prepared by adding 200 µL of 5% DMSO/0.1 M phosphate buffer (pH 7.4) to each well, and then 300 µL of 100 µM test compounds in 5% DMSO/0.1 M phosphate buffer (pH 6.4) was added to the donor wells. The acceptor plate was then placed on top of the donor plate and incubated at 37˚C without agitation for 4 h. At the end of the incubation, the plates were separated and the solutions from each well of both the acceptor plate and the donor plate were transferred to 96-well plates and mixed with acetonitrile and water. The final concentrations of compounds in both the donor wells and acceptor wells, as well as the concentrations of the initial donor solutions, were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The permeability of the compounds was calculated according to a previous report (4). The recovery of tested compounds was more than 90%. The permeabilities of Antipyrine (100 µM), Metoprolol (500 µM) and Sulfasarazine (500 µM) as reference compounds, with 100%, 95%, and 13% gastrointestinal absorptions in humans (4), were 11, 1.5 and 0.055×10^{-6} cm/s, respectively.

Hepatic microsomal stability assay: Disappearance of the parent compound over time was measured by using the amount of drug at time zero as a reference.

After 5 min of preincubation, 1 mM NADPH (final concentration, the same applies to the following) was added to a mixture containing 1 μM of the test compound, 0.2 mg/mL of human (pooled 200 individuals of mixed gender) or mouse (CD1 male) liver microsomes (purchased from Sekisui XenoTech LLC), 1 mM EDTA and 0.1 M phosphate buffer (pH 7.4) and incubated at 37˚C for 30 min by rotation at 60 rpm. An aliquot of 50 μL of the incubation mixture was sampled and added to 250 μL of chilled acetonitrile/internal standard (IS). After centrifuging for 15 min at 3,150 × *g* (4˚C), the supernatants were diluted with water and analyzed by LC-MS/MS. Hepatic microsomal stability (mL/min/kg, CL_{int}) was calculated according to a previous report (5), using 48.8 (human) or 45.4 (mouse) mg MS protein/g liver and 25.7 (human) or 87.5 (mouse) g liver/kg body weight as scaling factors.

Determination of the unbound fraction in human or mouse plasma: An equilibrium dialysis apparatus was used to determine the unbound fraction for each compound in human or mouse plasma. High Throughput Dialysis Model HTD96b and Dialysis Membrane Strips MWCO 12-14 kDa obtained from HTDialysis, LLC (Gales Ferry, CT) were used. Plasma was spiked with the test compound (1 μM), and 150 μL aliquots were loaded into the apparatus and dialyzed versus 150 μL of 0.1 M phosphate buffer (pH 7.4) at 37˚C for 6 h by rotation at 80 rpm. The unbound fraction was calculated as the ratio of receiver side (buffer) to donor side (plasma) concentrations.

In vivo pharmacokinetics assay

Five-week-old female BALB/c mice (purchased from Japan SLC) were treated with s2U by oral (150 mg/kg) or intravenous (20 mg/kg) administration. Blood was collected from the mouse tail with heparin 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h after administration, and plasma samples were isolated at 2,000 rpm for 5 min.

Plasma samples were precipitated with 4–8 volumes of acetonitrile/IS and centrifuged at 15,000 × *g* at 4˚C for 10 min. The supernatants were diluted with 7 volumes of water and analyzed by LC-MS/MS. Standard non-compartmental analysis was performed to determine the pharmacokinetic parameters and to simulate the repeated dose concentration time profiles using Phoenix Winnonlin ver 8.3 (Pharsight): the estimated initial concentration (C_0), maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}), elimination half-life ($t_{1/2}$), area under the concentration time curve from time zero to infinity (AUC_∞), total clearance (CL_{tot}), and volume of distribution at terminal phase (V_{dz}). The absolute bioavailability (BA) of the oral dose was calculated as AUC_∞(po)/AUC_∞(iv).

LC-MS/MS quantification method

A Qtrap 6500+ mass spectrometer (Sciex) equipped with a Shimadzu Nexera series LC system (Shimadzu) was used. All compounds were analyzed in multi-reaction monitoring mode under electron spray ionization conditions. The analytical column used was an Acquity UPLC HSS T3 (1.8 μm, 3 × 50 mm, Waters) at 40˚C. The gradient mobile phase consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B) at a total flow rate of 0.5 mL/min. The initial mobile phase composition was 2% B, which was held constant for 0.1 min, increased in a linear fashion to 90% B over 0.9 min, then held constant for 1.5 min, and finally brought back to the initial condition of 2% B over 0.01 min and re-equilibrated for 2.5 min. The transitions (precursor ion > product ion) of s2U and IS (antipyrine) were 261.1 > 129.0 and 189.0 > 56.0 (positive), respectively.

Bacterial reverse mutation test

Each strain was tested using the direct method (in the absence of the S9 metabolic activation system) and the metabolic activation method (in the presence of the S9 metabolic activation system). 0.1 mL of the s2U or control solution was added to 0.5 mL of 0.1 mol/L Na-phosphate buffer (pH 7.4) (direct method) or S9 mix (metabolic activation method). In addition, 0.1 mL of culture medium was added. The bacteria were then preincubated at 37ºC for 20 min. After the pre-incubation, 2 mL of medium for stratification containing 0.05 mM L-histidine and 0.05 mM D-biotin (Salmonella typhimurium) or 0.05 mM L-tryptophan (Escherichia coli) were mixed in and were incubated at 37ºC for 49 hours. All plates of each strain were checked for growth inhibition using a stereomicroscope. The presence or absence of precipitation of the s2U was also checked visually. Next, the number of reversion mutant colonies was measured using a colony analyzer with area correction. These tests were performed by the Drug Safety Testing Center Co., Ltd. (Saitama).

In vitro gene mutation test using the Hprt genes in V79 cells

Approximately 2 x 10^7 V79 cells in the logarithmic growth phase were mixed with s2U or positive control and tested with or without S9 metabolic activation system (direct method or metabolic activation method). Approximately 2 x 10^6 cells per plate (50 mL of culture medium/225 cm^2 flask) and incubated in a CO_2 incubator (37°C) for 7 days. Then, cells are divided for calculating the colony-forming capacity (abCE: absolute cloning efficiency) and the mutant frequency (MF: Mutant Frequency). For determining abCE, each cell suspension was diluted with culture medium and 200 cells per plate (5 mL of culture medium) were seeded into one 60 mm dia petri dish each and incubated for 7 days. The number of colonies counted, and abCE was calculated. For determining MF, the cell suspension was diluted with culture medium containing 6-TG (final 6-TG

concentration: approximately 5 μg/mL) to 2 × 10^5 cells per plate (10 mL of culture medium), five 90 mm dia petri dishes each, and then selectively cultured for 11 days. After the end of incubation, the number of colonies was counted and MF was calculated. These tests were performed by the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo).

Figure S1. Identification and cytotoxicity of s2U.
A, Schematic representation of the compound screening using BHK-21 cells and flaviviruses. **B**, Screening results of s2U. Antiviral assays were carried out as described in Supplementary Table S3. EC_{50} (50% effective concentration) values represent mean values (n = 2). **C**, Effect of s2U on cell proliferation. Cells were incubated with serial dilutions of the compound. Resazurin reduction assay or CellTiter-Glo assay was performed at 3- or 4-days post-treatment. Cytotoxicity (%) is expressed relative to the values for the DMSO-treated samples and cell-free samples. The EC_{50} and CC_{50} (50% cytotoxic concentration) values were defined in GraphPad Prism versions 8.4.3 and 9.5.1 with a variable slope (four parameters).

Figure S2. Dose-response inhibition of several RNA viruses by s2U.
Cells were infected with DENV2 (multiplicity of infection [MOI] = 0.05), ZIKV (MOI = 0.05), YFV (MOI = 0.05), JEV (MOI = 0.05), WNV (MOI = 0.05), CHIKV (MOI = 0.01), HCoV-229E (MOI = 0.005), HCoV-OC43 (MOI = 0.1), SARS-CoV (MOI = 0.01), MERS-CoV (MOI = 0.01) and several SARS-CoV-2 variants (MOI = 0.01) containing a serially diluted compound. Cell lysates were collected for viral RNA determination, and viral RNA levels were determined relative to *ACTB* transcripts. The 50% and 90% effective concentration (EC_{50} and EC_{90}) values were defined in GraphPad Prism versions 8.4.3 and 9.5.1 with a variable slope (Find ECanything; F = 90). Data are presented as mean values of biological triplicates from one of the experiments, and error bars indicate SD.

Figure S3. Antiviral activity of s2U against several RNA and DNA viruses.
A, Dose-response inhibition of viral protein expression in the HCoV-OC43-infected cells. Cells were stained with viral-specific antibodies (green, Nucleocapsid) and counterstained with Hoechst 33342 nuclear dye (blue). Scale bars indicate 200 µm. **B–E**, Dose-response inhibition of SARS-CoV-2 propagation by s2U. Supernatants of SARS-CoV-2-infected VeroE6/TMPRSS2 (**B**), VeroE6/ACE2/TMPRSS2 (**C**), and A549/ACE2/TMPRSS2 (**D, E**) cells were collected at 24- (**A**) or 48- (**C–E**) hours post-infection (hpi), and dilutions were used to inoculate VeroE6/ACE2/TMPRSS2 cells. Two days after inoculation, viral titers were determined by plaque assay. **F, G**, s2U did not inhibit RABV (**F**) and RVFV (**G**) virus replication. Cell lysates were collected for viral RNA determination; viral RNA levels were determined relative to *ACTB* or *18S rRNA* transcripts. **H**, s2U did not inhibit HSV-1 virus replication. Cell lysates were collected for viral DNA determination; viral DNA levels were determined relative to *ACTB* transcripts. Data are presented as mean values of biological triplicates from one of the experiments, and error bars indicate standard deviation (SD). Statistically significant differences were determined using a one-way ANOVA

followed by Dunnett's multiple comparisons test to compare with non-treated cells; ∗ $p < 0.01$, ∗∗ $p < 0.005$, ∗∗∗ $p < 0.0005$, and ∗∗∗∗ $p < 0.0001$.

Figure S4. Molecular target and mechanism of action of s2U.

A, Ribonucleotide competition for HCoV-229E inhibition by s2U. HCoV-229E (MOI = 0.005)-infected MRC5 cells were treated with 15 μM of s2U and serial dilutions of exogenous nucleosides. A resazurin reduction assay was performed at 3 days post-infection (dpi). Antiviral activities (%) are expressed relative to the values for the DMSO-treated, infected samples and non-infected samples. **B, C**, Effect of s2U resistance mutation on replication fitness. BHK-21 (**B**) and VeroE6 (**C**) cells were infected with rgDENV2-WT or rgDENV2-NS5-G605V (MOI = 0.01) for 1 h. Cell lysates were collected at 24, 48, 72, and 96 hpi, and viral RNA levels were determined relative to *18S rRNA* (BHK-21) or *ACTB* (VeroE6) transcripts. Data are presented as mean values, and error bars indicate SD. Statistically significant differences between wildtype and G605V viruses (**B–C**) were determined using a two-way ANOVA followed by Bonferroni's multiple comparisons tests; ∗ $p < 0.01$, ∗∗ $p < 0.005$, ∗∗∗ $p < 0.0005$, and ∗∗∗∗ $p < 0.0001$.

Figure S5. Effect of s2U on mitochondrial biogenesis.

A–D, HepG2 cells were assayed for a reduction in mitochondrial-encoded protein COX-I or nuclear-encoded protein SDH-A after 5 days of incubation with 3-fold serial dilutions of s2U (**A**), ribavirin (**B**), favipiravir (**C**) and chloramphenicol (**D**). Inhibitory effects (% of Control) are expressed relative to the values for the DMSO-treated samples. **D**, 50% inhibitory concentration (IC_{50}) values of these compounds against protein expression. The IC_{50} value was defined in GraphPad Prism version 8.4.3 with a variable slope (four parameters).

Figure S6. Establishment of mouse-adapted DENV2 strain (DENV2 AG-P10).

A, Schematic representation of the passage history of DENV2 in AG129 mice. Virus in serum from infected mice was propagated in C6/36 cells. **B**, Survival of DENV2 AG-P10-infected AG129 mice. Mice were intraperitoneally inoculated with 4×10^5 plaque-forming units [PFU] of a DENV2 clinical isolate (n = 3) and DENV2 AG-P10 (n = 5). Survival was monitored daily. **C**, Viral RNA copies/mL in organ samples were quantified using qRT-PCR. At 4 dpi, the infected mice (1×10^3 PFU of DENV2 AG-P10, n = 2) were euthanized under deep anesthesia by isoflurane inhalation, and serum and whole tissues (spleen, kidney, liver, small intestine, large intestine, and brain) were harvested and homogenized in PBS with a TissueRuptor. **D**, Amino acid substitutions occurred during the passage. Data are presented as mean values, and error bars indicate SD.

Figure S7. Establishment of mouse-adapted SARS-CoV-2 strain (SARS-CoV-2 MA-P10).

A, Schematic representation of the passage history of SARS-CoV-2 in BALB/c mice. **B**, Virus titers in lung homogenates from SARS-CoV-2-infected mice from passage 1 (P1) to P10 (n = 3–9). **C**, Survival of SARS-CoV-2 MA-P10-infected BALB/c mice. Young (5-week-old) and adult (30–50-week-old) female mice were intranasally inoculated with 2×10^5 $TCID_{50}$ of SARS-CoV-2 MA-P10 (n = 5 per group). Survival was monitored daily. **D, E**, Virus titers and viral RNA loads in lung from SARS-CoV-2-MA-P10-infected mice. Virus titers (**D**) were quantified by a standard 50% tissue culture infection dose ($TCID_{50}$) assay using VeroE6/TMPRSS2 cells. Viral RNA copies/mL (**E**) were quantified using qRT-PCR. **F**, Macroscopic appearance of lung tissue of SARS-CoV-2-MA-P10-infected mice at 1, 3, and 5 dpi. **G**, Amino acid substitutions occurred during the passage. Data are presented as mean values, and error bars indicate SD.

Figure S8. *In vivo* efficacy of s2U in the DENV2 and SARS-CoV-2 mouse model.

A, Schematic representation of the viremia study using AG129 mice and strain DENV2 AG-P10. **B–D**, Effect of s2U on viremia at 3 dpi in mice treated twice daily with s2U (50 or 150 mg/kg) compared with vehicle-treated mice (n = 5 per group). The relative viral RNA level (DENV2 copies/18S copies) of spleen (**B**), kidney (**C**), and liver (**D**) samples were quantified using qRT-PCR. **E**, Schematic representation of the study using BALB/c mice and SARS-CoV-2 MA-P10. **F–H**, Relative *Ifnb* (**F**), *Il6* (**G**), and *Cxcl10* (**H**) gene expression profiles in lungs from mice at 1 dpi with SARS-CoV-2. Cytokine RNA levels were determined relative to *18S rRNA* transcripts. **I**, Schematic representation of the viremia study using BALB/c mice and SARS-CoV-2 MA-P10. **J**, Effect of s2U on viremia at 1 dpi in mice orally administered 300 mg/kg s2U twice daily compared with vehicle-treated mice (n = 5 per group). Virus titers in lung samples were quantified by a standard $TCID_{50}$ assay using VeroE6/TMPRSS2 cells. Data are presented as mean values, and error bars indicate SD. Statistically significant differences between the s2U-treated and vehicle-treated groups were determined using a one-way ANOVA followed by Dunnett's multiple comparisons tests (**B–D**, **F–H**) or unpaired *t*-test (**J**); $* \ p < 0.01$, $** \ p < 0.005$, $*** \ p < 0.0005$, and $**** \ p < 0.0001$.

Figure S9. Close-up view of the catalytic site including the escape mutation site, residue 605, in crystal structures of DENV and SARS-CoV-2 RdRps. These are superimposed into MNV RdRp complexed with s2U.

G605 in motif B (left bottom and right) and located close to the active site, D663 in motif C, a catalytic region in DENV RdRp. The crystal structure of DENV RdRp showed the disordered region for residues 602-605 (cyan). s2U (white) binds to D346 of MNV RdRp, which corresponds to D663 in DENV and D760 in SARS-CoV-2. This residue is conserved in a wide range of viruses. s2U also binds to another catalytic residue D250, which corresponds to D539 in DENV and D623 in SARS-CoV-2. This residue is conserved in positive-sense RNA viruses but not in negative-sense RNA viruses.

Figure S10. Pharmacokinetic (PK) properties of s2U.
A, *In vitro* absorption, distribution, metabolism, and excretion (ADME) properties of s2U. **B–D**, Pharmacokinetic properties of s2U in mice after oral (**B**) and intravenous (**C**) dosing. s2U was administered to 5-week-old female BALB/c mice (n = 3 per group) *via* oral gavage as a solution formulated in 5% DMSO/0.5% methylcellulose at 150 mg/kg or intravenously as a saline solution at 20 mg/kg. C_0: initial concentration, C_{max}: maximum plasma concentration, T_{max}: time to reach C_{max}, $t_{1/2}$: terminal phase elimination half-life, AUC: area under the plasma concentration versus the time, AUC_∞: AUC curve to infinite time, CL_{tot}: total clearance, V_{dz}: volume of distribution at the terminal phase, BA (F): bioavailability. **E, F**, Simulation of twice-daily or once-daily doses of s2U by oral or intravenous administration derived from the single-dose PK experiment. Data are presented as mean values, and error bars indicate SD (**B, C**).

Figure S11. *In vivo* toxicity evaluation of s2U using BALB/c mice.
In vivo toxicity evaluation of s2U in mice after oral and intravenous dosing. s2U was administered to 5-week-old female BALB/c mice (n = 3 or 5 per group) *via* oral gavage (PO) as a solution formulated in 5% DMSO/0.5% methylcellulose at 150 or 300 mg/kg or intravenously (IV) as a saline solution at 25 or 50 mg/kg. Body weight changes were monitored daily for 7 days. Data are presented as mean values, and error bars indicate SD.

Table S1. Antiviral activity of reference compounds against various RNA viruses.

Antiviral assays were carried out as described in Supplementary Table S3 and S4. EC_{50}: 50% effective concentration. EC_{90}: 90% effective concentration.

a: EC_{50} values represent mean values from at least three independently performed experiments (n = 2).

b: EC_{90} values represent mean values from a single experiment with biological triplicates.

c: Fold change is calculated from the ratio of rgNS5-G605V/rgWT.

Table S2. Bacterial reverse mutation test and *in vitro* gene mutation test using the *Hprt* genes in V79 cells.

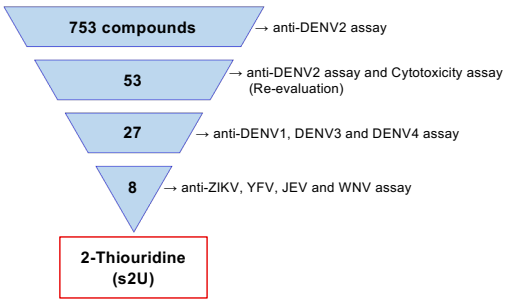
Table S3. *In vitro* assay conditions (CPE-based assay).

Table S4. *In vitro* assay conditions (qPCR assay, IFA, and Plaque assay).

Table S5. Sequence of primers and probes for the qPCR assays.

Figure S1

A



B

Screening Results			
Virus	Strain	Cell	EC_{50} (μM)
DENV1	D1/hu/PHL/10-07	BHK-21	2.6
DENV2	D2/hu/INDIA/09-74	BHK-21	0.58
DENV3	D3/hu/Thailand/00-40	BHK-21	2.1
DENV4	D4/hu/Solomon/09–11	BHK-21	1.8
ZIKV	MR766	BHK-21	5.0
YFV	17D-204	BHK-21	2.9
JEV	Beijing-1	BHK-21	4.8
WNV	NY99	BHK-21	5.3

C

Cell	Species	Organ	CC_{50} (μM) [a]
BHK-21	Hamster	Kidney	> 400 [b,c]
VeroE6	Monkey	Kidney	> 400 [b,c]
VeroE6/ TMPRSS2	Monkey	Kidney	282.8 [b]
MRC5	Human	Lung	> 400 [b]
293T	Human	Kidney	100.6 [c]
Huh7	Human	Liver	> 400 [b]
MOLT4	Human	T cell	> 50 [c]
THP-1	Human	Monocyte	> 50 [c]

a : CC_{50} values represent mean values from at least three independently performed experiments (n = 2).

b : CellTiter-Glo assay

c : Resazurin assay

Figure S2

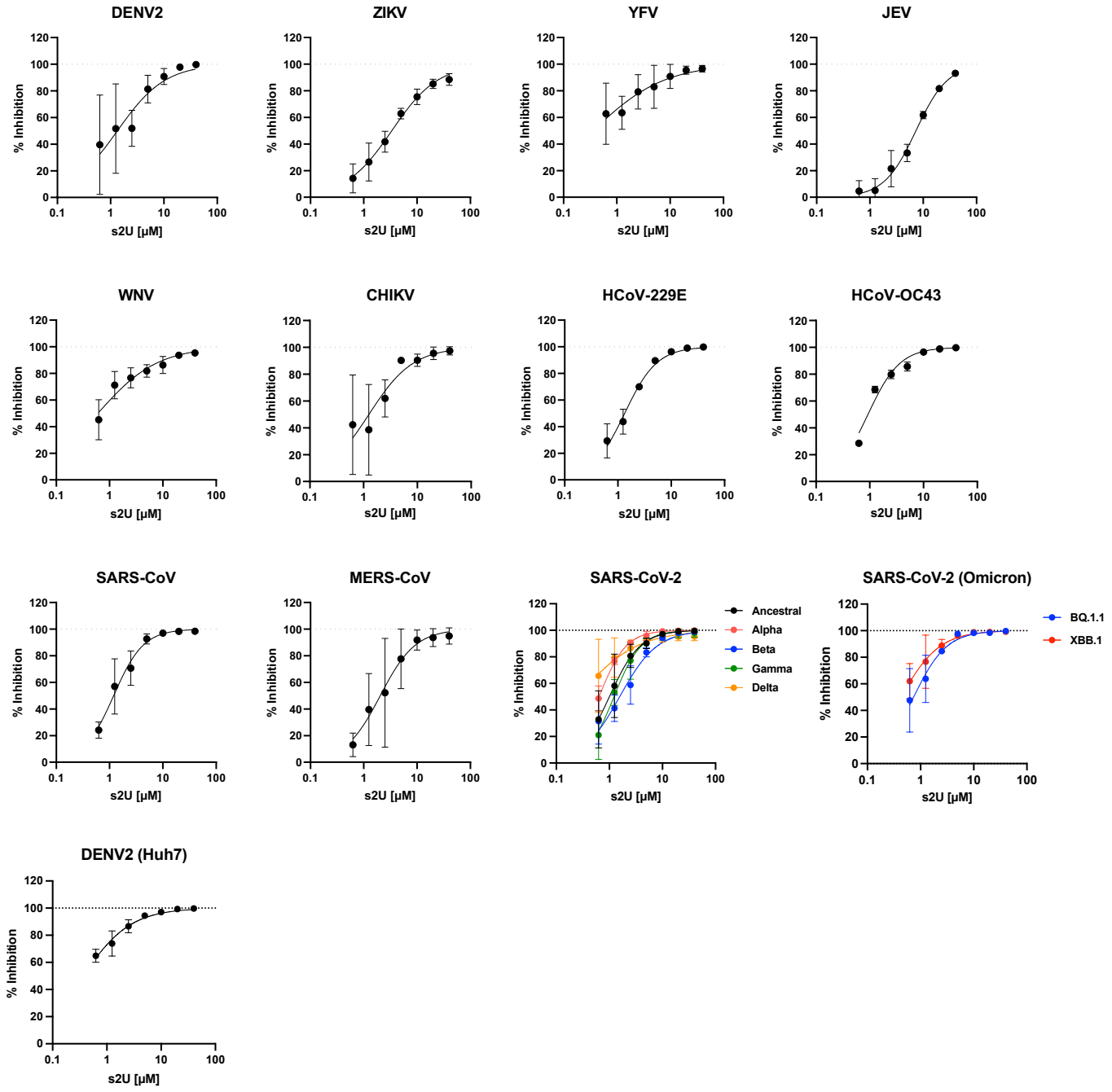

DENV2
ZIKV
YFV
JEV
WNV
CHIKV
HCoV-229E
HCoV-OC43
SARS-CoV
MERS-CoV
SARS-CoV-2
SARS-CoV-2 (Omicron)
DENV2 (Huh7)
% Inhibition
s2U [μM]
Ancestral
Alpha
Beta
Gamma
Delta
BQ.1.1
XBB.1

Figure S3

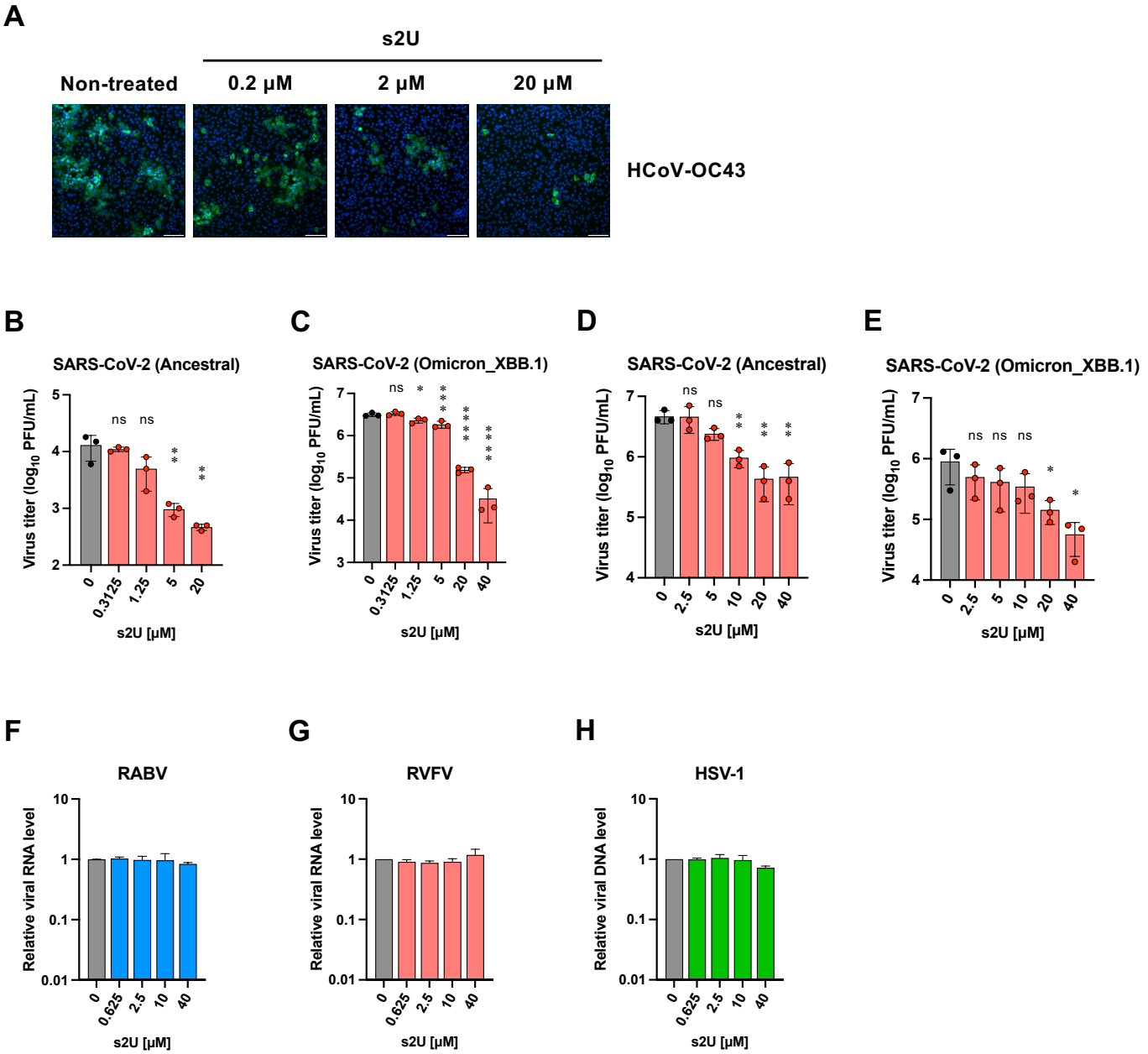

A
s2U
Non-treated
0.2 μM
2 μM
20 μM
HCoV-OC43
B
SARS-CoV-2 (Ancestral)
Virus titer (log10 PFU/mL)
s2U [μM]
C
SARS-CoV-2 (Omicron_XBB.1)
Virus titer (log10 PFU/mL)
s2U [μM]
D
SARS-CoV-2 (Ancestral)
Virus titer (log10 PFU/mL)
s2U [μM]
E
SARS-CoV-2 (Omicron_XBB.1)
Virus titer (log10 PFU/mL)
s2U [μM]
F
RABV
Relative viral RNA level
s2U [μM]
G
RVFV
Relative viral RNA level
s2U [μM]
H
HSV-1
Relative viral DNA level
s2U [μM]

Figure S4

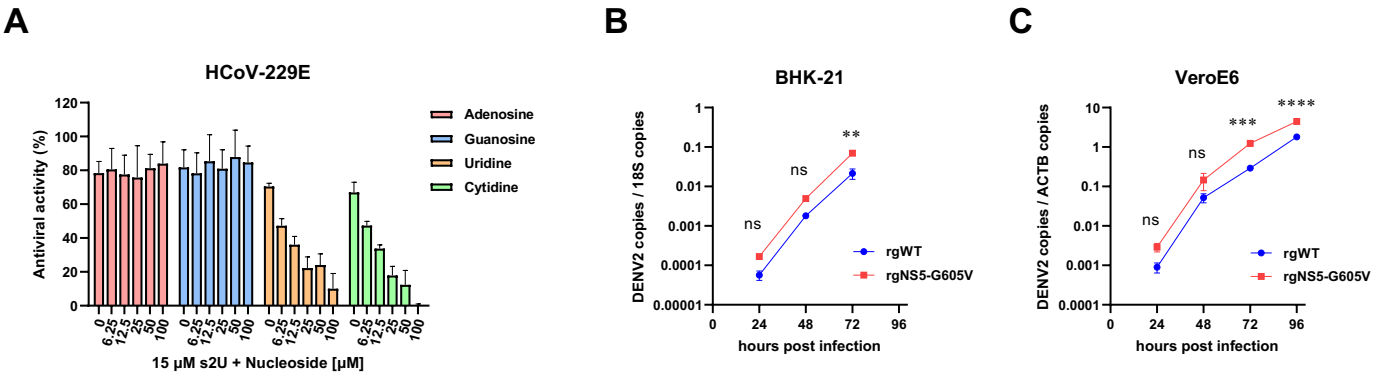
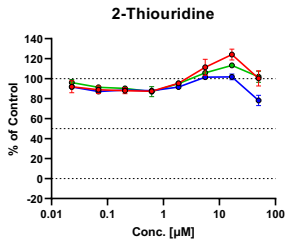
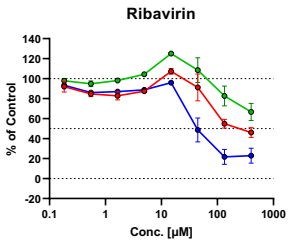

A
HCoV-229E
Antiviral activity (%)
120
100
80
60
40
20
0
Adenosine
Guanosine
Uridine
Cytidine
0
6.25
12.5
25
50
100
15 µM s2U + Nucleoside [µM]
B
BHK-21
DENV2 copies / 18S copies
1
0.1
0.01
0.001
0.0001
0.00001
ns
ns
**
rgWT
rgNS5-G605V
0
24
48
72
96
hours post infection
C
VeroE6
DENV2 copies / ACTB copies
10
1
0.1
0.01
0.001
0.0001
ns
ns
***
****
rgWT
rgNS5-G605V
0
24
48
72
96
hours post infection

Figure S5

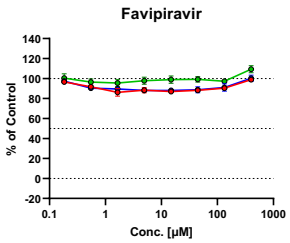
A



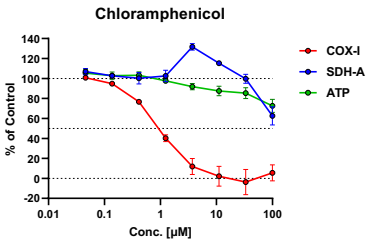
B



C



D



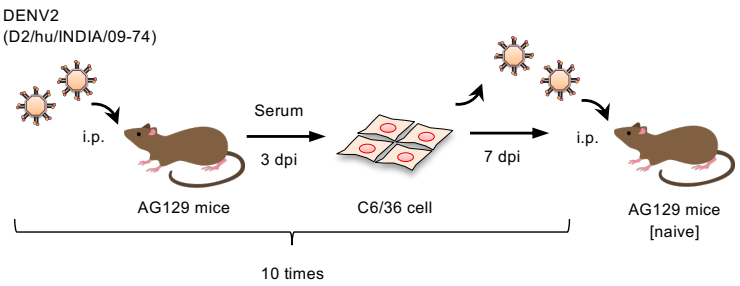
E

	IC_{50} (µM) [a]			
Protein	2-Thiouridine	Ribavirin	Favipiravir	Chloramphenicol [e]
COX-I [b]	>50	97 ± 33	>400	0.97 ± 0.060
SDH-A [c]	>50	45 ± 1.4	>400	>100
ATP [d]	>50	>400	>400	>100

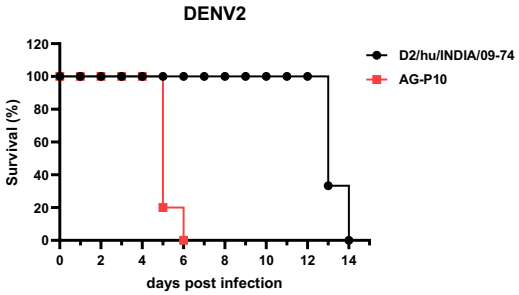
a : IC_{50} values represent mean values ± SEM from three independently performed experiments (n = 2).
b : Cytochrome *c* oxidase (COX-I) is the mitochondrial DNA-encoded protein.
c : Succinate dehydrogenase A subunit (SDH-A) is the nuclear DNA-encoded protein.
d : Cell viability monitored as ATP level was measured using Cell-Titer Glo® 2.0 Assay.
e : Chloramphenicol was used as control compound for specific inhibition of COX-I synthesis.

Figure S6

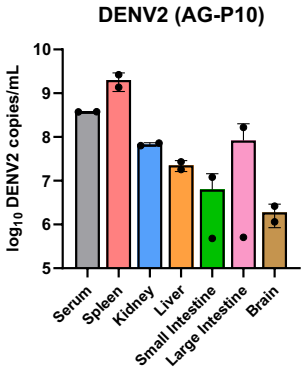
A



B



C

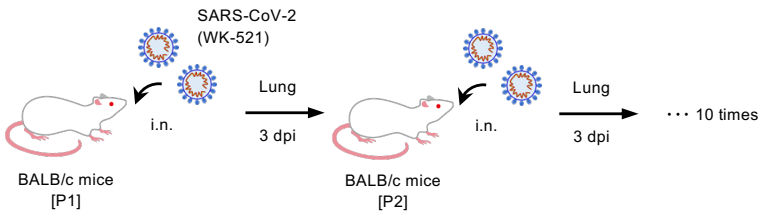


D

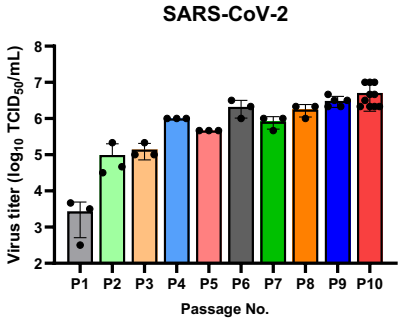
Viral protein	NS4B	NS5
Amino acid substitution	A119T	E802Q

Figure S7

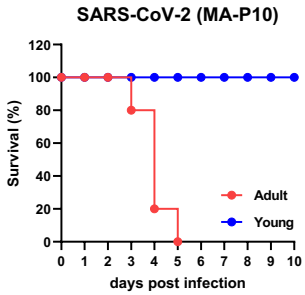
A



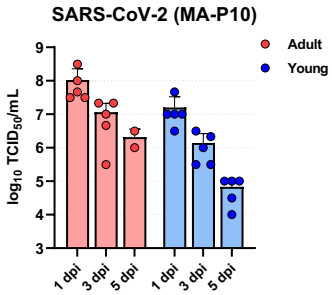
B



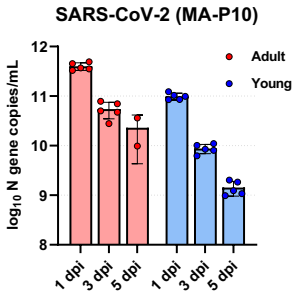
C



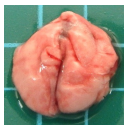
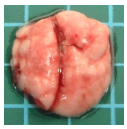
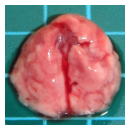
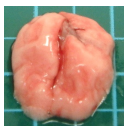
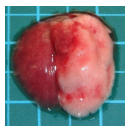
D



E



F

Group	1 dpi	3 dpi	5 dpi
Young (5 wks)			
Adult (35-45 wks)			dead

G

Viral protein	Amino acid substitution
nsp4	A457V
nsp5	E270G
nsp9	R39K
nsp16	K160R
Spike	Q498H
Nucleocapsid	T205I

Figure S8

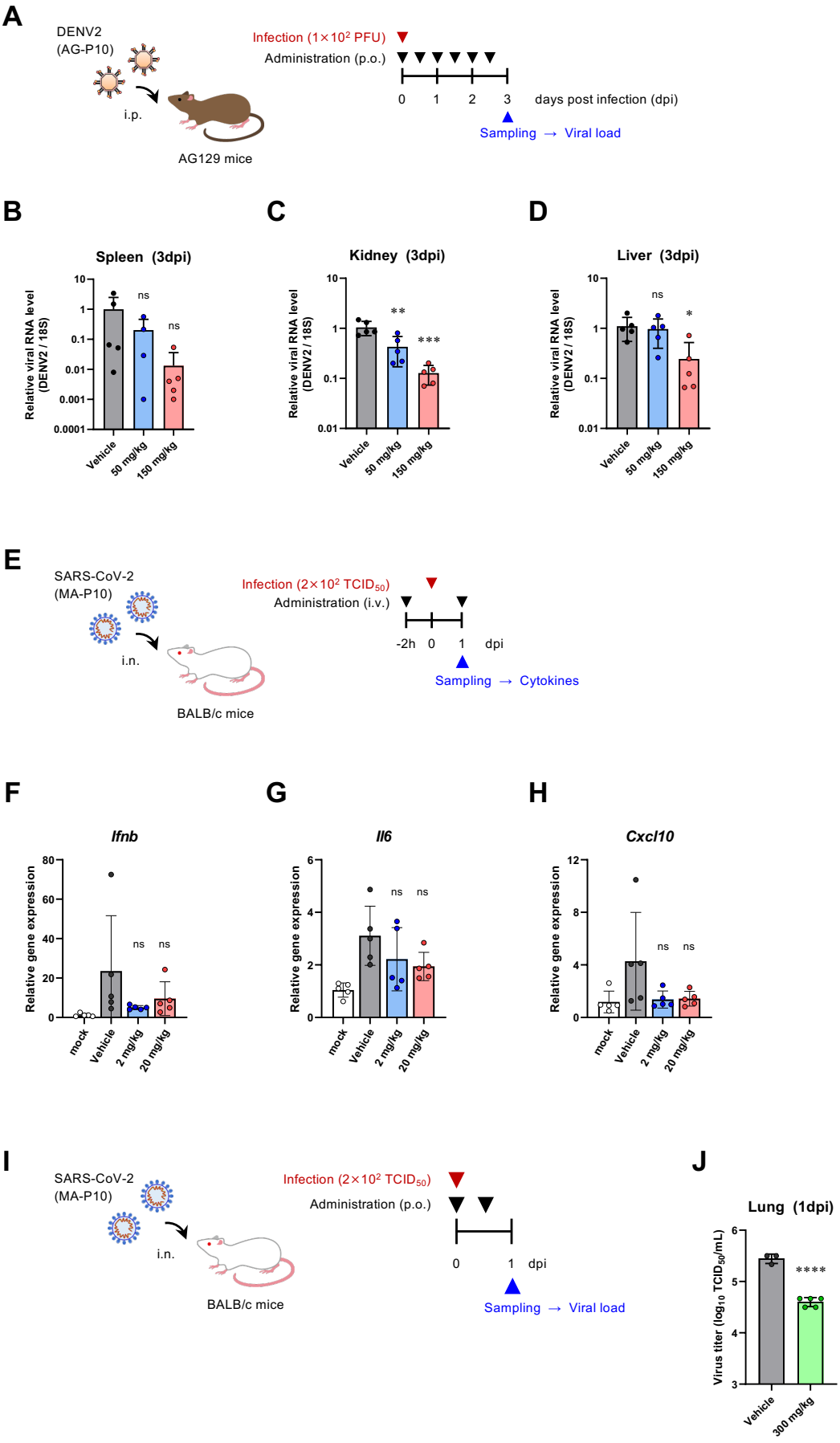

A
DENV2
(AG-P10)
i.p.
AG129 mice
Infection (1×10² PFU)
Administration (p.o.)
0 1 2 3 days post infection (dpi)
Sampling → Viral load
B
Spleen (3dpi)
Relative viral RNA level (DENV2 / 18S)
ns
ns
Vehicle
50 mg/kg
150 mg/kg
C
Kidney (3dpi)
**
***
D
Liver (3dpi)
ns
*
E
SARS-CoV-2
(MA-P10)
i.n.
BALB/c mice
Infection (2×10² TCID50)
Administration (i.v.)
-2h 0 1 dpi
Sampling → Cytokines
F
Ifnb
Relative gene expression
ns
ns
mock
Vehicle
2 mg/kg
20 mg/kg
G
Il6
H
Cxcl10
I
SARS-CoV-2
(MA-P10)
i.n.
BALB/c mice
Infection (2×10² TCID50)
Administration (p.o.)
0 1 dpi
Sampling → Viral load
J
Lung (1dpi)
Virus titer (log10 TCID50/mL)
****
Vehicle
300 mg/kg

Figure S9

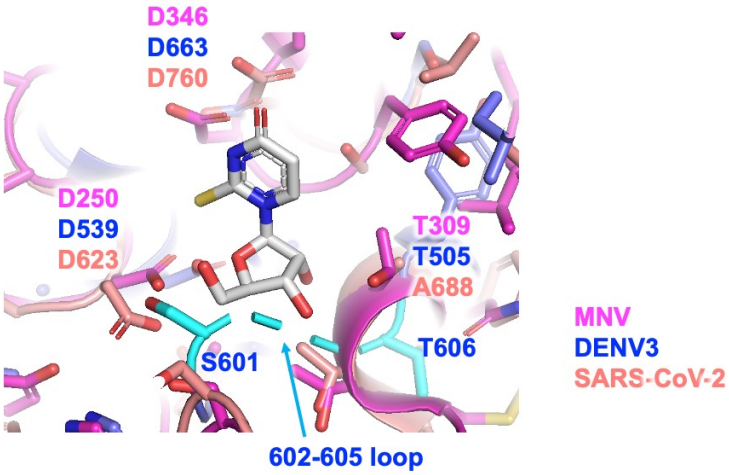
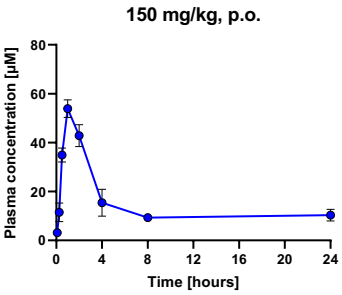

D346
D663
D760
D250
D539
D623
T309
T505
A688
S601
T606
602-605 loop
MNV
DENV3
SARS-CoV-2

Figure S10

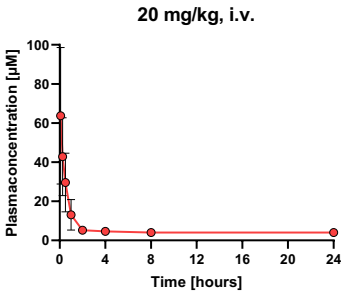
A

Compound	Solubility (μM)		Membrane Permeability ($x10^{-6}$ cm/sec)	Metabolic stability (mL/min/kg)		Protein binding (Unbound fraction)	
	JP1 (pH1.2)	JP2 (pH6.8)	PAMPA (pH6.5)	Human liver microsome	Mouse liver microsome	Human plasma	Mouse plasma
2-Thiouridine	78	>100	0.013	<22.0 (<17.6μL/min/mg)	<69.8 (<17.6μL/min/mg)	0.70	0.73

B



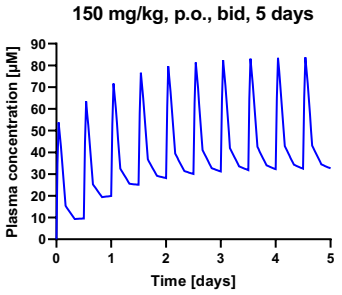
C



D

Compound	Route	Dose (mg/kg)	C_0 (μg/mL)	C_{max} (μg/mL)	T_{max} (hr)	$t_{1/2}$ (hr)	AUC∞ (μg/mL·hr)	CL_{tot} or CL_{tot}/F (mL/hr/kg)	V_{dz} or V_{dz}/F (mL/kg)	BA (F) (%)
2-Thiouridine	p.o.	150		14.0	1	31.2	198	757	34025	17.3
	i.v.	20	20			80.7	153	131.1	15260	

E



F

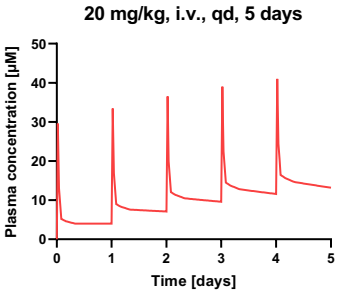


Figure S11

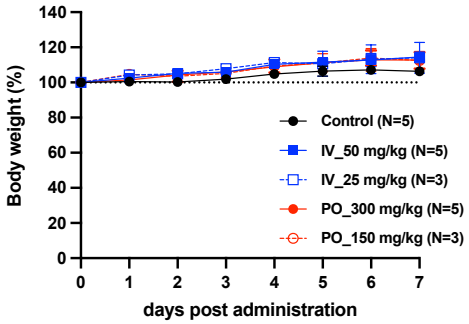

Body weight (%)
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
days post administration
Control (N=5)
IV_50 mg/kg (N=5)
IV_25 mg/kg (N=3)
PO_300 mg/kg (N=5)
PO_150 mg/kg (N=3)

Table S1

MTT / Resazurin Assay Results			EC_{50} (μM) [a]			
Virus	Strain	Cell	Ribavirin	Favipiravir	Remdesivir	GS-441524
DENV1	D1/hu/PHL/10-07	BHK-21	46	18		
DENV2	D2/hu/INDIA/09-74	BHK-21	32	20		
	AG-P10 (Mouse-adapted)	BHK-21	77	16		
DENV3	D3/hu/Thailand/00-40	BHK-21	61	13		
DENV4	D4/hu/Solomon/09–11	BHK-21	91	5.2		
ZIKV	MR766	BHK-21	18	39		
YFV	17D-204	BHK-21	15	24		
JEV	Beijing-1	BHK-21	33	80		
WNV	NY99	BHK-21	43	36		
CHIKV	SL10571	BHK-21	46	8.1		
HCoV	229E	MRC5			0.071	0.76
HCoV	OC43	MRC5			0.23	2.0
SARS-CoV-2	WK-521 (Ancestral)	VeroE6/TMPRSS2			1.5	
	QK002 (Alpha)	VeroE6/TMPRSS2			2.0	
	TY8-612 (Beta)	VeroE6/TMPRSS2			0.91	
	TY7-501 (Gamma)	VeroE6/TMPRSS2			0.91	
	TY11-927 (Delta)	VeroE6/TMPRSS2			1.7	
	TY38-873 (Omicron BA.1)	VeroE6/TMPRSS2			0.67	
	TY40-385 (Omicron BA.2)	VeroE6/TMPRSS2			1.1	
	TY41-703 (Omicron BA.4)	VeroE6/TMPRSS2			0.77	
	TY41-702 (Omicron BA.5)	VeroE6/TMPRSS2			1.0	
	TY41-796 (Omicron_BQ.1.1)	VeroE6/TMPRSS2			1.2	
	TY41-795 (Omicron_XBB.1)	VeroE6/TMPRSS2			0.78	
	MA-P10 (Mouse-adapted)	VeroE6/TMPRSS2			1.3	
RABV	HEP	BHK-21	16	5.9		
LACV	ATCC VR-1834	MDBK	2.5	20		
LPHV	11SB17	KB	5.3	>100		
LCMV	Armstrong	KB	1.8	21		
JUNV	Candid #1	293T	2.6	10		
SFTSV	ArtLN/2017	MDCK	5.4	7.3		
RVFV	MP12	MDCK	17	21		
TPMV	VRC-66412	VeroE6	6.7	31		
IAV H5N1	A/Hong Kong/483/97	A549	26	6.2		
IAV H7N9	A/Anhui/1/2013	MA104/TMPRSS2	3.7	12		
Resazurin Assay Results			EC_{50} (μM) [a]		Fold change [c]	
Virus	Strain	Cell	Ribavirin	Favipiravir	Ribavirin	Favipiravir
rgDENV2	Wild type	BHK-21	16	7.9		
rgDENV2	NS5-G605V	BHK-21	37	17	2.3	2.2
qRT-PCR Assay Results			EC_{90} (μM) [b]			
Virus	Strain	Cell	Remdesivir			
SARS-CoV	Hanoi	VeroE6	2.7			
SARS-CoV-2	WK-521 (Ancestral)	VeroE6/TMPRSS2	3.3			

a : EC_{50} values represent mean values from at least three independently performed experiments (n = 2).

b : EC_{90} values represent mean values from a single experiment with biological triplicates.

c : Fold change is calculated from the ratio of rgNS5-G605V/rgWT.

Table S2

Bacterial reverse mutation test of s2U

		dose μg/plate	Number of revertants/plate (Mean ± SD)				
			TA100	TA1535	WP*uvrA*	TA98	TA1537
S9 mix (–)	s2U	0	115 ± 17	11 ± 2	23 ± 5	16 ± 3	6 ± 1
		1,667	112 ± 11	6 ± 1	27 ± 6	19 ± 1	8 ± 1
		5,000	98 ± 2	10 ± 1	28 ± 2	11 ± 4	4 ± 1
S9 mix (+)	s2U	0	115 ± 17	14 ± 3	24 ± 3	24 ± 3	12 ± 1
		1,667	126 ± 6	11 ± 4	34 ± 6	37 ± 4	8 ± 2
		5,000	119 ± 8	11 ± 4	29 ± 2	13 ± 1	5 ± 1
S9 mix (–)	AF2 [*a]	[*b]	714 ± 31		102 ± 6	294 ± 4	
	NaN_3	0.5		432 ± 76			
	9-AA [*a]	80					546 ± 19
S9 mix (+)	2-AA [*a]	[*c]	1441 ± 89	326 ± 33	1158 ± 76	424 ± 30	241 ± 20

***In vitro* gene mutation test of s2U using the Hprt genes in V79 cells**

		dose μg/ml	relative cell survival (%)	mutation frequency (x 10^{-6}) [*d]
S9 mix (–)	s2U	0	100	12.72
		51.6	107	10.84
		77.3	107	10.11
		116	87	11.11
		174	102	14.94
		261	76	17.44
S9 mix (+)	s2U	0	100	9.95
		51.6	103	11.37
		77.3	121	12.31
		116	95	11.65
		174	86	10.89
		261	99	15.96
S9 mix (+)	3MC [*e]	5	77	598.74

*a : positive control: AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl)–acrylamide, 9-AA: 9-aminoacridine, 2-AA: 2-aminoanthracene .

*b : AF2 conc (μg/plate): 0.01 (TA100), 0.01 (WPuvrA), 0.1 (TA98).

*c : 2AA conc (μg/plate): 1 (TA100), 2 (TA1535), 10 (WPuvrA), 0.5 (TA98), 2 (TA1537).

*d : MF is determined as; [total of the number of mutant colonies / (abCE × total of the number of seeding cells)], where abCE is determined as the ratio of the number of colonies to the number of cells inoculated after phenotypic expression period.

*e : 3MC (3-methylcholanthrene).

Table S3

MTT / Resazurin Assay

Virus	Strain	Cell	MOI	Method	Period
DENV1	D1/hu/PHL/10-07	BHK-21	10 $TCID_{50}$	Resazurin	5 days
DENV2	D2/hu/INDIA/09-74	BHK-21	MOI=0.01	Resazurin	4 days
	AG-P10 (Mouse-adapted)	BHK-21	MOI=0.01	Resazurin	4 days
DENV3	D3/hu/Thailand/00-40	BHK-21	10 $TCID_{50}$	Resazurin	5 days
DENV4	D4/hu/Solomon/09–11	BHK-21	10 $TCID_{50}$	Resazurin	5 days
ZIKV	MR766	BHK-21	MOI=0.01	MTT	3 days
YFV	17D-204	BHK-21	MOI=0.01	MTT	3 days
JEV	Beijing-1	BHK-21	10 $TCID_{50}$	MTT	3 days
		VeroE6	MOI=0.01	MTT	
WNV	NY99	BHK-21	10 $TCID_{50}$	MTT	3 days
		VeroE6	MOI=0.01	MTT	
CHIKV	SL10571	BHK-21	10 $TCID_{50}$	MTT	3 days
		VeroE6	MOI=0.01	MTT	
HCoV	229E	MRC5	MOI=0.005	Resazurin	3 days
HCoV	OC43	MRC5	MOI=0.01	Resazurin	3 days
SARS-CoV-2	WK-521 (Ancestral)	VeroE6/TMPRSS2	10 $TCID_{50}$	MTT	2 days
	QK002 (Alpha)	VeroE6/TMPRSS2	10 $TCID_{50}$	MTT	2 days
	TY8-612 (Beta)	VeroE6/TMPRSS2	10 $TCID_{50}$	MTT	2 days
	TY7-501 (Gamma)	VeroE6/TMPRSS2	10 $TCID_{50}$	MTT	2 days
	TY11-927 (Delta)	VeroE6/TMPRSS2	10 $TCID_{50}$	MTT	2 days
	TY38-873 (Omicron BA.1)	VeroE6/TMPRSS2	10 $TCID_{50}$	MTT	3 days
	TY40-385 (Omicron BA.2)	VeroE6/TMPRSS2	10 $TCID_{50}$	MTT	3 days
	TY41-703 (Omicron BA.4)	VeroE6/TMPRSS2	10 $TCID_{50}$	MTT	3 days
	TY41-702 (Omicron BA.5)	VeroE6/TMPRSS2	10 $TCID_{50}$	MTT	3 days
	TY41-796 (Omicron_BQ.1.1)	VeroE6/TMPRSS2	10 $TCID_{50}$	MTT	2 days
	TY41-795 (Omicron_XBB.1)	VeroE6/TMPRSS2	10 $TCID_{50}$	MTT	3 days
	MA-P10 (Mouse-adapted)	VeroE6/TMPRSS2	10 $TCID_{50}$	MTT	2 days
RABV	HEP	BHK-21	10 $TCID_{50}$	MTT	3 days
LACV	ATCC VR-1834	MDBK	10 $TCID_{50}$	MTT	3 days
LPHV	11SB17	KB	10 $TCID_{50}$	MTT	4 days
LCMV	Armstrong	KB	10 $TCID_{50}$	MTT	4 days
JUNV	Candid #1	293T	10 $TCID_{50}$	MTT	4 days
SFTSV	ArtLN/2017	MDCK	10 $TCID_{50}$	MTT	4 days
RVFV	MP12	MDCK	10 $TCID_{50}$	MTT	3 days
TPMV	VRC-66412	VeroE6	10 $TCID_{50}$	MTT	4 days
IAV H5N1	A/Hong Kong/483/97	A549	10 $TCID_{50}$	MTT	3 days
IAV H7N9	A/Anhui/1/2013	MA104/TMPRSS2	10 $TCID_{50}$	MTT	3 days
rgDENV2	Wild type	BHK-21	MOI=0.1	Resazurin	4 days
rgDENV2	NS5-G605V	BHK-21	MOI=0.1	Resazurin	4 days

Table S4

qRT-PCR (qPCR) Assay

Virus	Strain	Cell	MOI	Period	Internal control
DENV2	D2/hu/INDIA/09-74	VeroE6	MOI=0.05	48 hpi	*ACTB*
		Huh7	MOI=0.05	48 hpi	*ACTB*
ZIKV	MR766	VeroE6	MOI=0.05	48 hpi	*ACTB*
YFV	17D-204	VeroE6	MOI=0.05	48 hpi	*ACTB*
JEV	Beijing-1	VeroE6	MOI=0.01	48 hpi	*ACTB*
WNV	NY99	VeroE6	MOI=0.05	24 hpi	*ACTB*
CHIKV	SL10571	VeroE6	MOI=0.01	24 hpi	*ACTB*
HCoV	229E	MRC5	MOI=0.005	48 hpi	*ACTB*
HCoV	OC43	VeroE6	MOI=0.1	48 hpi	*ACTB*
SARS-CoV	Hanoi	VeroE6/TMPRSS2	MOI=0.01	24 hpi	*ACTB*
MERS-CoV	EMC2012	VeroE6/TMPRSS2	MOI=0.01	24 hpi	*ACTB*
SARS-CoV-2	WK-521 (Ancestral)	VeroE6/TMPRSS2	MOI=0.01	24 hpi	*ACTB*
	QK002 (Alpha)	VeroE6/TMPRSS2	MOI=0.01	24 hpi	*ACTB*
	TY8-612 (Beta)	VeroE6/TMPRSS2	MOI=0.01	24 hpi	*ACTB*
	TY7-501 (Gamma)	VeroE6/TMPRSS2	MOI=0.01	24 hpi	*ACTB*
	TY11-927 (Delta)	VeroE6/TMPRSS2	MOI=0.01	24 hpi	*ACTB*
	TY41-796 (Omicron_BQ.1.1)	VeroE6/ACE2/TMPRSS2	MOI=0.01	24 hpi	*ACTB*
	TY41-795 (Omicron_XBB.1)	VeroE6/ACE2/TMPRSS2	MOI=0.01	24 hpi	*ACTB*
RABV	HEP	BHK-21	MOI=0.1	24 hpi	*18S rRNA*
RVFV	MP12	VeroE6	MOI=0.01	24 hpi	*ACTB*
HSV-1	F	VeroE6	MOI=0.1	24 hpi	*ACTB*

IFA

Virus	Strain	Cell	MOI	Period	Antibody
DENV2	D2/hu/INDIA/09-74	VeroE6	MOI=0.1	48 hpi	Envelope
CHIKV	SL10571	VeroE6	MOI=0.05	24 hpi	E1
HCoV	OC43	VeroE6/TMPRSS2	MOI=0.1	48 hpi	Nucleocapsid
SARS-CoV-2	WK-521 (Ancestral)	VeroE6/TMPRSS2	MOI=0.005	24 hpi	Nucleocapsid
	TY41-796 (Omicron_BQ.1.1)	VeroE6/ACE2/TMPRSS2	MOI=0.1	24 hpi	Nucleocapsid
	TY41-795 (Omicron_XBB.1)	VeroE6/ACE2/TMPRSS2	MOI=0.1	24 hpi	Nucleocapsid

Plaque Assay

Virus	Strain	Cell	Overlay	Period
DENV2	D2/hu/INDIA/09-74	BHK-21	1% Methylcellulose	4 days
SARS-CoV-2	WK-521 (Ancestral)	VeroE6/ACE2/TMPRSS2	0.5% Agar	2 days
	TY41-795 (Omicron_XBB.1)	VeroE6/ACE2/TMPRSS2	0.5% Agar	2 days

Table S5

Virus		Sequence (5'→3')
DENV2	Fw	AGTGGACACGAGAACCCAAGA
	Rv	TTCGGCCGTGATTTTCATTAG
	Pr	FAM/AAAAGAAGGCACGAAGAA/MGB
ZIKV	Fw	GACATGGCTTCGGACAG
	Rv	CTTTGCCAAAAAGTCCACA
YFV	Fw	GCTAATTGAGGTGYATTGGTCTGC
	Rv	CTGCTAATCGCTCAAMGAACG
	Pr	56-FAM/ATCGAGTTG/ZEN/CTAGGCAATAAACAC/3IABkFQ
JEV	Fw	AGCTGGGCCTTCTGGT
	Rv	CCCAAGCATCAGCACAAG
	Pr	56-FAM/CTTCGCAAG/ZEN/AGGTGGACGGCCA/3IABkFQ
WNV	Fw	AAGTTGAGTAGACGGTGCTG
	Rv	AGACGGTTCTGAGGGCTTAC
	Pr	56-FAM/GCTCAACCCCAGGAGGACTGG/MGBEQ
CHIKV	Fw	AAGCTYCGCGTCCTTTACCAAG
	Rv	CCAAATTGTCCYGGTCTTCCT
	Pr	56-FAM/CCAATGTCT/ZEN/TCAGCCTGGACACCTTT/3IABkFQ
HCoV-229E	Fw	CAGTCAAATGGGCTGATGCA
	Rv	CAAAGGGCTATAAAGAGAATAAGGTATTCT
	Pr	56-FAM/TGAACCACA/ZEN/ACGTGGTCGTCAGGG/3IABkFQ
HCoV-OC43	Fw	CGATGAGGCTATTCCGACTAGGT
	Rv	CTTCCTGAGCCTTCAATATAGTAACC
	Pr	56-FAM/TCCGCCTGG/ZEN/CACGGTACTCCCT/3IABkFQ
SARS-CoV	Fw	GGAGCCTTGAATACACCCAAAG
	Rv	GCACGGTGGCAGCATTG
	Pr	56-FAM/CCACATTGG/ZEN/CACCCGCAATCC/3IABkFQ
MERS-CoV	Fw	CAAAACCTTCCCTAAGAAGGAAAAG
	Rv	GCTCCTTTGGAGGTTCAGACAT
	Pr	56-FAM/ACAAAAGGC/ZEN/ACCAAAAGAAGAATCAACAGACC/3IABkFQ
SARS-CoV-2	Fw	CACATTGGCACCCGCAATC
	Rv	GAGGAACGAGAAGAGGCTTG
	Pr	56-FAM/ACTTCCTCA/ZEN/AGGAACAACATTGCCA/3IABkFQ
RABV	Fw	GCCACGGTTATTGCTGCAT
	Rv	CTCCCAAATAGCCCCCTAGAA
	Pr	FAM/CCCTCATGAGATGTC/MGB
RVFV	Fw	TTCTTTGCTTCTGATACCCTCTG
	Rv	GTTCCACTTCCTTGCATCATCTG
	Pr	56-FAM/TTGCACAAG/ZEN/TCCACACAGGCCCCT/3IABkFQ
HSV-1	Fw	GGGCCGTGATTTTGTTTGTC
	Rv	CCGCCAAGGCATATTTGC
	Pr	FAM/TAGTGGGCCTCCATGGG/MGB
ACTB (*C.aethiops*)	Fw	GCTGCCCTGAGGCTCTCTT
	Rv	TGATGGAGTTGAAGGTAGTTTCATG

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