3D8, a nucleic acid-hydrolyzing scFv, confers antiviral activity against SARS-CoV-2 and multiple coronaviruses *in vitro*

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Abstract

The current pandemic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pose a critical public health threat worldwide. Coronaviruses (subfamily Orthocoronavirinae, family Coronaviridae, order Nidovirales) are a group of enveloped positive-sense single-stranded RNA viruses. Six pathogenic human coronaviruses, likely zoonotic viruses, cause the common cold in humans. A new emerging coronavirus, SARS-CoV-2, become a crucial etiology for the Coronavirus-induced disease 19 (COVID-19). However, effective therapeutics and vaccines against multiple coronaviruses remain unavailable. This study aimed to investigate an antiviral molecule, single chain variable fragment (scFv), against SARS-CoV-2 and other coronaviruses. 3D8, a recombinant scFv, exhibits broad-spectrum antiviral activity against DNA and RNA viruses owing to its nucleic acid-hydrolyzing property. Here, we report that 3D8 scFv inhibited the replication of SARS-CoV-2, human coronavirus OC43 (HCoV-OC43), and porcine epidemic diarrhea virus (PEDV). Our results revealed the prophylactic and therapeutic effects of 3D8 scFv against SARS-CoV-2 in Vero E6 cells. Immunoblot and plaque assays showed the absence of coronavirus nucleoproteins and infectious particles in 3D8 scFv-treated cells, respectively. In addition, we observed the antiviral effects of 3D8 against HCoV-OC43 and PEDV. In conclusion, this study provides insights into the broad-spectrum antiviral agent of 3D8 scFv; thus, it could be considered a potential antiviral countermeasure against SARS-CoV-2 and zoonotic coronaviruses.

Key points (Main message):

1. 3D8, a nucleic acid-hydrolyzing scFv, exhibits potent prophylactic and therapeutic antiviral effects on SARS-CoV-2.

2. 3D8 exhibits broad-spectrum antiviral activity against multiple coronaviruses: hCoV OC43 and PEDV.
3. 3D8 potentially degrades viral RNA.

Introduction

Coronaviruses (subfamily Orthocoronavirinae in the family Coronaviridae of the order Nidovirales) belong a group of enveloped viruses containing a single-stranded positive-sense RNA genome (1, 2). Divergent coronaviruses constitute four genetic lineage groups, including alphacoronaviruses, betacoronaviruses, gammacoronaviruses, and deltacoronaviruses. These viruses infect a broad range of natural reservoir hosts, including humans, bats, rodents, pigs, and camels (3). Currently, six coronavirus species cause infectious diseases in humans. Four coronaviruses, human coronavirus OC43 (HCoV-OC43), 229E, NL63, and HKU1, induce flu-like common cold symptoms in immunocompromised individuals (4, 5). Two highly transmissible and pathogenic viruses, severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) and Middle East respiratory syndrome coronavirus (MERS-CoV), are associated with fatal illness involving pneumonia and respiratory disorders (6). In late December 2019, the city of Wuhan, in the Hubei province of China, reported a few cases of patients with severe pneumonia of an unknown etiological agent (7), which was identified as the novel coronavirus disease (COVID-19) caused by SARS-CoV-2 (8). SARS-CoV-2 has several similarities to SARS-CoV and binds to the common host cell receptor, angiotensin-converting enzyme 2 (ACE-2), and Transmembrane Serine Protease 2 (TMPRSS2) (9, 10); however, the novel strain is genetically distinct from SARS-CoV-1 (11). The novel coronavirus SARS-CoV-2 is transmitted through species barriers from bats to humans (12). COVID-19 is characterized by influenza-like symptoms ranging from mild to

severe lung injury as well as multi-organ failure, leading to death in patients with comorbidities (13). This novel virus has led to a global pandemic, which resulted in unparalleled public health emergencies (14). As of November 24, 2020, it has rapidly spread to 220 countries and territories, infecting over 58.7 million people including more than 1.38 million deaths (15) [https://covid19.who.int].

The rapid and widespread emergence of SARS-CoV-2 presents the urgent need for antiviral countermeasures (4). Currently, there are no available therapeutics against human coronaviruses. A variety of antivirals are repositioning on clinical trials Nucleoside analogues (remdesivir, favipiravir and ribavirin), protease inhibitors (disulfiram, lopinavir and ritonavir), antiparasitic drugs (chloroquine and hydrochloroquine), pegylated interferons, monoclonal antibodies, oligonucleotide-based therapies, peptides and small-molecule drugs are contemplated for possible therapeutic agents against SARS-CoV-2 (16, 17). In particular, remdesivir, which inhibits viral RNA polymerases, is proposed as a potent antiviral against SARS-CoV-2 and clinical improvement has been observed in patients under compassionate use (18, 19)

3D8, a 27-kDa recombinant antibody fragment, is a single chain variable fragment (scFv) that comprises a variable region of a heavy chain covalently linked to the corresponding variable region of a light chain. It was originally found in autoimmune-prone Murphy Roths Large (MRL) mice (20). The 3D8 scFv possessing the nucleic acid hydrolyzing activity degrades viral DNA and/or mRNA in the infected cells (21, 22). This protein has the broad-spectrum antiviral effects against herpes simplex virus (HSV), influenza virus, and pseudorabies (PRV) virus *in vitro*. 3D8 exhibited *in vivo* antiviral therapeutic effects against PRV in C57BL/6 mice. The transmission of avian influenza and bronchitis viruses was suppressed in transgenic chickens expressing the 3D8 scFv

(23-26). However, the antiviral activity of the 3D8 scFv against SARS-CoV-2 and other coronaviruses remains unknown.

Here, this study aimed to investigate the antiviral activity of 3D8 scFv against emerging coronaviruses *in vitro*. These data provide insight into a broad-spectrum antiviral agent of scFv against SARS-CoV-2 and multiplex coronaviruses.

Results

3D8 inhibits SARS-CoV-2 in a dose-dependent manner

To determine the antiviral activity of the 3D8 scFv against SARS-CoV-2, different concentrations of the scFv were applied to Vero E6 cells after virus challenge. SARS-CoV-2 replication in cultures treated with various doses of 3D8 was quantified using RT-qPCR **(Fig 1A)**. The replication of SARS-CoV-2 significantly decreased in a 3D8 dose-dependent manner. The 10 µM and 5 µM concentrations of 3D8 effectively inhibited viral replication by up to approximately 90% and 75%, respectively, compared to the non-treatment group. The production of infectious virus particles was quantified by performing the plaque assay **(Fig 1B)**. The viral titer of SARS-CoV-2 was reduced in a 3D8 dose-dependent manner. In particular, when treated with 10 µM of 3D8, the titer of the virus was reduced by 10 times compared to the non-treatment group. Continual treatment with 3D8 showed antiviral activity against SARS-CoV-2 at an effective concentration (EC50) of 4.25 µM **(Fig 1C)**. Moreover, this scFv did not show cytotoxicity in Vero E6 cells treated with the 3D8 scFv at concentrations ranging from 1 µM to 10 µM **(Fig 1D)**. However, cytotoxicity was noted at a dose of 40 µM.

3D8 effectively inhibits SARS-CoV-2 in pretreated cells (prophylactic effect)

We determined the prophylactic antiviral activity of the 3D8 scFv against SARS-CoV-2 in pretreated cell cultures. A significant reduction in the SARS-CoV-2 N gene copies was observed upon treatment with 10 μM 3D8 scFv **(Fig 2A)**. The gene copy number of the SARS-CoV-2 E gene and RdRp gene was reduced by 99.6% and 99.4%, respectively (data not shown). The N protein of SARS-CoV-2 was not observed after treatment with the 3D8 scFv **(Fig 2B)**. Furthermore, we determined the inhibitory effect of 3D8 on the production of infectious particles of SARS-CoV-2. The production of infectious virus particles was more than 10 times lower in the scFv-treated group than in the control group **(Fig 2C)**. Collectively, these data demonstrated that 3D8 has a prophylactic effect on SARS-CoV-2 infection.

3D8 effectively inhibits SARS-CoV-2 in post-treated cells (therapeutic effect)

To determine the therapeutic effect of 3D8 at 2 h post-infection (p.i.), we assessed the inhibitory activity of SARS-CoV-2 based on the reduction of the relative gene copy number **(Fig 2D)**. 3D8 treatment resulted in a decrease in the gene copy number of the N gene by 63.4%. Western blot analysis revealed the absence of N protein expression in the 3D8-treated samples **(Fig 2E)**. The production of infectious particles of SARS-CoV-2 in the 3D8-treated group was 10 times lower than that in the control group **(Fig 2F)**. The reduced viral gene copy number, lack of infectious virus particles, and absence of N protein expression indicated the therapeutic effect of 3D8 upon SARS-CoV-2 infection.

3D8 possesses broad-spectrum antiviral activity against multiple coronaviruses

To determine the antiviral activity of 3D8 against other coronaviruses, viral gene copies and infectious particles were examined after HCoV-OC43 and PEDV infections. The 3D8-treated group

138 revealed effective inhibition of viral replication upon HCoV-OC43 infection. The load of HCoV-
139 OC43 RNA was significantly reduced in a 3D8 dose-dependent manner **(Fig 3A)**. The expression
140 of viral proteins was inhibited upon treatment with 3D8 **(Fig 3B)**. 3D8 effectively inhibited the
141 replication of HCoV-OC43 with the EC_{50} value of 1.40 μM **(Fig 3C)**. Immunohistochemistry
142 analysis exhibited a reduction in HCoV-OC43 replication upon 3D8 treatment **(Fig 3D)**.
143 Treatment with 3D8 resulted in the effective inhibition of viral replication upon PEDV infection.
144 The load of PEDV RNA was significantly suppressed in a 3D8 dose-dependent manner **(Fig 4A)**.
145 The expression of viral proteins was reduced upon treatment with 3D8 **(Fig 4B)**. The EC_{50} value of
146 3D8 against PEDV was 1.10 μM **(Fig 4C)**. Immunohistochemistry analysis revealed a reduction in
147 PEDV replication upon 3D8 treatment **(Fig 4D)**. These data demonstrated the broad-spectrum
148 activity of 3D8 against multiple zoonotic coronaviruses.
149

150 ## Discussion

151 Three novel human coronaviruses have emerged during the past two decades (*3, 5*). The outbreak
152 of COVID-19 occurred in late December in Wuhan (China) and rapidly became a global pandemic
153 (*7*). The public health emergency caused by the SARS-CoV-2 outbreak presents the demand for
154 countermeasures against emerging and re-emerging zoonotic coronaviruses. As the virus
155 disseminates, efforts are being made to mitigate transmission via public health interventions
156 including social distancing, quarantine, and contact tracing. However, therapeutics and vaccines
157 against SARS-CoV-2 are urgently needed for the effective control of outbreaks. In this study, we
158 demonstrated that 3D8, a nucleic acid-hydrolyzing scFv, inhibited the replication of SARS-CoV-2
159 and multiple coronaviruses *in vitro*.
160

The scFv is a molecule derived from an antibody composed of variable region of heavy and light chains linked with peptides(*20*). scFv has applied for basic researches, biotechnological and medicinal applications such as cancer therapy and potential alternatives to conventional diagnostic approaches(*25, 26*). scFv has various advantages over traditional monoclonal antibodies such as ease of genetic manipulation, rapid molecular design and characterization, greatly reduced size, production of antibodies against viral proteins, and various expression systems. Neutralizing scFv against N protein protects piglets from PEDV infection (*27*). The orally administered piglets had no or mild clinical symptoms, intestinal lesions and significantly increased survival rates. 3D8 is a unique scFv that possesses a broad-spectrum nuclease activity and confers antiviral protection from a variety of viruses including DNA and RNA viruses **(Figure 5)** (*28, 29*). 3D8 scFv previously showed the antiviral effect against infectious Bronchitis virus, a member of gammacoronaviruses in transgenic chickens expressing 3D8 scFv (*24*). The antiviral activity of 3D8 scFv against SARS-CoV-2 and other coronaviruses remained to be examined.

Our study demonstrated that 3D8 confers effective antiviral activity against SARS-CoV-2, HCoV-OC43, and PEDV *in vitro*. The function of 3D8 inhibited the replication of multiple coronaviruses in a dose-dependent manner. The reduction of infectious virus particles accounted for the nuclease activity of 3D8, indicating the degradation of viral nucleic acids prohibited the production of viral genomes and proteins. Upon infection with HCoV-OC43 and PEDV, the therapeutic treatment of 3D8 scFv showed the inhibition of viral replication and protein expression, indicating the antiviral activity of multiple coronaviruses. The previous studies demonstrated the biochemical characteristics and robust antiviral activity of 3D8 scFv against classical swine fever virus and herpes simplex virus *in vitro* (*22, 28*). The 3D8 expressing transgenic mice and chickens exhibited the *in vivo* antiviral activity against influenza virus and PRV (*23, 29*). The cellular entry mechanism

of 3D8 revealed a caveolin-dependent manner without a carrier (*30*). The intranasal transfer of 3D8 scFv into a mouse described the presence of the protein in the epithelial barrier of lung tissues (*31*). Taken together, the 3D8, a nucleic-acid hydrolyzing mini-antibody, may be a potential candidate for antivirals due to the broad spectrum, the easy penetration to the cell, and the accessibility to the lung *in vivo*.

Infection of coronaviruses have significantly impacted on humans and livestocks (*32*). However, the effective antiviral countermeasures against the viruses are still unavailable. HCoV-OC43, belongs to lineage betacoronaviruses, is associated with mild common cold in humans. HCoV-OC43 infection occurs frequently in early childhood and causes acute respiratory tract illness, pneumonia and croup (*33*). PEDV, a member of alphacoronaviruses, is a highly contagious coronavirus that causes severe diarrhea and death in neonatal piglets (*27*). All of age groups are highly susceptible to PEDV infection with neonatal piglets under 2 weeks of age, showing the highest mortality rates. In this study, HCoV-OC43 and PEDV were effectively reduced for the replication and protein synthesis. These results suggest 3D8 may be a potential antiviral agent against viral threats for the public health and livestock industry.

In this study, there is the limitation. The absence of *in vivo* study is a major limitation in animal models. To address this, future studies are needed to illustrate the *in vivo* inhibitory effect of 3D8 scFv using the animal models. Although the previous studies presented the nuclease activity of 3D8 scFv, the precise cellular mechanism and *in vivo* effects of the administration remain to be further investigated.

In conclusion, 3D8 scFv confers effective antiviral activity against the SARS-CoV-2 and multiple coronaviruses. This study provides insights into the broad-spectrum antiviral countermeasure of scFv; thus, it can be a potential antiviral agent against the emerging virus outbreak.

Materials and methods

Ethics

An antiviral study of 3D8 against SARS-CoV-2 was performed at the Biosafety Level-3 facilities at Hallym Clinical and Translational Science Institute, Hallym University, Chuncheon, South Korea, under guidelines and protocols approved by institutional biosafety requirements. Experiments involving OC43 and PEDV were performed at Biosafety Level-2 facilities.

Cells and viruses

African green monkey kidney epithelial Vero cells (ATCC® CCL-81) and Vero E6 cells (ATCC® CRL-1586) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, USA 12-604F, BioWhittaker®) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 1% 10 mM HEPES in 0.85% NaCl (Lonza, USA, 17-737E, BioWhittaker®), and 100 U/mL penicillin and 100 µg/mL streptomycin (Pen Strep, Gibco, USA, 15070-063). Cell cultures were maintained at 37 °C and 5% CO_2. SARS-CoV-2 was obtained from the Korea Centers for Disease Control and Prevention (KCDC). The virus was propagated in Vero E6 cells, and the infectious titer was determined by plaque assay in Vero E6 cells. hCoV OC43 and PEDV were obtained from KCDC and the Korean Animal and Plant Quarantine Agency (KAPQA), respectively.

Plaque assay

Vero E6 cells were plated at 1×10^6 cells per well in 6-well plates (Corning) and incubated at 37 °C with 5% CO_2. A confluent monolayer of cells was washed with phosphate-buffered saline (PBS, Lonza, USA, BioWhittaker®), infected with ten-fold serial dilutions of viral suspension prepared in

serum-free maintenance media (DMEM only), and incubated at 37 °C. Following infection for 1 h with intermittent shaking at 15-min intervals, the viral inoculum was aspirated, and overlay media (DMEM/F12 media) containing 4% bovine serum albumin (BSA), 2 mM glutamine, 2.5% sodium bicarbonate ($NaHCO_3$), 10 mM HEPES, 50 mg/mL DEAE dextran, 100 U/mL penicillin, 100 µg/mL streptomycin (Pen Strep), and 0.6% immunodiffusion-grade Oxoid agar was added. After 4–5 days of incubation at 37 °C with 5% CO_2, fixation with 4% paraformaldehyde (Biosesang, F1119Z21 YR) was performed. After overnight incubation, the overlay agar media was flicked using a metal spatula, and the plates were stained with crystal violet (0.1% crystal violet in 20% methanol) for 10 min. Plaques were enumerated, and viral titers were quantified.

In vitro antiviral activity

Cells were seeded at 1×10^6 cells per well and allowed to adhere for 24 h at 37 °C with 5% CO_2 in 6-well plates (Corning). After incubation, the cells were washed twice with PBS, and the viruses were adsorbed at different multiplicity of infection (MOI) for 2 h at 37 °C. The plates were manually rocked to ensure uniform and efficient distribution of inoculum every 15–20 min. After adsorption, the cells were treated with 3D8 at different concentrations (2-h post-infection treatment of 3D8). About 1 mL of DMEM supplemented with 10% FBS and antibiotics was added to the cells, which were then incubated. In case of pre-treatment, Vero E6 cells were treated with 3D8 and incubated overnight before the virus challenge. At 48 hour post infection (hpi), supernatants and cells were harvested. The samples were stored at -80 °C until use.

Real-time quantitative polymerase chain reaction

Total RNA was extracted using TRIzol (Ambion, Life Technologies). Reverse transcription of RNA into cDNA was performed using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, 1 μg of RNA was used, and cDNA was synthesized using an oligo deoxythymine (dT) kit. The reaction was performed at 37 °C for 60 min, followed by 95 °C for 5 min.

Viral RNA was quantified via real-time quantitative PCR (RT-qPCR) using a Power SYBR® Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific) with primers for SARS-CoV-2 and other coronaviruses and GAPDH as an endogenous control. Details of the primers used are provided in the supplementary information.

Cell viability assay

Vero-E6 cells were seeded at 4×10^4 cells per well in 96-well plates and incubated for 24 h at 37 °C in a CO_2 atmosphere. NVG308 protein was applied from 1 μM to 40 μM; the cells were incubated for 48 h at 37 °C. After incubation, 10 μL of MTT solution (Intron) was added to each well, and the cells were incubated for 3 h. After adding 100 μL of DMSO, the viability of the Vero-E6 cells was measured using a microplate reader at 595 nm.

Immunoblot assays

Cells were lysed using RIPA lysis buffer (Santa Cruz Biotechnology, SC-24948). Cell lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane using a wet method. After transmembrane transfer, the lysates were incubated with primary antibodies (anti-SARS-CoV-2, Invitrogen, PA1-41098; anti-PEDV, MEDIAN, #9191; anti-hCoV OC43, LS-bio, LS-C79764; and anti-hGAPDH rabbit IgG,

Sigma-Aldrich, MFCD01322099 and Abcam, ab9485) overnight at 4 °C and then incubated with an anti-rabbit IgG-HRP conjugate for 1 h at room temperature. The membrane reaction with ECL solution (Bio-Rad, 170-5061) was observed and confirmed via the chemiluminescence mode using ImageQuant LAS 500 (GE).

Immunocytochemistry

Immunocytochemistry for identifying the antiviral effects of 3D8 against the coronaviruses was conducted as described previously [36]. Vero E6 cells were seeded at 1.5×10^4 cells in an 8-well chamber and incubated for 24 h. PEDV and hCoV-OC43 were infected at MOI = 0.002 and 0.02, respectively, for 2 h. About 5 μM (185 μg/mL) of purified 3D8 and 1% P/S antibiotics (Gibco,15140122) were added to DMEM media (Hyclone, SH30243.01) supplemented with 10% FBS (Welgene gold serum, S-001-07), and the media was incubated at 37 °C with 5% CO_2. The cells were washed with PBS and fixed for 15 min in ice-cold methanol at room temperature. The cells were then permeabilized with permeabilization buffer (Biolegend, #421002) for 10 min at room temperature. After blocking with 1% BSA + 0.3 M glycine (22.52 mg/mL) + PBST buffer for 1 h at room temperature, primary antibodies for detecting PEDV (mouse, anti-PEDV monoclonal Ab, Mybio, #MBS313516), hCoV-OC43 (mouse, anti-OC43 monoclonal Ab, LSBio, #LS-C79764), and 3D8 (polyclonal rabbit IgG Serum Ab) were incubated overnight at 4 °C. Following this, PEDV and hCoV-OC43 were incubated with TRITC-conjugated anti-mouse Ab (1:500) (Abcam, #ab6786), and 3D8 was incubated with Alexa 488-conjugated anti-rabbit Ab (1:1000) (Abcam, #ab150077). The nuclei were stained with Hoechst (Thermo Fisher, #62249) during the last 10 min of incubation at room temperature. Cells were mounted in mounting medium (VECTASHIELD, #H-1200) and observed using a NIKON A1R (Eclipse A1Rsi and Eclipse Ti-E).

298

299 **Statistical analysis**

300 Statistical analyses of data were performed in Graph Pad Prism 8.

301

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306

307 ## Conflict of interest

308 The authors declare that the research was conducted in the absence of any commercial or financial
309 relationships that could be construed as a potential conflict of interest.

310

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Figure captions

Fig 1. 3D8 exhibits antiviral activity against SARS-CoV-2 in a dose-dependent manner

(A) Dose-dependent inhibition of SARS-CoV-2 by 3D8. Vero E6 cells were infected with SARS-CoV-2 and treated with a range of 3D8 concentrations at 2 hpi. At 48 hpi, the cells were harvested, and the viral RNA level was determined using RT-qPCR. (B) Supernatants from the 3D8-treated samples were collected, and a plaque assay was performed to determine the infectious viral titer.

388 The clear zone indicates the plaques formed. (C) Percent inhibition of SARS-CoV-2 replication by
389 3D8 in Vero E6 cells. Vero E6 cells were infected with SARS-CoV-2, and 3D8 was added at 2 hpi.
390 Replication was measured through quantification of the virus RNA level. (D) Cytotoxicity testing
391 of 3D8 in Vero E6 cells was performed by applying a range of 3D8 concentrations in uninfected
392 cell cultures. Error bars indicate the standard deviation of triplicate measurements in a
393 representative experiment. (***$p<0.001$, One-way ANOVA test; ns: non-significant)
394

395 **Fig 2. Prophylactic and therapeutic antiviral effects of 3D8 against COVID-19**

396 (A) Inhibition of SARS-CoV-2 by 3D8 in pretreated cell cultures. Vero E6 cells pretreated with
397 3D8 were infected with SARS-CoV-2. At 48 hpi, the cells were harvested, and the viral copy
398 number was quantified based on the relative concentration of the N gene. (B) Vero E6 cells
399 pretreated with 3D8 were infected with SARS-CoV-2. At 48 hpi, the cells were lysed using RIPA
400 lysis buffer. Cell lysates were electrophoresed on SDS-PAGE and transferred to nitrocellulose
401 membranes. After incubation with primary antibodies, the membranes were observed using a
402 chemiluminescence reader. (C) Supernatants were harvested from 3D8-pretreated cell cultures
403 infected with SARS-CoV-2, and the infectious viral titer was determined using a plaque assay. The
404 clear zone indicates the plaques formed. (D) Inhibition of SARS-CoV-2 by 3D8 in post-treated cell
405 cultures. Vero E6 cells were infected with SARS-CoV-2 and treated with 3D8 at 2 hpi. At 48 hpi,
406 the cells were harvested, and the viral copy number was quantified based on the relative
407 concentration of the N gene. (E) Vero E6 cells were infected with SARS-CoV-2 and treated with
408 3D8 at 2 hpi. At 48 hpi, the cells were lysed with RIPA lysis buffer. Cell lysates were
409 electrophoresed on SDS-PAGE and transferred to nitrocellulose membranes. After incubation with
410 primary antibodies, the membranes were observed using a chemiluminescence reader. (F)

411 Supernatants were harvested from 3D8 post-treated cell cultures infected with SARS-CoV-2, and
412 the infectious viral titer was determined using a plaque assay. The clear zone indicates the plaques
413 formed. (***p<0.001; ****p<0.0001, One-way ANOVA test; ns: non-significant)
414
415 **Fig 3. *In vitro* antiviral effect of 3D8 against human coronavirus OC43**
416 (A) Dose-dependent inhibition of OC43 by 3D8. Vero E6 cells were infected with OC43 and treated
417 with a range of 3D8 concentrations at 2 hpi. At 48 hpi, the cells were harvested, and the virus copy
418 number was determined via qPCR. (B) Vero E6 cells were infected with OC43 and treated with
419 3D8 at 2 hpi. At 48 hpi, the cells were lysed with RIPA lysis buffer. Cell lysates were
420 electrophoresed on SDS-PAGE and transferred to nitrocellulose membranes. After incubation with
421 primary antibodies, the membranes were observed using a chemiluminescence reader. (C) Percent
422 inhibition of OC43 replication by 3D8 in Vero E6 cells. Vero E6 cells were infected with OC43,
423 and 3D8 was added at 2 hpi. Replication was measured through quantification of the virus RNA
424 level. (D) Vero E6 cells were treated with OC43 and treated with 3D8 at 2 hpi. At 48 hpi, the cells
425 were washed with PBS and fixed using methanol. Then, they were permeabilized with buffer and
426 blocked with BSA. The cells were then incubated with primary antibodies overnight. After
427 incubation, TRITC-conjugated anti-mouse and Alexa 488-conjugated anti-rabbit antibodies were
428 added. Hoechst was used to stain the nucleus. (*p< 0.05; ***p<0.001, One-way ANOVA test; ns:
429 non-significant)
430
431 **Fig 4. *In vitro* antiviral effect of different concentrations of 3D8 against PEDV**
432 (A) Dose-dependent inhibition of PEDV by 3D8. Vero E6 cells were infected with PEDV and
433 treated with a range of 3D8 concentrations at 2 hpi. At 48 hpi, the cells were harvested, and the

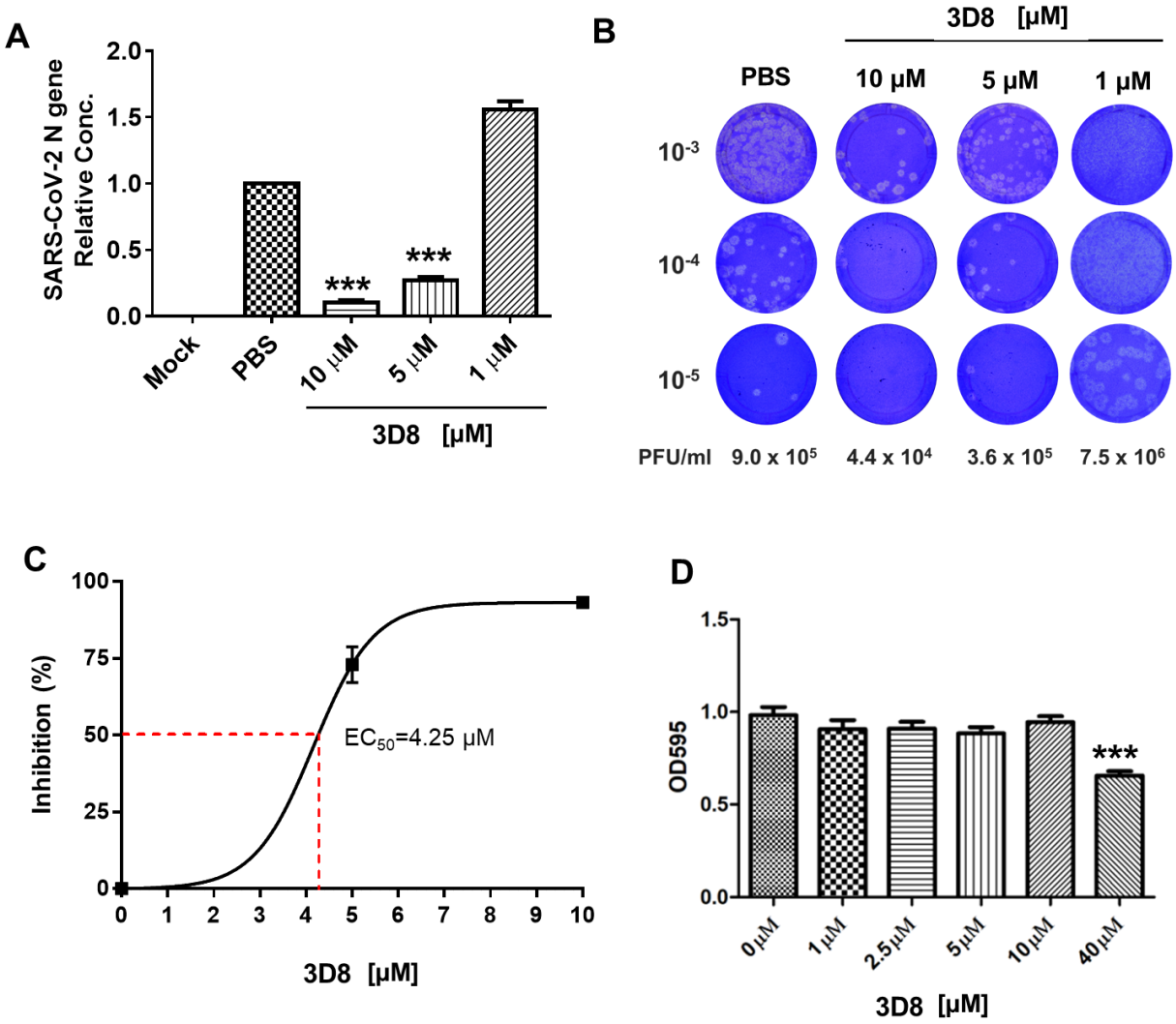
virus RNA level was determined via qPCR. (B) Vero E6 cells were infected with PEDV and treated with 3D8 at 2 hpi. At 48 hpi, the cells were lysed with RIPA lysis buffer. Cell lysates were electrophoresed on SDS-PAGE and transferred to nitrocellulose membranes. After incubation with primary antibodies, the membranes were observed using a chemiluminescence reader. (C) Percent inhibition of PEDV replication by 3D8 in Vero E6 cells. Vero E6 cells were infected with PEDV and treated with 3D8 at 2 hpi. Replication was measured through quantification of the virus RNA level. (D) Vero E6 cells were treated with PEDV and treated with 3D8 at 2 hpi. At 48 hpi, the cells were washed with PBS and fixed with methanol. Then, they were permeabilized with buffer and blocked with BSA. The cells were incubated with primary antibodies overnight. After incubation, TRITC-conjugated anti-mouse and Alexa 488-conjugated anti-rabbit antibodies were added. Hoechst was used to stain the nucleus. (*$p< 0.05$; **$p<0.01$, One-way ANOVA test; ns: non-significant)

Fig 5. Suggested mode of action for 3D8

3D8, a single chain variable fragment (scFv), is internalized into the cell through caveolae-mediated endocytosis. After release from the endosomal compartment, 3D8 localizes to the cytosol. In the cytosol, it binds to the viral nucleic acid and degrades it to prevent its amplification, thus inhibiting viral growth. 3D8 confers nuclease activity without sequence specificity and hydrolyzes viral RNA genomes or transcripts.

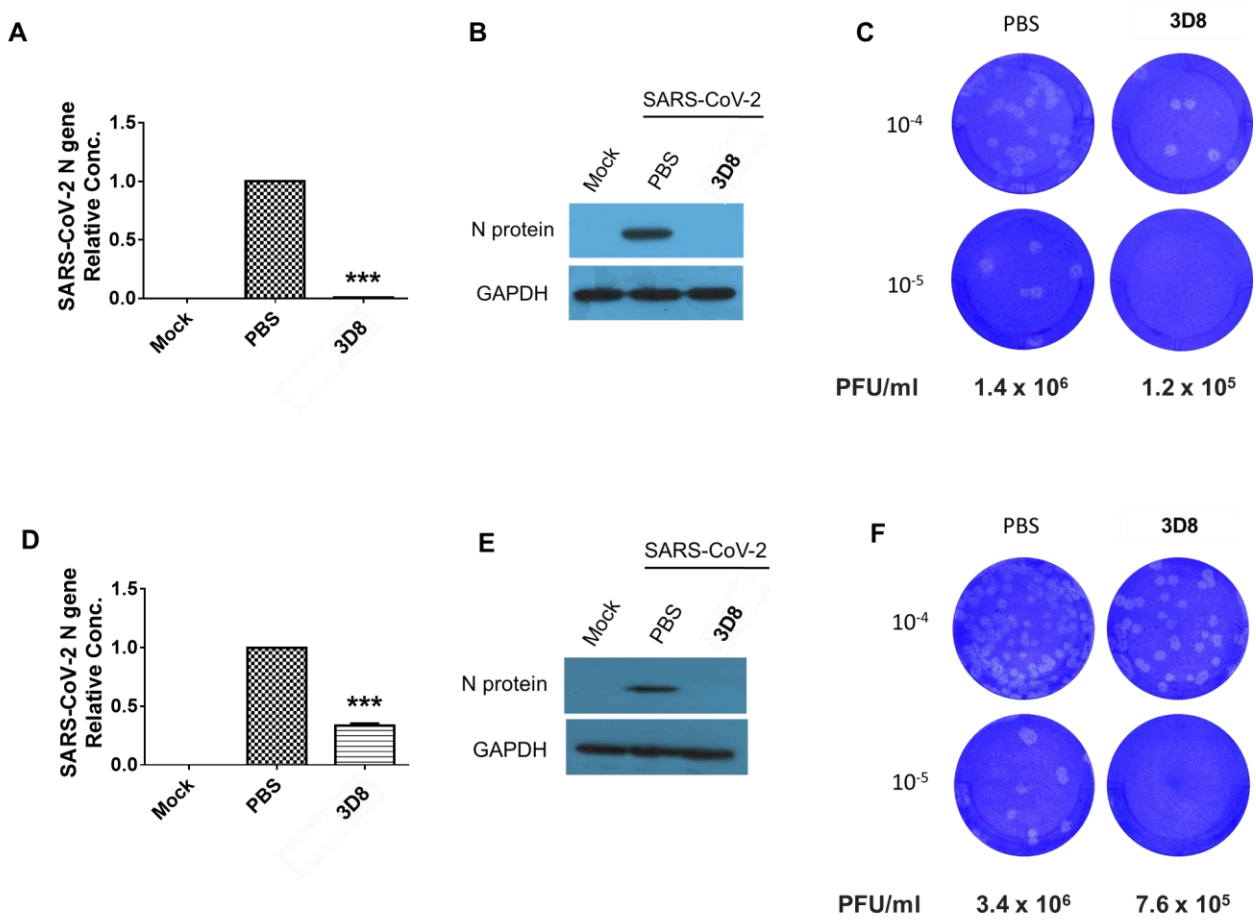
Figures.

Fig 1.



466 **Fig 2.**

467



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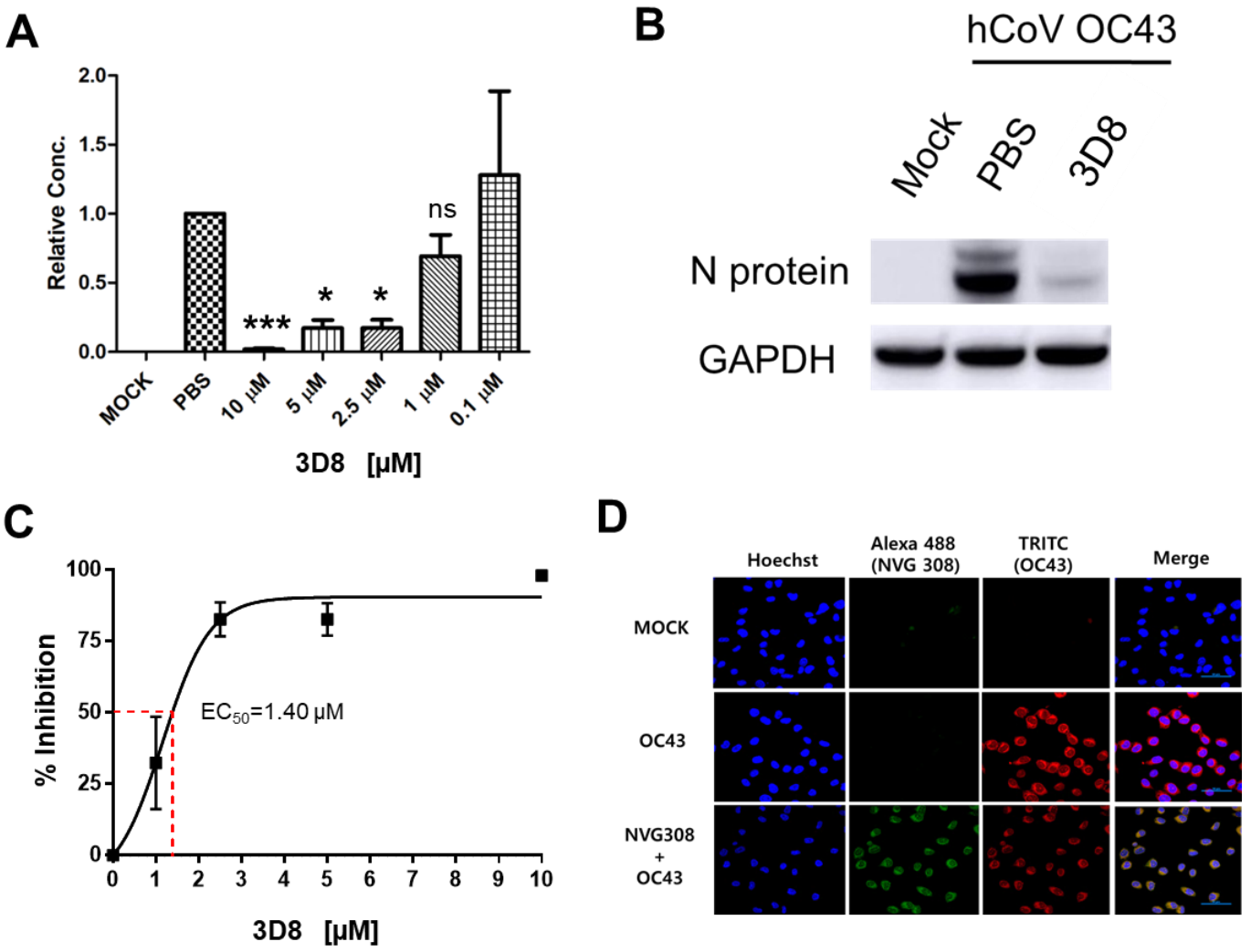
469 **Fig 3.**



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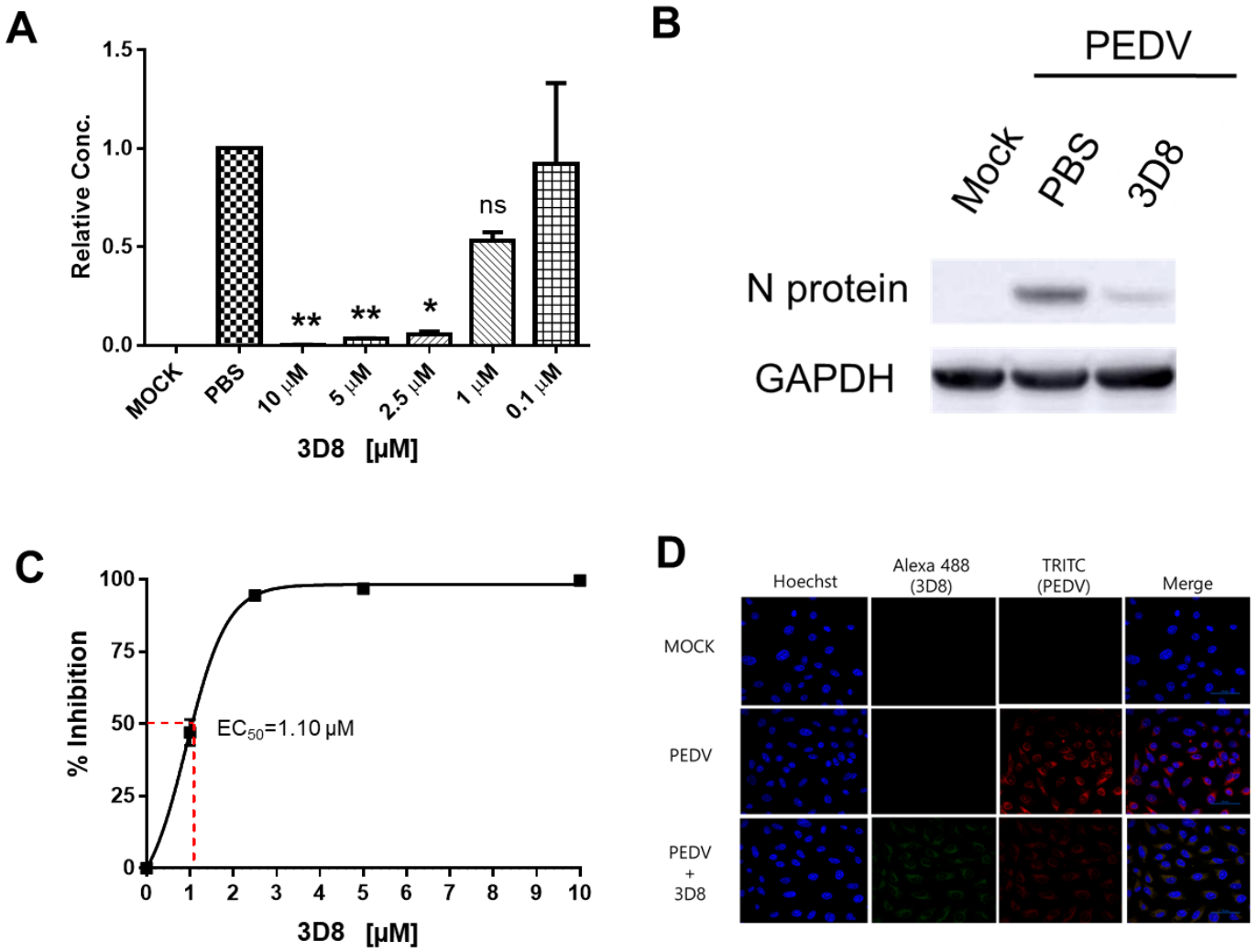
478 **Fig 4.**



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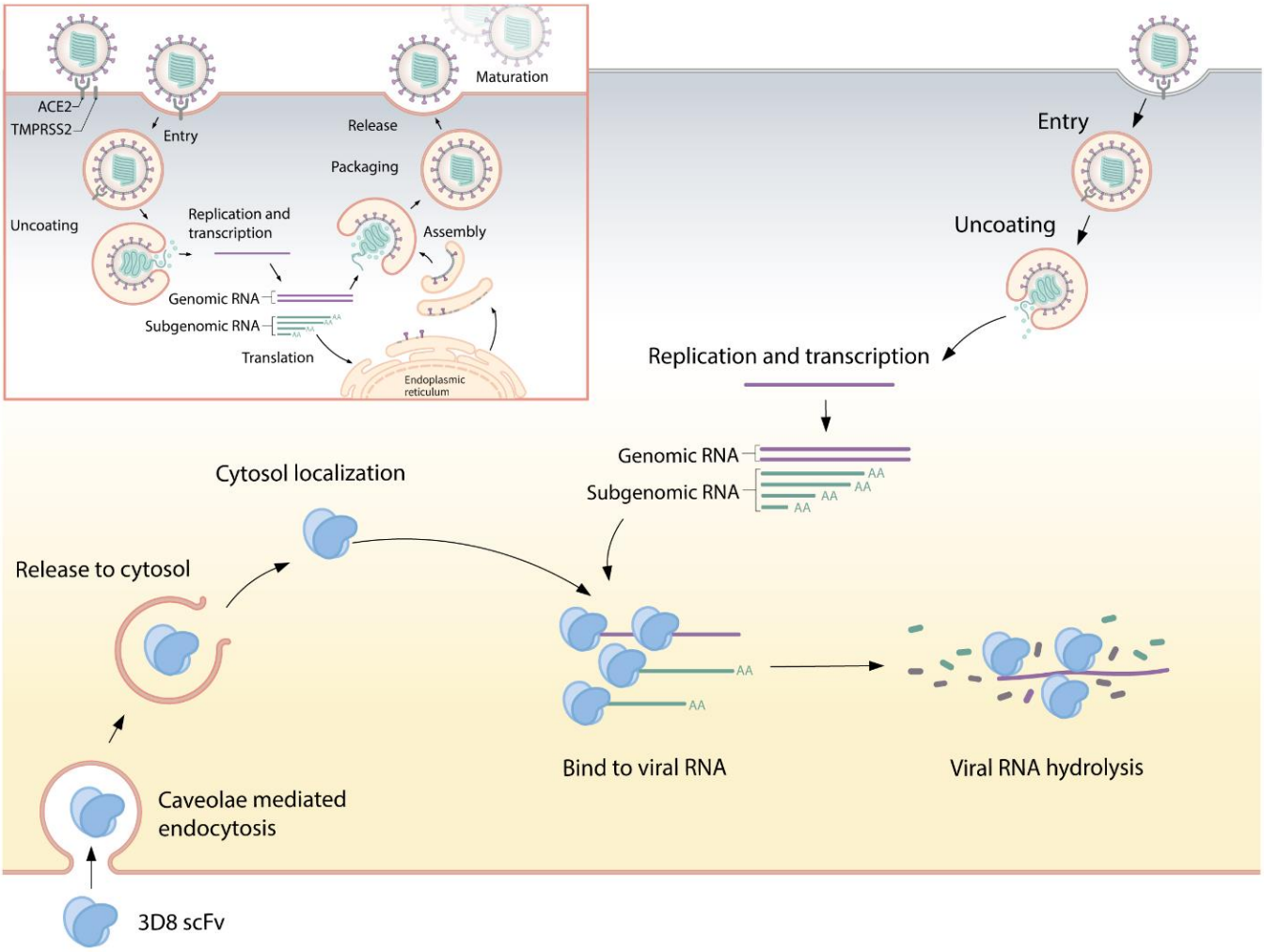
487 **Fig 5.**



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