Supplementary Information for

Phenothiazines inhibit SARS-CoV-2 cell entry via a blockade of spike protein binding to neuropilin-1

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SI Materials and Methods

Compounds

The US Food and Drug Administration (FDA)-approved drug library containing a total of 1,061 molecules (L4200) was purchased from TargetMol (Wellesley Hills, MA, USA). Ribavirin (CAS#36791-04-5) was purchased from FUJIFILM Wako Pure Chemical Corporation (Wako, Osaka, Japan). EG00229 (CAS# 1018927-63-3) was purchased from Tocris Bioscience (Bristol, UK). Alimemazine tartrate (CAS#4330-99-8), asenapine maleate (CAS#85650-56-2), chlorpromazine hydrochloride (CAS#69-09-0), fluphenazine dihydrochloride (CAS#146-56-5), perphenazine (CAS#58-39-9), prochlorperazine dimaleate (CAS#84-02-6), promazine hydrochloride (CAS#53-60-1), promethazine hydrochloride (CAS#58-33-3), thioridazine hydrochloride (CAS#130-61-0), and trifluprομazine hydrochloride (CAS#1098-60-8) were purchased from Selleck Chemicals (Houston, TX, USA). Acepromazine maleate (CAS#3598-37-6) and ethopropazine hydrochloride (CAS#1094-08-2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pipamazine (CAS#84-04-8), thiethylperazine dimaleate (CAS#1179-69-7), and α-isothipendyl hydrochloride (CAS#1225-60-1) were purchased from TRC-Canada (Toronto, ON, Canada). Cepharanthine (CAS# 481-49-2), levomepromazine (CAS#60-99-1), and mepazine hydrochloride (CAS#2975-36-2) were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Diethazine hydrochloride (CAS#341-70-8), methoxypromazine maleate (CAS#3403-42-7), and perazine dihydrochloride (CAS#5317-37-3) were purchased from LGC (Teddington, UK). Trifluoperazine hydrochloride (CAS#440-17-5) and thioproperazine (CAS#316-81-4) were purchased from Cayman Chemical (Ann Arbor, MI, USA) and AmBeed (Arlington Heights, IL, USA), respectively.

Plasmids

To generate the plasmid expressing the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein (NCBI accession: YP_009724390.1) with a deletion at the C-terminal 19 amino acids and a D614G substitution (SARS2-S) [pCMV3-SCoV2-d19S(D614G)], DNA fragments containing the corresponding mutations were amplified by PCR using a SARS-CoV-2 spike protein-expressing plasmid (VG40589-UT; Sino Biological, Beijing, China) as a template and then inserted in frame into the SARS-CoV-2 spike protein-expressing plasmid (VG40589-UT) using restriction enzyme sites, *KpnI* and *XbaI*. To generate the plasmid expressing the SARS-CoV-2 spike protein (SARS2-S) with deletions at the C-terminal 19 amino acids and at amino acid positions 69 and 70 as well as D614G, N501Y, and E484K substitutions, [pCMV3-SCoV2-d19S(D614G, 69-70del, N501Y, E484K)], DNA fragments containing the corresponding mutations were amplified by PCR using pCMV3-SCoV2-d19S(D614G) as a template and then inserted in frame into the SARS-CoV-2 spike protein-expressing plasmid (VG40589-UT) using restriction enzyme sites, *KpnI* and *XbaI*. To generate the plasmid expressing ZsGreen (pC-ZsG), a DNA fragment containing the full-length ZsGreen open reading frame (ORF) was inserted into the mammalian expression vector pCAGGS using restriction enzyme sites, *EcoRI* and *KpnI*.

Cells

HEK293T (American Type Culture Collection, ATCC, Manassas, VA, USA, CRL-1573) cells were grown in Dulbecco's modified Eagle medium (DMEM, Nacalai Tesque, Kyoto, Japan) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (10% FBS/DMEM) at 37 °C and 5% CO_2.

HEK293T cells constitutively expressing human angiotensin-converting enzyme 2 (ACE2) (SL221; GeneCopoeia, Rockville, MD, USA) were grown at 37 °C and 5% CO_2 in 10% FBS/DMEM containing 100 μg/ml hygromycin B. Vero E6 cells constitutively expressing human TMPRSS2 (VeroE6/TMPRSS2, Japanese Collection of Research Bioresources Cell Bank, Ibaraki, Osaka, Japan, JCRB 1819) were grown in 10% FBS/DMEM containing 1 mg/ml geneticin.

Viruses

The recombinant, replication-deficient vesicular stomatitis virus (VSV)-based pseudoviruses expressing green fluorescent protein (GFP) instead of the VSV glycoprotein (VSVG) (rVSV∆G-GFP) and bearing the C-terminal 19 amino acid-deleted SARS-CoV-2 spike protein containing a D614G substitution (SARS2-S) (rVSVΔG-GFP/SARS2-S) or VSVG (rVSVΔG-GFP/VSVG) were generated using procedures similar to those we have described previously (Hashizume et al., 2021). The SARS-CoV-2 TY7-501 strain (GISAID ID, EPI_ISL_833366) was obtained from National Institute of Infectious Diseases (NIID, Tokyo, Japan) and amplified twice using VeroE6/TMPRSS2 cells (Matsuyama et al., 2020) as the cell substrate before use in experiments.

Screening of the US FDA-approved drug library using rVSV∆G-GFP/SARS2-S

Each compound of the US FDA-approved drug library was added separately (10 μM, final concentration) to HEK293T/ACE2 cells that had been seeded in 96-well plates at a density of 5×10^4 cells per well and cultured overnight, and they were incubated at 37 °C and 5% CO_2 for 90 min. The cells were then inoculated with rVSVΔG-GFP/SARS2-S or rVSVΔG-GFP/VSVG [3×10^3 focus-forming units (FFU) per well].

Cepharanthine (2.5 μM) and ribavirin (100 μM) were used as positive controls for rVSVΔG-GFP/SARS2-S and rVSVΔG-GFP/VSVG, respectively (Ohashi et al., 2021). Compounds were present throughout the experimental endpoint. At 16 hpi, the cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; 4% PFA/PBS) (Nacalai Tesque) and permeabilized with dilution buffer (DB; 0.3% Triton X-100 in PBS containing 3% bovine serum albumin), and their nuclei were stained with Hoechst 33342 (Nacalai Tesque). Fluorescent images were captured with the CQ1 Confocal Quantitative Image Cytometer (Yokogawa Electric Corporation, Tokyo, Japan), and the GFP-positive cell numbers were determined using the high-content analysis software CellPathfinder (Yokogawa Electric Corporation).

Determination of the 50% effective concentration (EC_{50}) in VSV-based pseudovirus infection assays

HEK293T/ACE2 cells that had been seeded in 96-well plates at a density of 5×10^4 cells per well and cultured overnight were treated with two-fold serial compound dilutions at 37 °C and 5% CO_2 for 90 min, followed by inoculation with rVSVΔG-GFP/VSVG or rVSVΔG-GFP/SARS2-S (3×10^3 FFU per well). Compounds were present throughout the experimental endpoint. After 16 h, the cells were fixed with 4% PFA/PBS and permeabilized with DB, and their nuclei were stained with Hoechst 33342. Fluorescent images were captured with the CQ1 Confocal Quantitative Image Cytometer (Yokogawa Electric Corporation), and the numbers of GFP-positive cells were determined using the high-content analysis software, CellPathfinder (Yokogawa Electric Corporation). The mean GFP-positive cell number of DMSO-treated and VSV-based pseudovirus-inoculated cells was set to 100%. The EC_{50} concentrations were determined using GraphPad Prism 9 software (GraphPad, San Diego, CA, USA).

Determination of the 50% cytotoxic concentration (CC_{50})

HEK293T/ACE2 or VeroE6/TMPRSS2 cells that had been seeded in 96-well plates at a density of 5×10^4 cells per well or 2×10^4 cell per well, respectively, and cultured overnight were treated with two-fold serial compound dilutions at 37 °C and 5% CO_2. After 17.5 h, CellTiter 96 AQ_{ueous} One Solution Reagent (Promega, Madison, WI, USA) was added. Thereafter, the assay was performed in accordance with the manufacturer's recommendations, and the absorbance at 490 nm was obtained using a multi-mode microplate reader (SpectraMax iD5, Molecular Devices, San Jose, CA, USA). The mean value obtained from DMSO-treated cells was set to 100%. The CC_{50} concentrations were determined using GraphPad Prism 9 software.

Fusion assay

HEK293T cells that had been seeded in 12-well plates at a density of 4×10^5 cells per well and cultured overnight were transfected with 0.5 μg of pCMV3-SCoV2-d19S(D614G) or pCMV3-SCoV2-d19S(D614G, 69-70del, N501Y, E484K) together with 0.5 μg of pC-ZsG. At 24 h post-transfection, the cells were detached with 0.5 mM ethylenediaminetetraacetic acid in PBS (Nacalai Tesque) containing 0.005% trypsin (Nacalai Tesque) and overlaid on HEK293T/ACE2 cells at a ratio of approximately one SARS2-S-expressing plasmid-transfected cell to three HEK293T/ACE2 cells. After being incubated for 4 h, the cells were fixed with 4% PFA/PBS and permeabilized with DB, and their nuclei were stained with Hoechst 33342. Fluorescent images were captured with the CQ1 Confocal Quantitative Image Cytometer, and the average number of nuclei in each syncytium was determined using the high-content analysis software, CellPathfinder.

Assessment of cell surface expression of ACE2

HEK293T/ACE2 cells that had been seeded in 12-well plates at a density of 5×10^5 cells per well and cultured overnight were treated with compounds for 90 min. The cell surface proteins on the cells were biotin-labeled with 1 mg/mL Biotin-SS-Sulfo-Osu (Dojindo, Kumamoto, Japan) for 30 min at 4°C. The biotin-labeled cells were washed twice with ice-cold quenching buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 150 mM NaCl) and once with ice-cold PBS. The cells were then lysed with 150 µL of 0.3% n-Decyl-β-D-maltopyranoside (DDM; Dojindo) in PBS supplemented with the Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 20 min at 4°C. The lysate was clarified by centrifugation at 15,000 rpm and 4°C for 5 min to remove the cell debris (Input samples). The cleared cell lysate was mixed with 20 µL of streptavidin-conjugated magnetic beads (Dynabeads M-280 Streptavidin; Thermo Fisher Scientific) and incubated overnight at 4°C. The beads were washed four times with 0.3% DDM in PBS. Next, 20 µL of loading buffer was added to the beads and the beads were incubated at 37°C for 30 min, followed by boiling for 5 min. Biotinylated proteins eluted into the loading buffer (pull-down samples) were separated using a magnetic stand. ACE2 levels in the Input and pull-down samples were analyzed by western blotting.

Detection of cell surface ACE2 by flow cytometry

HEK293T/ACE2 cells that had been seeded in 12-well plates at a density of 5×10^5 cells and cultured overnight were detached in 5 mM EDTA in PBS and washed with 0.5% BSA in PBS. Anti-ACE2 antibody conjugated with AlexaFluor 488 (FAB9332G-100UG; R&D Systems, Minneapolis, MN, USA) was added to 10^6 cells, and the

mixture was incubated on ice for 30 min. 7-AAD (Invitrogen, Waltham, MA, USA) was added to the cells and incubated on ice for 30 min. Cells were washed with 0.5% BSA and resuspended in 2% FBS in PBS. Cell surface expression of ACE2 was analyzed by flow cytometry using SH800S (SONY, Tokyo, Japan) and data were analyzed with Cell Sorter Software (SONY).

ELISA-based SARS2-S–ACE2 binding assay

The effects of the test compounds and the neutralizing antibody R001 on SARS2-S binding to ACE2 were analyzed with a SARS-CoV-2 Spike:ACE2 Inhibitor Screening Assay Kit (BPS Bioscience, San Diego, CA, USA). Recombinant SARS2-S receptor-binding domain (rRBD)-coated microplate was incubated with 5 μg/ml anti-S1 Ab (ShinoBiological, Wayne, PA, USA; 40592-R001) or compounds (10 or 100 μM) at room temperature for 1 h under gentle agitation. Recombinant ACE2 fused with a His tag (rACE2-His) was added to the plate, and the plate was incubated at room temperature for another 1 h. The microplate was washed four times to remove unbound ACE2-His. The rRBD-binding rACE2-His was reacted with HRP-conjugated anti-His antibody followed by incubation with an HRP substrate to elicit chemiluminescence. Chemiluminescence signal intensities were measured using a multi-mode microplate reader (SpectraMax iD5).

Small-interfering RNA (siRNA)-mediated gene knockdown of neuropilin-1 (NRP-1)

Chemically synthesized siRNA against NRP-1 (siNRP-1) and a non-targeting siRNA control (siControl; MISSION siRNA Universal Negative Control #1) were purchased from Sigma-Aldrich (Hellec et al., 2018). HEK293T and HEK293T/ACE2 cells that had been seeded in 12-well plates at a density of 2.5×10^5 cells per well and cultured

overnight were transfected with 50 nM of siNRP-1 or siControl using 2 μl of Lipofectamine RNAi MAX Reagent (Invitrogen). At 48 h post-transfection, the NRP-1 levels in the siRNA-treated cells were examined by western blotting, or the siRNA-treated cells were used in a fusion assay.

Western blotting

The siRNA-transfected cells were washed twice with ice-cold PBS and lysed with PD buffer (0.5% Triton-X-100, 250 mM NaCl, 50 mM Tris-HCl, 10% glycerol, 1 mM $MgCl_2$, 1 μM $CaCl_2$) containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). The resulting cell lysate was clarified by centrifugation at 20,600 ×*g* and 4 °C for 5 min. The clarified cell lysate was mixed at a 3:1 ratio with 4× Laemmli sample buffer [277.8 mM Tris-HCl, 44.4% glycerol, 4.4% sodium dodecyl sulfate (SDS), 0.02% bromophenol blue] containing 2-mercaptoethanol and denatured at 98 °C for 5 min. Protein samples were fractionated by SDS-polyacrylamide gel electrophoresis using a 4%–20% Mini-PRO TEAN TGX Gel (BioRad, Hercules CA, USA), and the resolved proteins were transferred by electroblotting onto polyvinylidene difluoride membranes (Immobilon-P PVDF Transfer Membranes; Millipore, Burlington, MA, USA). To detect specific proteins, the membranes were incubated first with a mouse monoclonal antibody to ACE2 (AC18F; Cayman Chemical, Ann Arbor, MI, USA), rabbit polyclonal antibodies to GAPDH (ABS16; Millipore), and a sheep monoclonal antibody to neuropilin-1 (RN009M; MBL) and then with horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, or anti-sheep IgG antibodies as appropriate. The Chemi-Lumi One L or Chemi-Lumi One Ultra chemiluminescent substrate (Nacalai Tesque) was used to generate chemiluminescent signals that were visualized with a chemiluminescent imager

(Amersham ImageQuant 800, Cytiva, Tokyo, Japan).

Virus titration

SARS-CoV-2 titers were determined by a 50% tissue culture infective dose ($TCID_{50}$) assay. VeroE6/TMPRSS2 cells that had been seeded in 96-well plates at a density of 1.0×10^4 cells/well and cultured overnight were inoculated with 10-fold serial dilutions of samples. After a three-day incubation at 37 °C and 5% CO_2, the cells were fixed with 10% formalin neutral buffer solution (FUJIFILM Wako Pure Chemical Corporation) and stained with crystal violet. The virus $TCID_{50}$ was determined by the Reed-Muench calculation method.

SARS-CoV-2 infection in vitro

HEK293T/ACE2 or VeroE6/TMPRSS2 cells that had been seeded in a 24-well plate at a density of 1.25×10^5 cells per well or a 96-well plate at a density of 2×10^4 cells per well, respectively, and cultured overnight were treated with alimemazine or vehicle control (DMSO). After 90 min, the cells were inoculated with SARS-CoV-2 TY7-501 strain. At 24 hpi, the cells in the tissue culture supernatant were harvested and frozen at −80 °C. At the time of virus titration, the cells in the tissue culture supernatant were thawed and clarified by centrifugation at 20,600 ×*g* and 4 °C for 5 min. Viral titers of the clarified supernatants were determined by a $TCID_{50}$ assay.

Mice and in vivo SARS-CoV-2 infection

All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University. BALB/c mice (8 weeks of age) obtained from Japan SLC (Hamamatsu, Japan) were orally administrated with

alimemazine (10 mg/kg, dissolved in saline) or vehicle (100 µl of saline) by gavage. One hour later, the mice were intranasally inoculated with SARS-CoV-2 TY7-501 strain (2×10^4 $TCID_{50}$). At 24 hpi, the mice were again treated by gavage with alimemazine (10 mg/kg) or vehicle (saline). The oral administration of alimemazine or vehicle control and the intranasal inoculation of virus were performed under anesthesia. At 2 days post-inoculation, lung tissues were harvested from euthanized mice, weighed, and frozen at −80 °C. At the time of virus titration, the lung tissues were thawed, homogenized in PBS, and clarified by centrifugation at 20,600 × *g* and 4 °C for 5 min. Clarified supernatants were used for titration by a $TCID_{50}$ assay.

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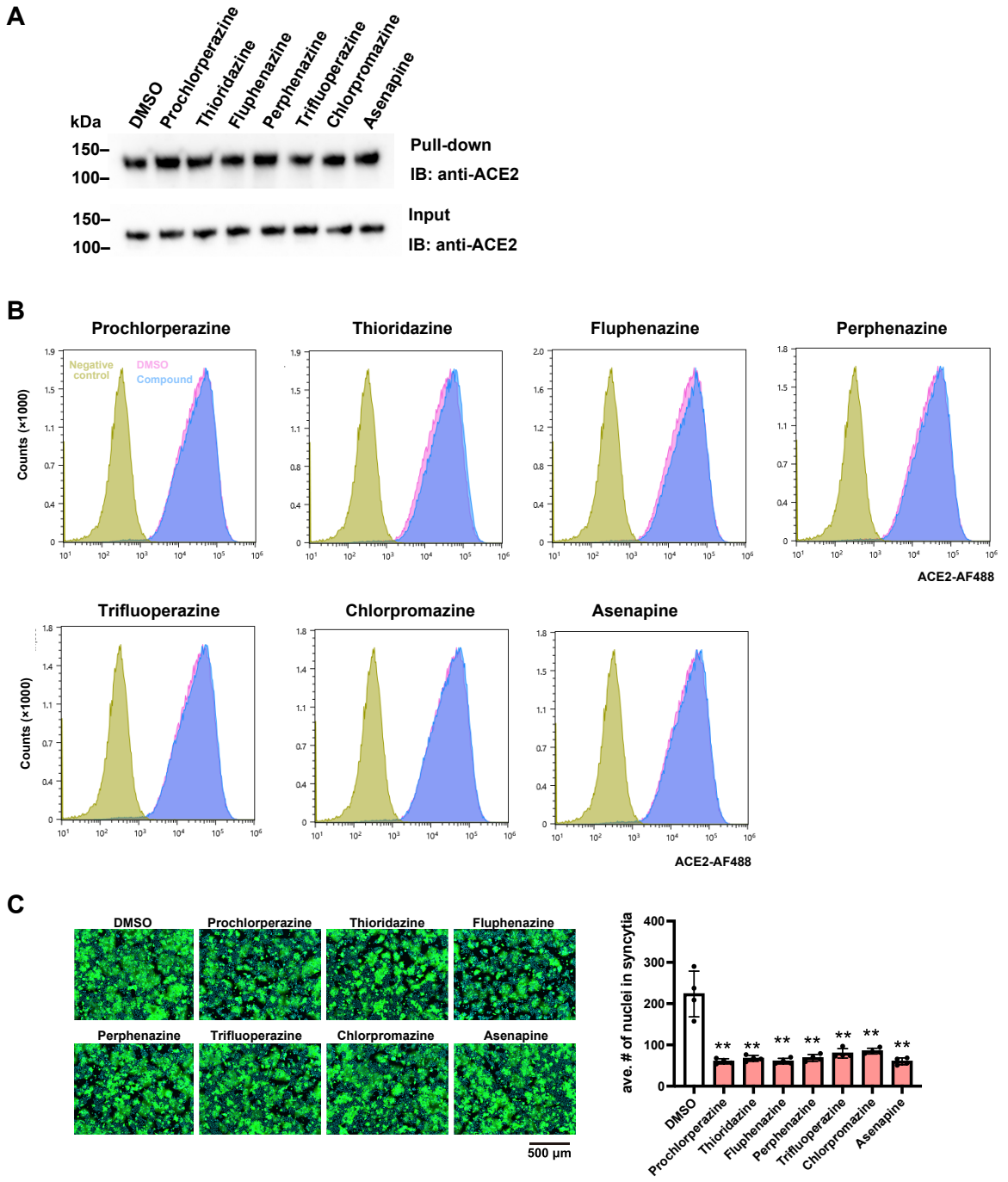


Fig. S1. The DRD2 antagonists do not affect cell surface ACE2 levels. (A) HEK293T/ACE2 cells treated with 10 μM DRD2 antagonists or with vehicle control (DMSO) for 90 min were biotin-labeled and the cell surface proteins were pulled down with streptavidin beads. The levels of ACE2 on the cell surface (Pull-down) and in the total cell lysate (Input) were analyzed by western blotting. (B) HEK293T/ACE2 cells treated with 0.1% DMSO or one of the DRD2 antagonists (10 μM) for 90 min were stained with anti-ACE2 antibody conjugated with AlexaFluor 488 (ACE2-AF488) and the cell surface ACE2 levels were analyzed by flow cytometry. (C) HEK293T cells transfected with plasmids expressing SARS2-S and ZsGreen (ZsG) were detached with trypsin and overlaid on HEK293T/ACE2 cells immediately after 0.1% DMSO or one of the DRD2 antagonists (10 μM) had been added to HEK293T/ACE2 cells.

DMSO and the DRD2 antagonists were present throughout the experimental period. After 4-h incubation, the cells were fixed and their nuclei were stained with Hoechst 33342. Fluorescent images of the cells were captured (left), and the average number (ave. #) of nuclei in each syncytium was determined with a high-content imaging system (right). The presented data are the mean ± SD of the results of four independent experiments. Statistical significance was determined by comparing the average number of nuclei of the DRD2 antagonist-treated samples with that of the DMSO-treated samples. $^{**}p < 0.01$.

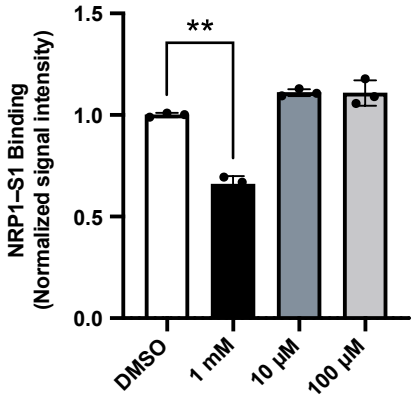


Fig. S2. Effects of EG00229 on the binding of SARS2-S to NRP-1. An ELISA-based SARS2-S–NRP-1 binding assay was performed in the presence of 0.1% DMSO or the indicated concentration of EG00229. The mean absorbance value of the DMSO-treated samples was set to 1. The presented data are the mean ± SD of the results of three replicates. $^{**}p < 0.01$.

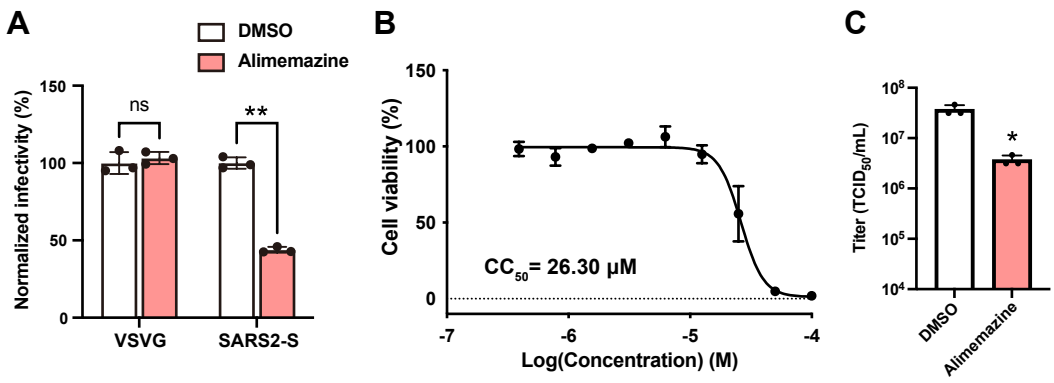
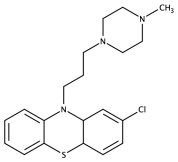
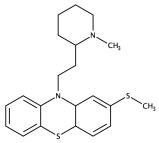
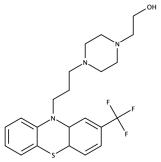
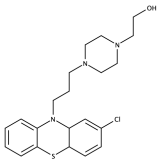
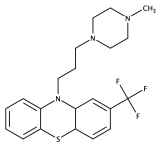
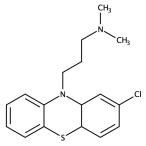
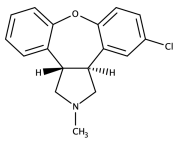


Fig. S3. Effect of alimemazine on SARS2-S-mediated entry in TMPRSS2 expressing cells. (A) VeroE6/TMPRSS2 cells that had been treated with 0.1% DMSO or 10 μM alimemazine for 90 min were inoculated with vesicular stomatitis virus (VSV)-based pseudovirus bearing SARS2-S (rVSVΔG-GFP/SARS2-S, SARS2-S) or the VSV glycoprotein (rVSVΔG-GFP/VSVG, VSVG). Alimemazine and DMSO were present throughout the experimental period. At 16 h post-inoculation, the cells were fixed, and their nuclei were stained with Hoechst 33342. The GFP-positive (virally infected) cells were quantified with a high-content imaging system. The mean value of vehicle-treated, VSV-based pseudovirus-inoculated cells was set to 100%. The presented data are the mean ± SD of the results of three replicates. Statistical significance was determined by a Student's *t*-test. ns, $p > 0.05$, not significant; $^{**}p < 0.01$. (B) The cell viability of VeroE6/TMPRSS2 cells treated with two-fold serial dilutions of alimemazine or with vehicle (0.1% DMSO) for 17.5 h was determined with CellTiter 96 AQ_{ueous} One Solution Reagent. The mean value of vehicle-treated cells was set to 100%. The presented data are the mean ± SD of the results of four replicates. (C) VeroE6/TMPRSS2 cells that had been pretreated with 0.1% DMSO or 14.4 μM alimemazine for 90 min were inoculated (MOI = 0.01) with SARS-CoV-2 TY7-501 strain. Alimemazine and DMSO were present throughout the experimental period. After 24 h, the cells in the tissue culture supernatant were collected, and the virus titers were determined by a $TCID_{50}$ assay. The presented data are the mean and SD of the results of three independent experiments. Statistical significance was determined by a Student's *t*-test. $^{*}p < 0.05$.

Table S1

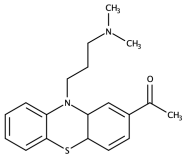
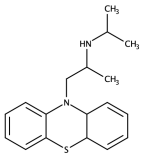
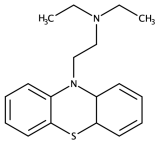
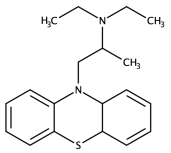
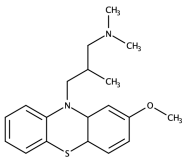
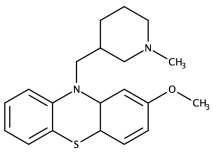
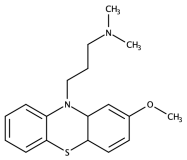
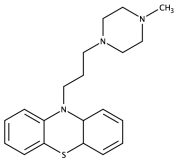
Inhibitory effects of the DRD2 antagonists on rVSVΔG-GFP/SARS2-S infection.

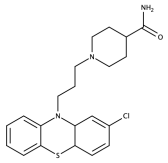
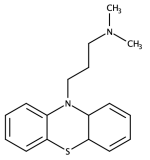
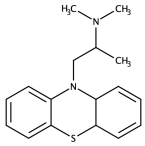
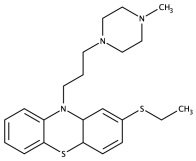
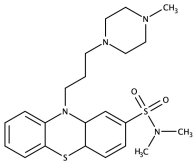
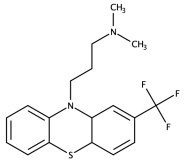
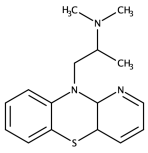
CAS No.	Compound	Structure[a]	EC_{50} (μM)	CC_{50} (μM)	SI (CC_{50}/EC_{50})
84-02-6	Prochlorperazine dimaleate		6.06	32.33	5.33
130-61-0	Thioridazine hydrochloride		4.63	18.92	4.08
146-56-5	Fluphenazine dihydrochloride		3.49	30.42	8.72
58-39-9	Perphenazine		6.06	27.37	4.51
440-17-5	Trifluoperazine hydrochloride		3.80	24.60	6.48
69-09-0	Chlorpromazine hydrochloride		4.15	26.56	6.41
85650-56-2	Asenapine maleate		2.93	42.45	14.50

[a]Counter ion structures are not shown.

Table S2

Inhibitory effects of phenothiazines on rVSVΔG-GFP/SARS2-S infection.

CAS No.	Compound	Structure[a]	EC_{50} (μM)	CC_{50} (μM)	SI (CC_{50}/EC_{50})
3598-37-6	Acepromazine maleate		5.96	77.11	12.94
4330-99-8	Alimemazine tartrate		2.42	29.50	12.20
341-70-8	Diethazine hydrochloride		4.81	> 100	> 20.79
1094-08-2	Ethopropazine hydrochloride		3.89	80.79	20.76
60-99-1	Levomepromazine		5.71	42.23	7.39
2975-36-2	Mepazine hydrochloride		3.49	57.00	16.31
3403-42-7	Methoxypromazine maleate		7.65	58.06	7.59
5317-37-3	Perazine dihydrochloride		5.44	71.00	13.06

CAS No.	Compound	Structure[a]	EC_{50} (μM)	CC_{50} (μM)	SI (CC_{50}/EC_{50})
84-04-8	Pipamazine		3.25	43.97	13.51
53-60-1	Promazine hydrochloride		5.85	60.09	10.27
58-33-3	Promethazine hydrochloride		3.85	66.96	17.42
1179-69-7	Thiethylperazine dimaleate		6.62	34.37	5.19
316-81-4	Thioproperazine		3.52	51.74	14.71
1098-60-8	Trifluprom azine hydrochloride		3.11	50.27	16.15
1225-60-1	α-isothipendyl hydrochloride		14.08	> 100	> 7.10

[a]Counter ion structures are not shown.