Supplementary Appendix

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This appendix has been provided by the authors to give readers additional information about the work.

Efficacy of antiviral agents against the SARS-CoV-2 Omicron BA.2 variant

Supplementary Appendix

Table of Contents

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Supplementary Materials

Cells.

VeroE6/TMPRSS2 (JCRB 1819)[1] cells were propagated in the presence of 1 mg/ml geneticin (G418; Invivogen) and 5 μg/ml plasmocin prophylactic (Invivogen) in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Calf Serum (FCS), and maintained at 37 °C with 5% CO_2. Chinese hamster ovary (CHO) cells were maintained in DMEM containing 10% FCS and antibiotics at 37 °C with 5% CO_2. Expi293F cells (Thermo Fisher Scientific) were maintained in Expi293 expression medium (Thermo Fisher Scientific) at 37 °C under 8% CO_2. The cells were regularly tested for mycoplasma contamination by using PCR, and confirmed to be mycoplasma-free.

Clinical specimens.

After informed consent was obtained, specimens were collected from individuals with SARS-CoV-2 infection. The research protocol was approved by the Research Ethics Review Committee of the Institute of Medical Science of the University of Tokyo (approval number 2019–71–0201).

Viruses.

hCoV-19/Japan/UT-NCD1288-2N/2022 (Omicron/BA.2; NCD1288), hCoV-19/Japan/NC928-2N/2021 (Omicron/BA.1; NC928)[2], hCoV-19/Japan/NC929-1N/2021 (Omicron/BA.1.1; NC929)[3], SARS-CoV-2/UT-NC002-1T/Human/2020/Tokyo (NC002), SARS-CoV-2/UT-HP127-1Nf/Human/2021/Tokyo (Alpha; HP127), hCoV-19/USA/MD-HP01542/2021 (Beta; HP01542), hCoV-19/Japan/TY7-503/2021 (Gamma; TY7-503), and hCoV-19/USA/WI-UW-5250/2021 (Delta; UW5250)] were propagated in VeroE6/TMPRSS2 cells in VP-SFM (Thermo Fisher Scientific). The sequence of hCoV-19/Japan/UT-NCD1288-2N/2022 (Omicron/BA.2; NCD1288) was deposited in the Global Initiative on Sharing All Influenza Data (GISAID) database with Accession ID: EPI_ISL_9595604.

All experiments with SARS-CoV-2 were performed in enhanced biosafety level 3 (BSL3) containment laboratories at the University of Tokyo and the National Institute of Infectious Diseases, Japan, which are approved for such use by the Ministry of Agriculture, Forestry and Fisheries, Japan.

Antibodies.

Amino acid sequences for the variable region of the heavy and light chains of the following human monoclonal antibodies against the S protein were used for gene synthesis: clones tixagevimab (COV2-2196/AZD8895; GenBank accession numbers QLI33947 and QLI33948), casirivimab (REGN10933; PDB accession numbers 6XDG_B and 6XDG_D), cilgavimab (COV2-2130/AZD1061; GenBank accession numbers QKY76296 and QKY75909), imdevimab (REGN10987; PDB accession numbers 6XDG_A and 6XDG_A), and S309 (PDB accession numbers 6WS6_A and 6WS6_F). An artificial signal sequence and the constant gamma heavy (IgG1, UniProtKB/Swiss-Prot accession number P01857) and kappa (UniProtKB/Swiss-Prot accession number P01834) or lambda (UniProtKB/Swiss-Prot accession number P0DOY2) light chain coding sequences were added before and after each variable

region. Codon usage was optimized for expression in CHO cells. The synthesized genes were cloned into a plasmid for protein expression and transfected into CHO cells. Cell culture media were harvested after incubation for 10–14 days at 37 °C. Monoclonal antibodies were purified by using MabSelect SuRe LX (Cytiva) or a protein A column. Purity was confirmed by SDS-PAGE and/or HPLC before use. The reactivities of these antibodies against SARS-CoV-2, including the Alpha, Beta, Delta, Gamma, and Omicron variants, have been tested previously[2].

Antiviral compounds.

Active components of remdesivir and molnupiravir (i.e., GS-441524 and EIDD-1931), and nirmatrelvir (PF-07321332) were purchased from MedChemExpress. All compounds were dissolved in dimethyl sulfoxide.

Supplementary Methods

Whole genome sequencing.

Viral RNA was extracted by using a QIAamp Viral RNA Mini Kit (QIAGEN). The whole genome of SARS-CoV-2 was amplified by using a modified ARTIC network protocol in which some primers were replaced or added[4,5]. Briefly, viral cDNA was synthesized from the extracted RNA by using a LunarScript RT SuperMix Kit (New England BioLabs). The DNA was amplified by multiplexed PCR in two pools using the ARTIC-N1 primers v4 or v5[6] and the Q5 Hot Start DNA polymerase (New England BioLabs). The DNA libraries for Illumina NGS were prepared from pooled amplicons by using a QIAseq FX DNA Library Kit (QIAGEN) and were then analyzed by using the iSeq 100 System (Illumina). The reads were assembled by the CLC Genomics Workbench (version 21, Qiagen) with the Wuhan/Hu-1/2019 sequence (GenBank accession no. MN908947) as a reference. The sequence of hCoV-19/Japan/UT-NCD1288-2N/2022 (Omicron/BA.2; NCD1288) was deposited in the Global Initiative on Sharing All Influenza Data (GISAID) database[7] with Accession ID: EPI_ISL_9595604.

Focus reduction neutralization test (FRNT).

Neutralization activities of SARS-CoV-2 were determined by using a focus reduction neutralization assay as previously described.[8] Serial dilutions of monoclonal antibodies (starting concentration, 50,000 ng/ml) were mixed with 1000 focus-forming units (FFU) of virus/well and incubated for 1 h at 37 °C. The antibody-virus mixture was inoculated on VeroE6/TMPRSS2 cells in 96-well plates in duplicate and incubated for 1 h at 37 °C. An equal volume of 1.2% Avicel RC-581 (DuPont Nutrition USA) in culture medium was added to each well. The cells were incubated for 24 h at 37 °C and then fixed with formalin. After the formalin was removed, the cells were immunostained with a mouse monoclonal antibody against SARS-CoV-1/2 nucleoprotein [clone 1C7C7 (Sigma-Aldrich)], followed by a horseradish peroxidase-labeled goat anti-mouse immunoglobulin (SeraCare Life Sciences). The infected cells were stained with TrueBlue Substrate (SeraCare Life Sciences) and then washed with distilled water. After cell drying, the focus numbers were quantified by using an ImmunoSpot S6 Analyzer, ImmunoCapture software, and BioSpot software (Cellular Technology). The results are expressed as the 50% focus reduction neutralization titer ($FRNT_{50}$). The $FRNT_{50}$ values were calculated by using GraphPad Prism (GraphPad Software).

Inhibitory effect of compounds against SARS-CoV-2 *in vitro.*

Antiviral susceptibilities of SARS-CoV-2 were determined by applying a focus reduction assay as previously reported for influenza virus.[9] VeroE6/TMPRSS2 cells in 96-well plates were infected with 1000 FFU of virus/well. Virus adsorption was carried out for 1 h at 37 °C and then an equal volume of 1.2% Avicel RC-581 (DuPont Nutrition USA) in culture medium containing serial dilutions of antiviral compounds was added to each well in triplicate. The cells were incubated for 24 h at 37 °C and then fixed with formalin. After the formalin was removed, immunostaining was performed as described for the FRNT. The results are expressed as the 50% inhibitory concentration (IC_{50}). The IC_{50} values were calculated by using GraphPad Prism (GraphPad Software).

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Table S1. Amino Acid Substitutions in the hCoV-19/Japan/UT-NCD1288-2N/2022 stock virus used in this study.*

Gene	hCoV-19/Japan/UT-NCD1288-2N/2022
ORF1a	Ser135Arg, Thr842Ile, Gly1307Ser, Leu3027Phe, Thr3090Ile, Leu3201Phe, Thr3255Ile, Pro3395His, Ser3675del, Gly3676del, Phe3677del, Phe3753Val(20%), Leu4391Phe(19%)
ORF1b	Pro314Leu, Gly662Val, Ser959Pro, Pro1161Ser(27%), Arg1315Cys, Ile1566Val, Thr2163Ile, Ala2513Val(18%)
S†	Thr19Ile, Leu24del, Pro25del, Pro26del, Ala27Ser, Gly142Asp, Val213Gly, **Gly339Asp, Ser371Phe, Ser373Pro, Ser375Phe, Thr376Ala, Asp405Asn, Arg408Ser, Lys417Asn, Asn440Lys, Ser477Asn, Thr478Lys, Glu484Ala, Gln493Arg, Gln498Arg, Asn501Tyr, Tyr505His**, Asp614Gly, His655Tyr, Asn679Lys, Pro681His, Asn764Lys, Asp796Tyr, Gln954His, Asn969Lys
ORF3a	Thr223Ile
E	Thr9Ile
M	Gln19Glu, Ala63Thr
ORF6	Asp61Leu
ORF9b	Pro10Ser, Glu27del, Asn28del, Ala29del
N	Pro13Leu, Glu31del, Arg32del, Ser33del, Arg203Lys, Gly204Arg, Ser413Arg

*Substitutions based on a comparison with the Wuhan/Hu-1/2019 sequence.

†Substitutions in the Receptor-Binding Domain of S are indicated in boldface type.

Table S2. Efficacy of Monoclonal Antibodies and Antiviral Drugs against SARS-CoV-2 Variants in Vitro.*

Monoclonal Antibody or Antiviral Drug	SARS-CoV-2 Variant							
	SARS-CoV-2/UT-NC002-1T/Human/2020/Tokyo (An ancestral strain/A)	SARS-CoV-2/UT-HP127-1Nf/Human/2021/Tokyo (Alpha/B.1.1.7)	hCoV-19/USA/MD-HP01542/2021 (Beta/B.1.351)	hCoV-19/Japan/TY7-503/2021 (Gamma/P.1)	hCoV-19/USA/WI-UW-5250/2021 (Delta/B.1.617.2)	hCoV-19/Japan/NC928-2N/2021 (Omicron/BA.1)	hCoV-19/Japan/NC929-1N/2021 (Omicron/BA.1.1)	hCoV-19/Japan/UT-NCD1288-2N/2022 (Omicron/BA.2)
Neutralization activity of monoclonal antibody — ng/ml†								
LY-CoV016, etesevimab	18.19 ± 9.10	150.38 ± 83.51	>50,000	>50,000	15.37 ± 9.78	>50,000	>50,000	>50,000
LY-CoV555, bamlanivimab	4.69 ± 1.43	2.65 ± 1.30	9554.88 ± 926.53	1601.65 ± 896.02	641.73 ± 324.79	>50,000	>50,000	>50,000
REGN10987, imdevimab	3.05 ± 0.93	1.87 ± 1.60	2.17 ± 1.30	1.04 ± 0.68	3.95 ± 1.78	>50,000	>50,000	68.65 ± 8.84
REGN10933, casirivimab	2.79 ± 1.87	2.74 ± 1.84	757.13 ± 287.91	187.69 ± 128.88	2.89 ± 1.78	14110.70 ± 1782.13	11998.94 ± 2604.70	1666.19 ± 771.77
COV2-2196, tixagevimab	1.92 ± 0.28	1.34 ± 0.67	18.98 ± 1.42	6.56 ± 1.56	4.05 ± 2.60	1299.94 ± 406.58	880.47 ± 68.08	395.78 ± 62.37
COV2-2130, cilgavimab	7.70 ± 2.20	3.60 ± 1.62	10.03 ± 3.05	4.00 ± 2.70	12.76 ± 2.93	443.87 ± 167.96	13558.20 ± 4646.95	4.44 ± 2.72
S309, sotrovimab precursor	27.33 ± 3.24	44.91 ± 22.76	100.98 ± 22.27	28.38 ± 1.86	111.43 ± 58.22	373.47 ± 159.49	384.52 ± 65.98	1359.05 ± 269.23
LY-CoV016 plus LY-CoV555	12.60 ± 1.91	15.26 ± 3.98	>10,000	2545.04 ± 625.72	10.28 ± 3.33	>10,000	>10,000	>10,000
REGN10987 plus REGN10933	3.53 ± 0.66	1.55 ± 0.78	5.18 ± 1.45	2.11 ± 0.48	1.91 ± 0.79	>10,000	>10,000	222.59 ± 64.47
COV2-2196 plus COV2-2130	3.42 ± 0.92	1.94 ± 0.34	10.30 ± 1.17	1.79 ± 0.87	5.50 ± 2.75	255.86 ± 45.31	1374.90 ± 14.47	14.48 ± 2.04
Viral susceptibility to drug — μM‡								
GS-441524§	1.04 ± 0.32	0.83 ± 0.19	0.63 ± 0.20	0.91 ± 0.33	1.12 ± 0.20	1.28 ± 0.42	1.63 ± 0.30	2.85 ± 0.31
EIDD-1931¶	0.51 ± 0.14	0.95 ± 0.17	0.60 ± 0.21	0.41 ± 0.13	0.83 ± 0.41	0.43 ± 0.08	1.09 ± 0.13	0.67 ± 0.22
PF-07321332‖	3.59 ± 0.96	4.23 ± 1.04	2.03 ± 0.96	4.57 ± 1.14	3.90 ± 0.50	4.26 ± 0.36	3.63 ± 0.42	6.76 ± 0.69

*The monoclonal antibody neutralization activity data and viral drug susceptibility were obtained as part of this study (all data with Omicron/BA.2 and with PF-07321332, shaded green) and previous studies (references 2 and 3). The antibodies used in this work were produced in the authors' laboratory and are not identical to the commercially available products. Plus–minus values are means ± SD. The viral variants of SARS-CoV-2 are listed according to the World Health Organization labels for the Pango lineage.

†The individual monoclonal antibodies were tested at a starting concentration of 50,000 ng per milliliter as a 50% focus reduction neutralization titer. The monoclonal antibody combinations were tested at a starting concentration of 10,000 ng per milliliter for each antibody.

‡The value presented is the 50% inhibitory concentration of the mean micromole value of triplicate reactions.

§GS-441524 is the main metabolite of remdesivir, an RNA-dependent RNA polymerase inhibitor.

¶EIDD-1931 is the active form of molnupiravir, an RNA-dependent RNA polymerase inhibitor.

‖PF-07321332, nirmatrelvir, a protease inhibitor.

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