Supplementary Appendix

Supplement to: Imai M, Ito M, Kiso M, et al. Efficacy of antiviral agents against omicron subvariants BQ.1.1 and XBB. N Engl J Med. DOI: 10.1056/NEJMc2214302

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

Efficacy of Antiviral Agents against Omicron BQ.1.1 and XBB Subvariants

Supplementary Appendix

Table of Contents

Supplementary Materials

Cells.

Vero E6-TMPRSS2-T2A-ACE2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Calf Serum (FCS), 100 U/mL penicillin–streptomycin, and 10 μg/mL puromycin. VeroE6/TMPRSS2 (JCRB 1819) cells were propagated in the presence of 1 mg/ml geneticin (G418; Invivogen) and 5 μg/ml plasmocin prophylactic (Invivogen) in DMEM containing 10% FCS. Vero E6-TMPRSS2-T2A-ACE2 and VeroE6/TMPRSS2 cells were 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.

Viruses.

hCoV-19/Japan/TY41-796/2022 (Omicron BQ.1.1), hCoV-19/Japan/TY41-795/2022 (Omicron XBB), hCoV-19/Japan/UT-NCD1288-2N/2022 (Omicron BA.2)[1], hCoV-19/Japan/TY41-702/2022 (Omicron BA.5)[2], and SARS-CoV-2/UT-NC002-1T/Human/2020/Tokyo were propagated in VeroE6/TMPRSS2 cells.

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), S309 (PDB accession numbers 6WS6_A and 6WS6_F), and bebtelovimab (LYCoV1404; PDB accession numbers 7MMO_D and 7MMO_E). 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[3].

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. Briefly, viral cDNA was synthesized from the extracted RNA by using a LunarScript RT SuperMix Kit (New England BioLabs). The DNA was amplified by performing a multiplexed PCR in two pools using the ARTIC-N5 primers 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). To determine the virus sequences, the reads were assembled by CLC Genomics Workbench (version 22, Qiagen) with the Wuhan/Hu-1/2019 sequence (GenBank accession no. MN908947) as a reference. The sequences of BQ.1.1 (hCoV-19/Japan/TY41-796/2022) and XBB (hCoV-19/Japan/TY41-795/2022) were deposited in the Global Initiative on Sharing All Influenza Data (GISAID) database with Accession IDs: EPI_ISL_15516893 and EPI_ISL_15516892, respectively.

Focus reduction neutralization test.

Neutralization activities of monoclonal antibodies were determined by using a focus reduction neutralization test as previously described.[4] Serial dilutions of monoclonal antibodies (starting concentration, 50,000 ng/ml) were mixed with 100–400 focus-forming units (FFU) of virus/well and incubated for 1 h at 37 °C. The antibody-virus mixture (50 µl) was then inoculated onto Vero E6-TMPRSS2-T2A-ACE2 cells in 96-well plates in triplicate. After a 1-h incubation at 37 °C, 100 µl of 1.5% Methyl Cellulose 400 (FUJIFILM Wako Pure Chemical Corporation, Japan) in culture medium was added to each well. The cells were incubated for 14–18 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-2 nucleoprotein [N45 (TAUNS Laboratories, Inc., Japan)], followed by a horseradish peroxidase-labeled goat anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories Inc.). 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 test ($FRNT_{50}$) titer. 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.[5] Vero E6-TMPRSS2-T2A-ACE2 cells in 96-well plates were infected with 100–400 FFU of virus/well. Virus adsorption was carried out for 1 h at 37 °C; then, the inoculum was removed and 1% Methyl Cellulose 400 (FUJIFILM Wako Pure Chemical Corporation) in culture medium containing serial dilutions of antiviral compounds was added to each well in triplicate. The cells were incubated for 18 h at 37 °C and then fixed with formalin. After the formalin was removed, immunostaining was performed as described for the focus reduction neutralization test. The results are expressed as the 50% inhibitory concentration (IC_{50}). The IC_{50} values were calculated by using GraphPad Prism (GraphPad Software).

Acknowledgements
We thank Susan Watson for scientific editing.

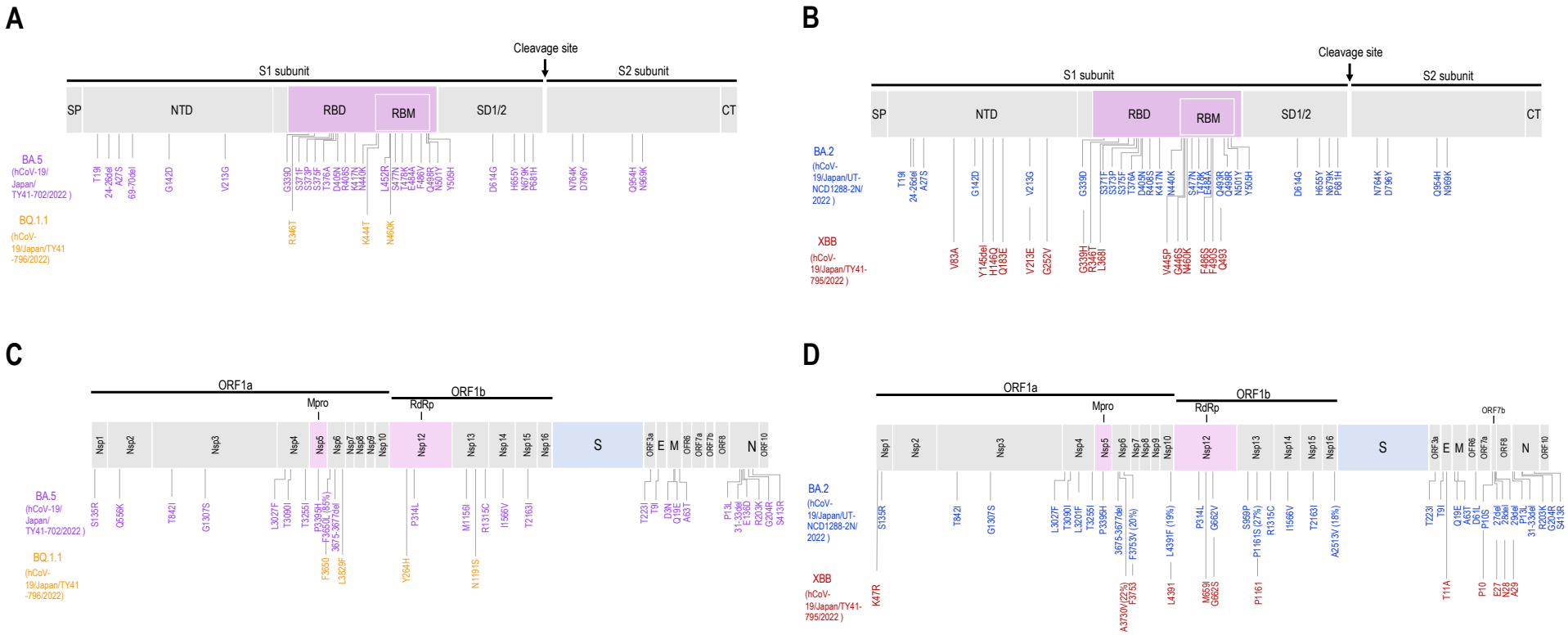

Figure S1. Mutations of Omicron subvariants.

Panels A and B. The spike (S) protein mutations in the BQ.1.1 and XBB clinical isolates used in this study. The BA.5 (hCoV-19/Japan/TY41-702/2022) and BA.2 (hCoV-19/Japan/UT-NCD1288-2N/2022) isolates possess 34 and 31 amino acid changes in their S proteins relative to the reference strain Wuhan/Hu-1/2019, respectively. Compared with BA.5 (hCoV-19/Japan/TY41-702/2022), these mutations are shown in orange for BQ.1.1 (hCoV-19/Japan/TY41-796/2022). Compared with BA.2 (hCoV-19/Japan/UT-NCD1288-2N/2022), these mutations are shown in red for XBB (hCoV-19/Japan/TY41-795/2022). The S protein comprises two subunits, S1 and S2. The arrow indicates the S1/S2 proteolytic cleavage site. SP, signal peptide; NTD, N-terminal domain; RBD, receptor-binding domain; RBM, receptor-binding motif; SD1/2, subdomain 1 and 2; and CT, cytoplasmic tail. Panels C and D. The non-spike protein mutations in the BQ.1.1 and XBB clinical isolates used in this study. Compared with the reference strain Wuhan/Hu-1/2019, the BA.5 (hCoV-19/Japan/TY41-702/2022) and BA.2 (hCoV-19/Japan/UT-NCD1288-2N/2022) isolates possess 30 and 37 amino acid changes in regions other than their S proteins, respectively. Compared with BA.5 (hCoV-19/Japan/TY41-702/2022), these mutations are shown in orange for BQ.1.1 (hCoV-19/Japan/TY41-796/2022). Compared with BA.2 (hCoV-19/Japan/UT-NCD1288-2N/2022), these mutations

are shown in red for XBB (hCoV-19/Japan/TY41-795/2022). ORF, open reading frame; Mpro, main protease; RdRp, RNA-dependent RNA polymerase; S, Spike; E, Envelope; M, Membrane; and N, Nucleocapsid.

Table S1. Efficacy of Monoclonal Antibodies and Antiviral Drugs against Omicron Subvariants In Vitro.*

WHO Label (Pango Lineage): Virus Strain	Neutralization Activity of Monoclonal Antibody†								Viral Susceptibility to Drug‡		
	$FRNT_{50}$ (ng/ml)								IC_{50} (μmol/liter)		
	REGN10987 imdevimab	REGN10933 casirivimab	COV2-2196 tixagevimab	COV2-2130 cilgavimab	S309 sotrovimab precursor	LYCoV1404 bebtelovimab	REGN10987 plus REGN10933	COV2-2196 plus COV2-2130	GS-441524 remdesivir§	EIDD-1931 molnupiravir¶	PF-07321332 nirmatrelvir ‖
Ancestral strain (A): SARS-CoV-2/UT-NC002-1T/Human/2020/Tokyo	34.4	28.7	29.1	79.0	1304.6	16.5	29.4	37.7	1.8	2.5	2.2
Omicron BA.2: hCoV-19/Japan/UT-NCD1288-2N/2022	1840.6	>50,000	12867.9	76.9	>50,000	12.5	2606.9	188.1	2.3	3.5	3.2
Omicron BA.5: hCoV-19/Japan/TY41-702/2022	876.5	>50,000	>50,000	258.0	>50,000	14.3	2982.0	633.6	2.3	4.6	2.9
Omicron BQ.1.1: hCoV-19/Japan/TY41-796/2022	>50,000	>50,000	>50,000	>50,000	>50,000	>50,000	>50,000	>50,000	1.0	2.8	2.6
Omicron XBB: hCoV-19/Japan/TY41-795/2022	>50,000	>50,000	>50,000	>50,000	>50,000	>50,000	>50,000	>50,000	1.4	1.2	2.9

* SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; WHO, World Health Organization.

† The antibodies used in this analysis were produced in the authors' laboratories and are not identical to the commercially available products. The individual monoclonal antibodies were tested at a starting concentration of 50,000 ng per milliliter as a 50% focus reduction neutralization test ($FRNT_{50}$) titer. The monoclonal antibody combinations were tested at a starting concentration of 25,000 ng per milliliter for each antibody. The $FRNT_{50}$ levels were determined by performing a focus reduction neutralization test in Vero E6-TMPRSS2-T2A-ACE2 cells. Data are the mean values for triplicate experiments.

‡ The drugs used were purchased from MedChemExpress. The in vitro 50% inhibitory concentration (IC_{50}) values were determined by performing a focus reduction assay in Vero E6-TMPRSS2-T2A-ACE2 cells. Data are the mean values for triplicate experiments.

§ 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, is an inhibitor of the main protease of SARS-CoV-2 and is also called 3-chymotrypsin-like protease.

Supplementary References

1. Takashita E, Kinoshita N, Yamayoshi S, et al. Efficacy of Antiviral Agents against the SARS-CoV-2 Omicron Subvariant BA.2. N Engl J Med. 2022;386:1475-1477.
2. Takashita E, Yamayoshi S, Simon V, et al. Efficacy of Antibodies and Antiviral Drugs against Omicron BA.2.12.1, BA.4, and BA.5 Subvariants. N Engl J Med. 2022;387:468-470.
3. Takashita E, Kinoshita N, Yamayoshi S, et al. Efficacy of Antibodies and Antiviral Drugs against Covid-19 Omicron Variant. N Engl J Med. 2022;386:995-998.
4. Vanderheiden A, Edara VV, Floyd K, et al. Development of a Rapid Focus Reduction Neutralization Test Assay for Measuring SARS-CoV-2 Neutralizing Antibodies. Current Protocols in Immunology. 2020;131:e116.
5. Takashita E, Morita H, Ogawa R, et al. Susceptibility of Influenza Viruses to the Novel Cap-Dependent Endonuclease Inhibitor Baloxavir Marboxil. Frontiers in Microbiology. 2018;9:3026.