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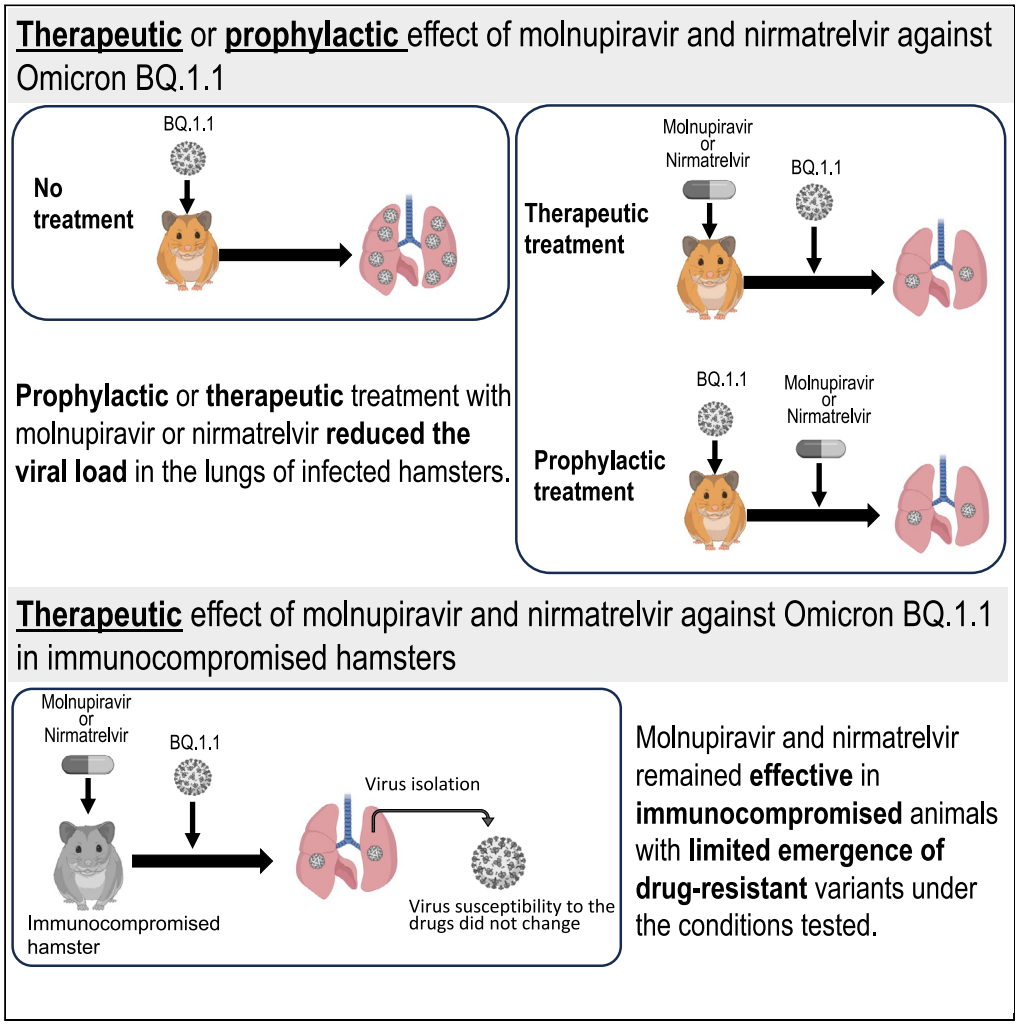

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Highlights

Molnupiravir and nirmatrelvir reduced viral loads in BQ.1.1-infected hamsters

Both drugs also reduced viral titers in immunocompromised hamsters

Immunocompromised hosts could not completely clear the virus with either drug

The emergence of drug-resistant viruses was limited under the conditions tested

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Drug susceptibility and the potential for drug-resistant SARS-CoV-2 emergence in immunocompromised animals

Maki Kiso,[1,6] Ryuta Uraki,[1,2,3,6] Seiya Yamayoshi,[1,2,3,4] Masaki Imai,[2,3,4] and Yoshihiro Kawaoka[1,2,3,5,7,*]

SUMMARY

The reduced susceptibility of mRNA vaccines and diminished neutralizing activity of therapeutic monoclonal antibodies against Omicron variants, including BQ.1.1, XBB, and their descendants, highlight the importance of antiviral therapies. Here, we assessed the efficacy of two antivirals, molnupiravir, targeting a viral RNA-dependent RNA polymerase, and nirmatrelvir, targeting a main protease, against BQ.1.1 in hamsters. We found that prophylactic or therapeutic treatment with either drug significantly reduced the viral load in the lungs of infected hamsters. We also evaluated the risk of emergence of drug-resistant viruses in immunocompromised hamsters. Although 13 days of drug treatment reduced viral titers, the immunocompromised hosts could not completely clear the virus. Viruses isolated from drug-treated immunocompromised hamsters did not show reduced susceptibility to the drugs. Molnupiravir and nirmatrelvir remain effective *in vivo* against variants with reduced susceptibility to monoclonal antibodies and mRNA vaccine-induced antibodies, with limited emergence of drug-resistant variants under the conditions tested.

INTRODUCTION

It has been more than three years since SARS-CoV-2 spread worldwide. At the onset of the pandemic, nonpharmaceutical interventions, including social distancing and face mask use, played an important role in preventing SARS-CoV-2 infection and transmission. However, a dedicated global effort to devise preventive and treatment strategies for COVID-19 led to the developments of effective vaccines, monoclonal antibodies, and antiviral drugs, which have significantly reduced the severity and fatality rate following infection.

During the COVID-19 pandemic, SARS-CoV-2 underwent frequent accumulation of amino acid substitutions, leading to the emergence of numerous variants of concern (VOCs), which caused multiple waves of infection (https://nextstrain.org/ncov/gisaid/global/all-time). Among these variants, the Omicron variant (lineage B.1.1.529) emerged in late November 2021 and spread rapidly around the world.[1] The omicron variants have more than 30 amino acid substitutions, deletions, or insertions in the spike protein, resulting in evasion from vaccines and therapeutic monoclonal antibodies.[2–6] In particular, the subvariants BQ.1.1 and XBB and their descendants escape from all monoclonal antibodies that the Food and Drug Administration (FDA) had authorized for emergency use, that is, casirivimab (REGN10933) plus imdevimab (REGN10987), bamlanivimab (LY-CoV555) plus etesevimab (LY-CoV016), tixagevimab (COV2-2196) plus cilgavimab (COV2-2130), and sotrovimab (S309). In addition, these variants are less susceptible to bebtelovimab (LY-CoV1404), which showed high neutralizing activity against Omicron variants before the emergence of BQ.1.1 and XBB.[7–9] Moreover, the neutralizing activities of plasma from individuals who received the COVID-19 mRNA vaccine are considerably lower against these latest variants, including BQ.1.1 and XBB, than against the ancestral strain, suggesting that the latest subvariants effectively evade current humoral immunity induced by mRNA vaccines.[8–10]

In addition to vaccines and monoclonal antibodies, therapeutic options to combat COVID-19 include antiviral drugs against SARS-CoV-2 such as molnupiravir and nirmatrelvir, both of which have been authorized for the treatment of COVID-19 by the FDA. Molnupiravir targets RNA-dependent RNA polymerase (RdRp, also known as nonstructural protein 12 [Nsp12]) and inhibits the RNA-dependent translation and transcription of viral RNA by causing a lethal accumulation of mispaired nucleobases in the viral RNA genome.[11,12] Nirmatrelvir inhibits the 3CL protease (3CLpro, also known as main protease [Mpro] and nonstructural protein 5 [Nsp5]), leading to the inhibition of viral replication.[13] These antiviral drugs are effective against not only the ancestral strain but also the recent circulating strains including BQ.1.1 and XBB *in vitro*.[3–10,14,15] However, the therapeutic and prophylactic efficacy of these drugs against these strains *in vivo* remains unknown.

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While vaccination is generally an effective method or protection against COVID-19, it is often ineffective in immunocompromised patients, including patients with human immunodeficiency virus (HIV) infection, transplants, primary immunodeficiencies and those treated with immunosuppressants.[16–19] Therefore, given the lack of effectiveness of available therapeutic monoclonal antibodies against recent SARS-CoV-2 variants, antiviral drugs are important options to combat the virus infection in these patients. As immunocompromised patients cannot eliminate viruses effectively,[20–22] longer treatment with antiviral drugs is required in these patients compared with immunocompetent patients. However, longer antiviral treatment in such patients has resulted in the emergence of antiviral-resistant viruses.[23–28]

Accordingly, here, we examined the efficacy of antiviral therapies for COVID-19 against BQ.1.1 in a hamster model. We also evaluated the risk of emergence of antiviral-resistant viruses after prolonged antiviral treatment in an immunocompromised hamster model.

RESULTS

Therapeutic and prophylactic effects of antivirals on the BQ.1.1 variant

We previously showed that the susceptibility of BQ.1.1 (hCoV-19/Japan/TY41-796/2022; TY41-796) to molnupiravir and nirmatrelvir was comparable to that of an early SARS-CoV-2 strain (SARS-CoV-2/UT-NC002-1T/Human/2020/Tokyo; NC002) *in vitro*.[7] Here, we assessed the therapeutic efficiency of these antivirals in hamsters infected with BQ.1.1 (TY41-796). Hamsters intranasally infected with 10^5 plaque-forming units (p.f.u.) of virus were treated from day 1 or 2 post-infection by oral gavage twice daily (at 12-h intervals) for 3 or 2 days, respectively, with molnupiravir (250 mg/kg/12 h) or nirmatrelvir (250 mg/kg/12 h) (Figures 1A and 1C). On day 4 post-infection, the animals were euthanized and their nasal turbinates and lungs were collected for virus titration. Both compounds dramatically reduced lung viral titers when treatment was initiated on day 1 post-infection (Figure 1B). Treatment with nirmatrelvir from day 2 post-infection resulted in a significant reduction in lung viral titers (mean reduction in viral titer, 2.8 $\log_{10}$ (p.f.u. g^{-1})), whereas treatment with molnupiravir did not (Figure 1D). No differences in the virus titers in the nasal turbinates were observed among the animals that were treated with molnupiravir or nirmatrelvir and the untreated hamsters on day 4 post-infection (Figures 1B and 1D).

Although these compounds are approved for therapeutic use, they have not yet been approved for preventive use. Prophylactic administration of antiviral drugs such as oseltamivir is effective and approved for influenza virus infection. To examine whether prophylactic administration of molnupiravir or nirmatrelvir can protect hamsters against SARS-CoV-2 infection, hamsters intranasally infected with 10^5 p.f.u. of virus were treated from 1 day before infection by oral gavage twice daily (at 12-h intervals) for 5 days, respectively, with molnupiravir (250 mg/kg/12 h) or nirmatrelvir (250 mg/kg/12 h) (Figure 2A). Although neither molnupiravir nor nirmatrelvir altered the virus replication in the nasal turbinates, both compounds significantly reduced the virus titers in the lungs on Day 4 post-infection (Figure 2B); no virus was recovered from the lungs of three of the five animals in both groups. These results suggest that prophylactic administration of molnupiravir or nirmatrelvir is effective against recently circulating SARS-CoV-2.

Effects of antivirals on the BQ.1.1 variant in immunocompromised animals

To assess whether treatment of immunosuppressed hosts with molnupiravir or nirmatrelvir is effective against recently circulating SARS-CoV-2, we used immunocompromised hamsters that intraperitoneally received cyclophosphamide (CPA).[29] CPA has been used successfully in the clinic for over 50 years; it affects T and B cells, decreasing immune responses and blocking DNA production, leading to immunosuppression.[30–32] We injected hamsters with CPA on days −3, 1, 5, and 9 relative to BQ.1.1 infection under the same conditions as we used previously to demonstrate the effectiveness of CPA[33] and monitored the hamsters' body weights (Figures 3A and 3B). Consistent with our previous study,[33] virus titers were below the detection limit on day 14 post-infection in both the nasal turbinates and lungs of hamsters that were not treated with CPA (Figure 3C). In contrast, high titers of virus persisted in the respiratory tract of the CPA-treated animals (control) on day 14 post-infection. The CPA-treated hamsters showed a smaller increase in body weight compared to the untreated hamsters (Figure 3B). Thirteen days of treatment with either molnupiravir or nirmatrelvir inhibited viral replication in the lungs of the immunosuppressed animals on day 14 post-infection (mean reduction in viral titer, 3.0 and 3.8 $\log_{10}$ [p.f.u. g^{-1}], respectively), although the difference between the molnupiravir-treated group and the vehicle control group was not statistically significant (Figure 3C). However, neither molnupiravir nor nirmatrelvir suppressed the viral titers in the nasal turbinates of the hamsters on day 14 post-infection despite the prolonged treatment with these compounds (Figure 3C). Regarding body weight, CPA-treated hamsters that received either molnupiravir or nirmatrelvir tended to gain more weight than control CPA-treated hamsters, although the difference was not statistically significant (Figure 3B).

To examine the emergence of resistant variants after prolonged antiviral treatment in immunosuppressed hosts, we isolated viruses from the virus-positive samples in VeroE6/TMPRSS2-T2A-ACE2 cells and then propagated them in Vero E6/TMPRSS2; the virus-positive samples included two lung samples and four nasal turbinate samples from five molnupiravir-treated hamsters, as well as from one lung sample and five nasal turbinate samples from five nirmatrelvir-treated hamsters on day 14 post-infection. Because the target viral proteins of molnupiravir and nirmatrelvir are Nsp12 and Nsp5, respectively, we performed deep sequencing analysis of the coding regions of these proteins. Deep sequencing analysis revealed that the viruses isolated from the lungs of one of the molnupiravir-treated animals (#51) and from the nasal turbinate of a molnupiravir-treated animal (#55) on day 14 post-infection possess the T225I (21%) and V166I (76%) substitutions in Nsp12, respectively. Among the nirmatrelvir-treated animals, the T21I (81%) or T304I (32%) substitution in Nsp5 was detected in viruses isolated from the nasal turbinates of animal #58 and animal #59, respectively, on day 14 post-infection (Table 1).

To gain further insight into the susceptibility of the isolated viruses against the antiviral compounds, we picked and propagated viruses from 5 to 10 single plaques from these four samples, that is, the lungs (#51) or nasal turbinates (#55) of the molnupiravir-treated animals and the nasal turbinates (#58 and # 59) of the nirmatrelvir-treated animals. Of the 10 picked viruses isolated from the lungs of the

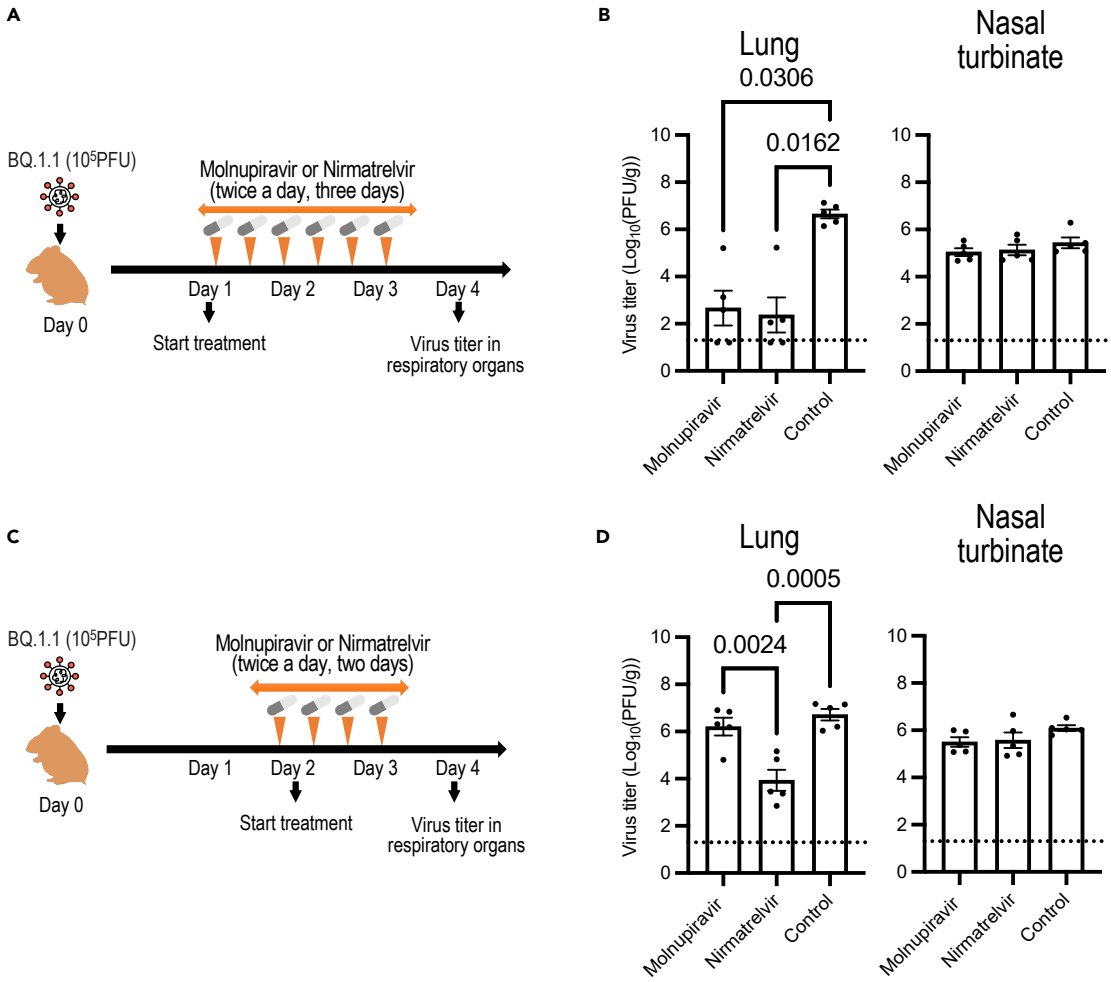

Figure 1. Therapeutic effects of antiviral compounds on the replication of the SARS-CoV-2 Omicron BQ.1.1 variant

(A and C) Schematic diagram of the experimental workflow for assessing the therapeutic effects of antiviral compounds.

(B and D) Syrian hamsters were intranasally inoculated with 10^5 p.f.u. of BQ.1.1. At 24 h (B) or 48 h (D) post-infection (hpi), the hamsters were treated with 250 mg/kg molnupiravir orally twice daily or with 250 mg/kg nirmatrelvir orally twice daily. Methylcellulose served as a control for oral treatment. Hamsters were euthanized on day 4 post-infection for virus titration. Vertical bars show the mean ± SEM. Points indicate data from individual hamsters (n = 5 per group). The lower limit of detection is indicated by horizontal dashed lines. To compare the lung and nasal turbinate titers of the different groups of BQ.1.1-infected hamsters, we used a Kruskal-Wallis test followed by Dunn's multiple comparisons test (B: lung), or a one-way ANOVA followed by Dunnett's multiple comparisons test (B and D: nasal turbinate, D: lung). $p < 0.05$ was considered statistically significant.

molnupiravir-treated animal (#51), two viruses possessed the T225I substitution, one virus possessed both the T225I and T582I substitutions, one virus possessed the V693I substitution in Nsp12, and the remaining six viruses did not contain any mutations in Nsp12. Although the plaque-picked virus #2 from animal #51 with the Nsp12 V693I mutation exhibited the highest IC_{50} value (7.2 μM) among the tested plaque-picked viruses, the difference between the IC_{50} value of virus #2 from animal #51 and the average IC_{50} value (4.0 μM) from plaque-picked viruses without mutations in Nsp12 was less than 2-fold. This suggests that the V693I mutation in virus #2 has minimal impact on the sensitivity of molnupiravir. Among the 5 picked viruses isolated from the nasal turbinates of the molnupiravir-treated animal (#55), all five viruses possessed only the V166I substitution in Nsp12. Overall, regardless of the presence or absence of mutations, the susceptibility of these picked viruses to molnupiravir was similar (Table 1).

Among the 15 viruses isolated from the nasal turbinates of the nirmatrelvir-treated animals (#58 and #59), only two viruses, both of which were isolated from animal #58, has acquired a single substitution of either T257I or T304I in Nsp5. Although the T21I substitution was detected in the propagated virus from the nasal turbinate of animal #58, we did not detect or isolate a virus with this substitution in Nsp5. The T257I or T304I substitution had little to no effect on nirmatrelvir susceptibility, given that the difference between the highest IC_{50} value from individual plaque-picked viruses and the average IC_{50} value (2.4 μM) from plaque-picked viruses was less than 2-fold (Table 1). We mapped the identified substitutions in the Nsp12 and Nsp5 molecules (Figures 3D and 3E). Although we found that V166I, T582I, and V693I in Nsp12 are close to the

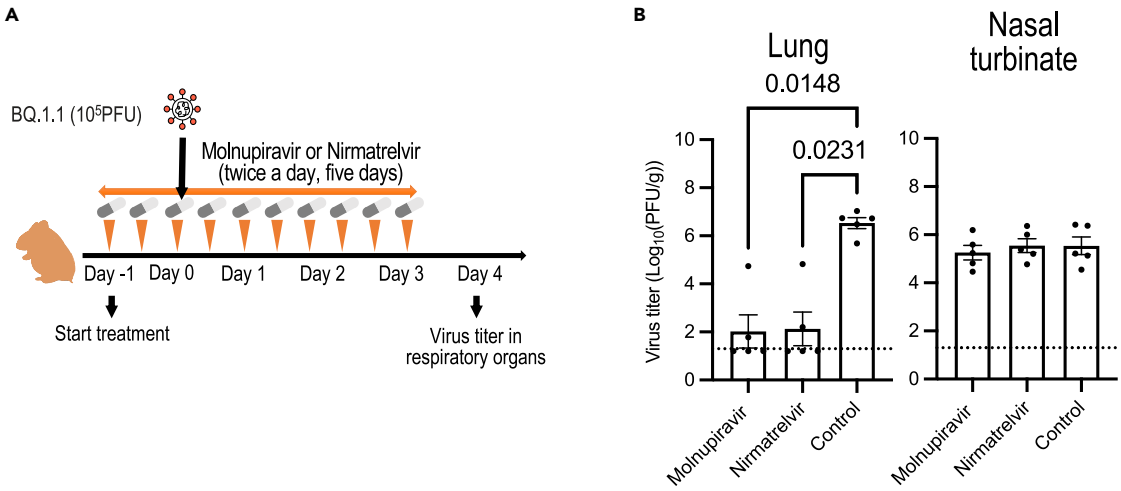

Figure 2. Prophylactic effects of antiviral compounds on the replication of the SARS-CoV-2 Omicron BQ.1.1 variant

(A) Schematic diagram of the experimental workflow for assessing the prophylactic effects of antiviral compounds.

(B) Syrian hamsters were intranasally inoculated with 10^5 p.f.u. of BQ.1.1. At 24 h before infection, hamsters were treated with 250 mg/kg molnupiravir orally twice daily for 5 days or with 250 mg/kg nirmatrelvir orally twice daily for 5 days. Methylcellulose served as a control for oral treatment. The hamsters were euthanized on day 4 post-infection for virus titration. Vertical bars show the mean ± SEM. Points indicate data from individual hamsters (n = 5 per group). The lower limit of detection is indicated by horizontal dashed lines. To compare the lung and nasal turbinate titers of the different groups of BQ.1.1-infected hamsters, we used a Kruskal-Wallis test followed by Dunn's multiple comparisons test or a one-way ANOVA followed by Dunnett's multiple comparisons test, respectively. $p < 0.05$ was considered statistically significant.

active cavity of Nsp12 and that T21I in Nsp5 is close to the active center of Nsp5, further analysis is needed, through the use of AI strategies such as AlphaFold prediction, to evaluate whether these identified substitutions impact the activities of drugs.

Overall, our results suggest that the emergence of resistant variants in immunosuppressed hamsters treated with molnupiravir or nirmatrelvir for 13 days is limited.

DISCUSSION

Given the decreased effectiveness of mRNA vaccines and the loss of neutralizing activity of monoclonal antibodies against recently circulating SARS-CoV-2 variants, including BQ.1.1 or XBB,[7–9,14,15] the importance of antiviral drugs targeting virus proteins has increased. However, the effectiveness of these drugs against recently circulating viruses that are resistant to monoclonal antibodies *in vivo* remains uncertain. Furthermore, it is important to determine whether the use of these drugs leads to the emergence of viruses with reduced sensitivity to such compounds, especially in immunocompromised hosts.

Here, we examined the efficacy of molnupiravir and nirmatrelvir against BQ.1.1, which has reduced susceptibility to all monoclonal antibodies at one time authorized for emergency use by the FDA, in a hamster model. We found that both drugs suppressed viral titers in the lungs at 4 dpi when given 24 h before or after BQ.1.1 infection. Nirmatrelvir, but not molnupiravir, reduced the virus burden in the lungs of infected hamsters when administered at 2 dpi, but its effectiveness in suppressing viral loads in the lungs was attenuated compared to its effectiveness when administered at 1 dpi. Of note, neither molnupiravir nor nirmatrelvir reduced the virus titers in the nasal turbinates. These results suggest that early treatment is essential for high efficacy of these drugs.

The emergence of drug-resistant viruses is a public health concern when using antiviral therapies. The neutralizing activity of therapeutic monoclonal antibodies (i.e., the effectiveness of these antibodies for COVID-19) has become remarkably attenuated due to the mutations in the spike protein of recently circulating strains.[7,8,14,15] In contrast, antivirals targeting viral proteins other than the spike protein, such as molnupiravir and nirmatrelvir, remain effective against recently circulating strains including BQ.1.1, XBB, and their descendants.[7–9] Previous studies have demonstrated that antiviral treatment for persistent virus infection in immunocompromised patients increases the risk of emergence of antiviral drug resistance.[34,35] To understand antiviral effectiveness and the potential for drug-resistant virus emergence, we examined whether prolonged treatment of immunocompromised hamsters with molnupiravir or nirmatrelvir could result in the emergence of resistant variants. Immunosuppressive treatment with CPA led to prolonged viral shedding in our model. Both molnupiravir and nirmatrelvir treatment suppressed viral load in the lungs of the immunosuppressed animals on day 14 post-infection, with varying degrees of suppression. However, neither molnupiravir nor nirmatrelvir affected the virus titers in the nasal turbinates of the animals on day 14 post-infection, suggesting that viral clearance cannot be achieved in immunocompromised hosts even if the drugs are given for longer than the five days that is usually prescribed for patients without underlying conditions. To examine whether the prolonged drug treatment led to the emergence of drug-resistant virus, we isolated viruses by plaque picks from the lung or nasal turbinate samples from the immunosuppressed animals treated

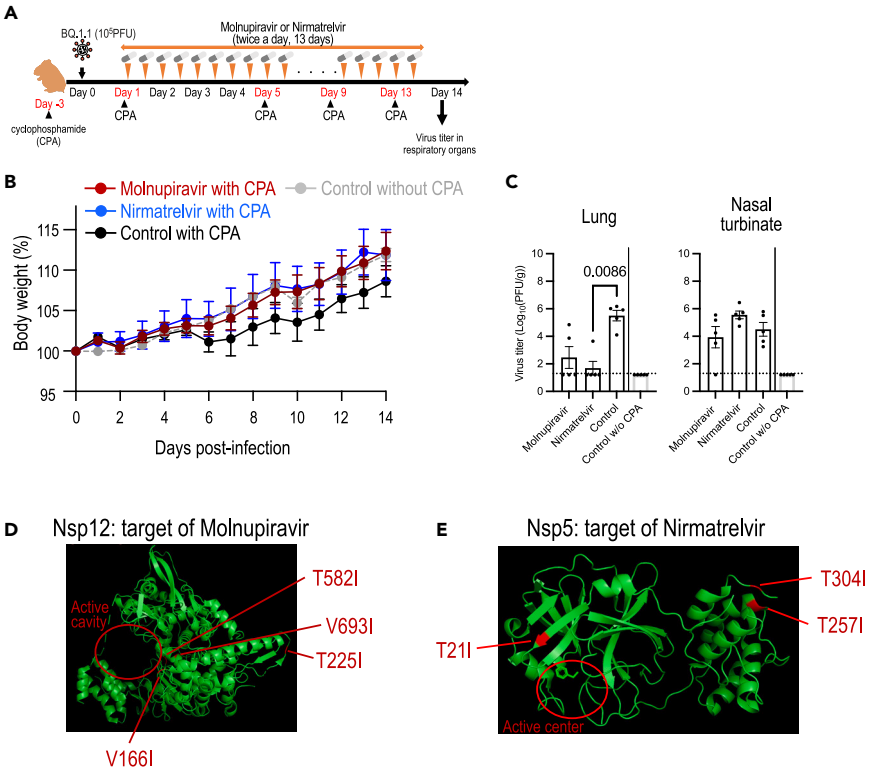

Figure 3. Therapeutic effects of antiviral compounds on the replication of the SARS-CoV-2 Omicron BQ.1.1 variant and the risk of emergence of antiviral-resistant viruses in immunocompromised animals

(A) Schematic diagram of the experimental workflow for assessing the therapeutic effects of antiviral compounds in immunocompromised hamsters.

(B and C) Syrian hamsters were intranasally inoculated with 10^5 p.f.u. of BQ.1.1. CPA was administered intraperitoneally to hamsters on days −3, 1, 5, 9, and 13 relative to infection. At 24 h post infection, the hamsters were treated with 250 mg/kg molnupiravir orally twice daily for 13 days or with 250 mg/kg nirmatrelvir orally twice daily for 13 days. Methylcellulose served as a control for oral treatment. (B) Body weights of virus-infected hamsters were monitored daily for 14 days after viral infection. Data are mean percentages ± SEM. of the starting weight. (C) Hamsters were euthanized on day 14 post-infection for virus titration. Vertical bars show the mean ± SEM. Points indicate data from individual hamsters (n = 5 per group). The lower limit of detection is indicated by horizontal dashed lines. To compare the lung and nasal turbinate titers of the different groups of BQ.1.1-infected hamsters, we used a Kruskal-Wallis test followed by Dunn's multiple comparisons test. $p < 0.05$ was considered statistically significant.

(D and E) Positions of identified substitutions on the Nsp12 (D) and Nsp5 (E) molecules. Nsp12 (D, PDB ID: 6NUS) and Nsp5 (E, PDB ID: 6lu7) are indicated in green. Red indicates the substituted residues after drug treatment.

with either compound on day 14 post-infection. None of the viruses isolated from the drug-treated immunosuppressed animals showed reduced susceptibility to molnupiravir or nirmatrelvir.

Previous studies have shown that some substitutions in Nsp5 lead to a reduction in susceptibility to nirmatrelvir.[13,36–46] In our study, the viruses we isolated did not contain E166V or H172Y, which can cf. a high degree of nirmatrelvir resistance.[13,46] Regarding the T21I and T304I substitutions in Nsp5 found in our study, it has been reported that these substitutions also emerge or are selected during serial passaging *in vitro* and each amino acid substitution has a low to modest impact on resistance to nirmatrelvir. Additional substitutions with T21I and/or T304I, such as T21I + S144A, T21I + E166V, T21I + A173V, T21I + T304I, L50F + T304I, T21I + S144A + T304I, and T21I + A173V + T304I, are reported to lead to reduced susceptibility to nirmatrelvir.[13,42,46] In addition, a recent preprint study demonstrated that the mutation E166V conferred strong resistance to nirmatrelvir, approximately 55-fold, with a significant reduction in replicon fitness of the ancestral strain (nearly 20-fold), but not the BA.1 subvariant (2-fold), suggesting that the Omicron variant may possess a lower resistance barrier compared to the variants that circulated before the emergence of Omicron.[47] Therefore, further studies may be required to ascertain the generalizability of drug-resistant mutations to the Omicron variants.

Thus far, there have been no reports linking amino acid substitutions to molnupiravir resistance. Although some of the isolates recovered from the drug-treated animals in our study had substitutions in Nsp12, which is a target for molnupiravir, the susceptibilities were similar among these isolates regardless of their substitutions.

In conclusion, the two small-molecule antiviral drugs molnupiravir and nirmatrelvir are effective against the BQ.1.1 variant of SARS-CoV-2, in wild-type and immunocompromised hamsters. However, complete viral clearance eluded the immunocompromised hamsters even with prolonged drug treatment. Our results indicate that the emergence of drug-resistant variants is limited under the conditions we tested,

Table 1. Drug Susceptibility and substitutions in viruses isolated from the respiratory organs of immunosuppressed hamsters after treatment with antiviral compounds

				Bulk sample		Plaque-picked individual samples		
Sample collection time point:	Treatment	Animal ID #	Sample	IC_{50}[a] (μM)	Substitutions in the target protein	Plaque-picked individual No.	IC_{50} (μM)	Substitutions in the target protein
Day 14	Molnupiravir	51	Lung	2.1	Nsp12 T225I (21.3%)	1	5.6	ND[b]
						2	7.2	Nsp12 V693I (99.6%)
						3	4.6	ND
						4	4.2	Nsp12 T225I (99.8%)
						5	4.9	ND
						6	3.9	Nsp12 T225I (99.8%)
						7	2.4	ND
						8	3.1	ND
						9	1.7	Nsp12 T225I (99.3%), T582I (82.9%)
						10	3.6	ND
		55	Nasal tubinate	1.8	Nsp12 V166I (76.0%)	1	2.5	Nsp12 V166I (91.1%)
						2	4.1	Nsp12 V166I (89.5%)
						3	2.9	Nsp12 V166I (92.3%)
						4	3.0	Nsp12 V166I (95.7%)
						5	3.3	Nsp12 V166I (95.4%)
	Nirmatrelvir	58	Nasal tubinate	2.2	Nsp5 T304I (32.5%)	1	1.0	Nsp5 T257I (98.7%)
						2	2.5	ND
						3	3.4	ND
						4	2.2	ND
						5	1.7	ND
						6	1.8	ND
						7	3.5	Nsp5 T304I (99.3%)
						8	2.4	ND
						9	2.0	ND
						10	2.5	ND
		59	Nasal tubinate	3.0	Nsp5 T21I (80.9%)	1	1.4	ND
						2	1.5	ND
						3	1.9	ND
						4	2.2	ND
						5	3.0	ND

[a]50% inhibitory concentration.
[b]Not detected.

but we should closely monitor the amino acid mutations in viruses isolated from immunocompromised patients after drug treatment to detect such viruses as soon as possible after emergence.

Limitations of the study

Although the emergence of resistant variants after molnupiravir or nirmatrelvir treatment appears to be limited in immunocompromised animals under the condition we tested, nirmatrelvir-resistant viruses have been detected in immunocompromised patients treated with nirmatrelvir/ritonavir.[26,28] Unlike our hamster experiments, these patients were treated with nirmatrelvir/ritonavir multiple times and/or for longer (e.g., three separate times for a total of 11 days[26] or five separate times for a total of approximately 9 weeks[28]). Further evaluation is needed to determine whether drug treatments in animal models designed to mirror to those in clinical settings can lead to the emergence of nirmatrelvir-resistant viruses.

RESOURCE AVAILABILITY

Lead contact

Further information or requests should be directed to and will be fulfilled by the lead contact, Yoshihiro Kawaoka (yoshihiro.kawaoka@wisc.edu).

Materials availability

All materials can be obtained directly from the authors or through commercially available sources.

Data and code availability

- Data: All data used in this paper are available in the main text and are clearly definite.
- Code: This paper does not contain any original code.
- Additional Information: Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

M. K.: data curation, formal analysis, and methodology. R.U.: conceptualization, data curation, formal analysis, validation, visualization, and writing of the first draft. S.Y.: conceptualization, data curation, formal analysis, and methodology. M. I.: conceptualization and data curation. Y. K.: conceptualization, supervision, writing (review and editing), and funding acquisition. M. K. and R.U. contributed equally.

DECLARATION OF INTERESTS

Y. Kawaoka has received unrelated funding support from Daiichi Sankyo Pharmaceutical, Toyama Chemical, Tauns Laboratories, Inc., Shionogi & Co. Ltd, Otsuka Pharmaceutical, KM Biologics, Kyoritsu Seiyaku, Shinya Corporation, and Fuji Rebio.

STAR★METHODS

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
SARS-CoV-2 nucleoprotein (clone N45)	TAUNS Laboratories, Inc.	N/A
Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L)	Jackson ImmunoResearch Laboratories Inc.	Cat#115-035-003; RRID: AB_10015289
Bacterial and virus strains		
hCoV-19/Japan/TY41-796/2022	Imai et al.[7]	N/A
Chemicals, peptides, and recombinant proteins		
Dulbecco's modified Eagle's medium	SIGMA	Cat #D5796
Fetal calf serum	gibco	Cat #10437-028
Penicillin–streptomycin	FUJIFILM Wako Pure Chemical Corporation	Cat #168-23191
Puromycin	InvivoGen	Cat # ant-pr-1
Geneticin	InvivoGen	Cat # ant-gn-5
Deposited data		
hCoV-19/Japan/TY41-796/2022	GISAID	EPI_ISL_16355655
Wuhan/Hu-1/2019 sequence	GenBank	MN908947
Experimental models: Cell lines		
VeroE6/TMPRSS2 cells	JCRB Cell Bank	JCRB1819
Vero E6-TMPRSS2-T2A-ACE2 cells	Graham laboratory	NA
Experimental models: Organisms/strains		
Syrian hamsters (male, 6 weeks old)	Japan SLC Inc.	http://www.jslc.co.jp/pdf/data/2013/syrian2013.pdf
Software and algorithms		
GraphPad Prism 9.3.0	GraphPad Software, Inc.	https://www.graphpad.com/scientific-software/prism/
BioSpot software	Cellular Technology	https://immunospot.com/plaque-colony-counting
Other		
QIAamp Viral RNA Mini Kit	QIAGEN	Cat# 52926
LunarScript RT SuperMix Kit	New England BioLabs	Cat# E3010
Q5 High-Fidelity DNA polymerase	New England BioLabs	Cat# M0491
Q5 Hot Start DNA polymerase	New England BioLabs	Cat# M0493
QIAseq FX DNA Library Kit	QIAGEN	Cat# 180477

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Experiment Committee of the Institute of Medical Science, the University of Tokyo (approval number PA19-75). Virus inoculations were performed under isoflurane, and all efforts were made to minimize animal suffering. *In vivo* studies were not blinded, and animals were randomly assigned to infection groups. No sample-size calculations were performed to power each study. Instead, sample sizes were determined based on prior *in vivo* virus challenge experiments.

METHOD DETAILS

Cells

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 Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Calf Serum (FCS). Vero E6-TMPRSS2-T2A-ACE2 cells (provided by Dr. Barney Graham, NIAID Vaccine Research Center, available at BEI Resources, NR-54970) were cultured in DMEM supplemented with 10% FCS, 10 mM HEPES pH 7.3, 100 U/mL penicillin–streptomycin, and 10 μg/mL puromycin. VeroE6/TMPRSS2 and Vero E6-TMPRSS2-T2A-ACE2 cells were maintained at 37°C with 5% CO_2. The cells were regularly tested for mycoplasma contamination by using PCR, and confirmed to be mycoplasma-free.

Viruses

The SARS-CoV-2 variant hCoV-19/Japan/TY41-796/2022 (Omicron BQ.1.1) was propagated in VeroE6/TMPRSS2 cells.[7] 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.

Antiviral compounds

Components of molnupiravir (i.e., EIDD-1931 for *in vitro*, EIDD-2801 for *in vivo*), and nirmatrelvir (PF-07321332) were purchased from MedChemExpress. All compounds were dissolved in dimethyl sulfoxide for *in vitro* experiments and dissolved in 0.5% methylcellulose prior to use in *in vivo* experiments.

Evaluation of the efficacy of the antiviral compounds in syrian hamsters

Six-week-old male Syrian hamsters (Japan SLC Inc., Shizuoka, Japan) were used in this study. For the efficacy evaluation of the antiviral compounds in hamsters, five hamsters per group, under *isoflurane* anesthesia, were inoculated intranasally with 10^5 PFU (in 30 μl) of BQ.1.1. Hamsters were treated with the following antiviral compounds, at 24 h or 48h post-infection to examine the therapeutic effect, or at 24 h before virus infection to assess the prophylactic effect: (1) molnupiravir, 250 mg/kg (in 1 ml) administered orally twice daily; (2) nirmatrelvir, 250 mg/kg (in 1 ml) administered orally twice daily; or (3) methylcellulose (1 ml) as a control for oral treatment. The animals were euthanized on Day 4 post-infection, and the virus titers in the nasal turbinates and lungs were determined by use of plaque assays on Vero E6-TMPRSS2-T2A-ACE2 cells.

For the evaluation of the emergence of antiviral-resistant virus in hamsters, cyclophosphamide (CPA) was administered intraperitoneally to hamsters on Day -3 (140 mg/kg), 1 (100 mg/kg), 5 (100 mg/kg), and 9 (100 mg/kg) relative to infection. Under *isoflurane* anesthesia, five hamsters per group were inoculated intranasally with 10^5 PFU (in 30 μl) of BQ.1.1 on Day 0. At 24 h after inoculation, the hamsters were treated with the following antiviral compounds for 13 days: (1) molnupiravir, 250 mg/kg (in 1 ml) administered orally twice daily; (2) nirmatrelvir 250 mg/kg (in 1 ml) administered orally twice daily; or (3) methylcellulose (1 ml) as a control for oral treatment. The animals were euthanized on Day 14 post-infection, and the virus titers in the nasal turbinates and lungs were determined by use of plaque assays on Vero E6-TMPRSS2-T2A-ACE2 cells. Viruses were also isolated from homogenates of lungs or nasal turbinates to determine 50% inhibitory concentration (IC_{50}) values and for viral genome analysis.

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.[3–5,48] Briefly, 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 and then the inoculum was removed and replaced with 1% Methyl Cellulose 400 (FUJIFILM Wako Pure Chemical Corporation) in culture medium containing serial dilutions of antiviral compounds, which 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, 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 IC_{50} values, which were calculated by using GraphPad Prism (GraphPad Software).

Deep sequence analysis

Viral RNA was extracted by using a QIAamp Viral RNA Mini Kit (QIAGEN). The coding regions of Nsp5 and Nsp12 were amplified by using primers of a modified ARTIC network protocol in which some of the primers were replaced or added.[49] 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-N6 primers and the Q5 High-Fidelity DNA polymerase or 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 sequences, the reads were analyzed by CLC Genomics Workbench (version 23, Qiagen) with the Wuhan/Hu-1/2019 sequence (GenBank accession no. MN908947) as a reference. Briefly, reads with quality scores of more than 0.05 were used for the analysis. The adaptor sequences were trimmed and trimmed sequences of less than 50 bases were

discarded. The reads were then mapped to the reference sequence. Local realignment and primer trimming were conducted. Amino acid substitutions that occurred at a rate of ≥10% are listed. The average coverage was between 3161.00 and 8228.08.

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism was used to analyze all of the data. Statistical analysis included a one-way ANOVA with Tukey's multiple comparisons test, and the Friedman test followed by Dunn's test. Differences among groups were considered significant for $P < 0.05$.