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Ensitrelvir is effective against SARS-CoV-2 3CL protease mutants circulating globally

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ABSTRACT

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been a public health concern worldwide. Ensitrelvir (S-217622) has been evaluated as an antiviral treatment for COVID-19, targeting SARS-CoV-2 3C-like protease ($3CL^{pro}$). Ensitrelvir has been reported to have comparable antiviral activity against some of the SARS-CoV-2 variants: alpha, beta, gamma, delta, and omicron (BA.1.18). In this paper, we describe that ensitrelvir is effective against newly emerging SARS-CoV-2 variants and globally prevalent $3CL^{pro}$ mutations. Ensitrelvir exhibited comparable antiviral activity against SARS-CoV-2 variants, including recently emerging ones: omicron (BA1.1, BA.2, BA.2.75, BA.4, BA.5, BQ.1.1, XBB.1, and XE), mu, lambda, and theta. Genetic surveillance of SARS-CoV-2 $3CL^{pro}$, the target of ensitrelvir, was conducted using a public database and identified 11 major $3CL^{pro}$ mutations circulating globally (G15S, T21I, T24I, K88R, L89F, K90R, P108S, P132H, A193V, H246Y, and A255V). The $3CL^{pro}$ mutation from proline to histidine at amino acid position 132 was especially identified in the omicron variant, with prevalence of 99.69%. Enzyme kinetic assay revealed that these $3CL^{pro}$ mutants have enzymatic activity comparable to that of the wild type (WT). Next, we assessed the inhibitory effect of ensitrelvir against mutated $3CL^{pro}$, with it showing inhibitory effects similar to that against the WT. These in vitro data suggest that ensitrelvir will be effective against currently circulating SARS-CoV-2 variants, including omicron variants and those carrying $3CL^{pro}$ mutations, which emerging novel SARS-CoV-2 variants could carry.

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has infected over 633 million people with 6.6 million deaths across the world as of November 20, 2022 [1]. Genetic lineages of SARS-CoV-2 have been emerging and circulating around the world since the beginning of this pandemic. Some COVID-19 treatment agents, especially monoclonal antibodies, lost their efficacy against SARS-CoV-2 variants because of SARS-CoV-2 S protein mutations, which restricted their clinical value [2–4]. COVID-19 treatment agents against novel SARS-CoV-2 variants and assessment of their efficacy are thus required [5].

The whole-genome sequence (WGS) of clinically isolated SARS-CoV-2 is often analyzed by next-generation sequencing and registered in public databases, which is useful for monitoring emerging genetic lineages of SARS-CoV-2. Global Initiative on Sharing Avian Influenza Data (GISAID) is one of the SARS-CoV-2 WGS public databases. SARS-CoV-2 WGS and information on SARS-CoV-2 mutations circulating around the world are used for various kinds of analyses, such as on the prevalence of SARS-CoV-2 variants and the spread of such variants in specific regions [6,7].

SARS-CoV-2 $3CL^{pro}$, also known as the main protease or nonstructural protein 5 (nsp5), is a homodimeric cysteine protease that mainly catalyzes the maturation of viral polyproteins pp1a and

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Abbreviations

COVID-19	coronavirus disease 2019
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
$3CL^{pro}$	3C-like protease
WT	wild type
nsp5	nonstructural protein 5
WGS	whole-genome sequence
GISAID	Global Initiative on Sharing Avian Influenza Data
VOC	variants of concern
VOI	variants of interest
VUM	variants under monitoring
VeroE6T	VeroE6 cells expressing transmembrane serine protease 2
MEM	minimum essential medium
$TCID_{50}$	50% tissue culture infectious dose
FRET	fluorescence resonance energy transfer
MS	mass spectrometry
IS	internal standard

pp1ab [8,9]. No human cysteine protease that cleaves after glutamine has been characterized, and thus SARS-CoV-2 $3CL^{pro}$ is an attractive therapeutic target for COVID-19 [10,11]. Ensitrelvir is the first nonpeptidic clinical candidate for treating COVID-19 and acts as a noncovalent SARS-CoV-2 $3CL^{pro}$ inhibitor, thereby blocking its replication [12].

In this study, the efficacy of ensitrelvir against emerging SARS-CoV-2 variants, including omicron variants, was examined. Next, genetic surveillance in SARS-CoV-2 $3CL^{pro}$ was conducted among variants of concern (VOC), variants of interest (VOI), and variants under monitoring (VUM) as designated by the World Health Organization (WHO), using GISAID data as of February 2022. The enzymatic activity of frequently identified $3CL^{pro}$ mutants was measured and the inhibitory effects of ensitrelvir against these mutants were assessed.

2. Materials and Methods

2.1. Purification of SARS-CoV-2 $3CL^{pro}$ mutants

SARS-CoV-2 $3CL^{pro}$ mutants were purified from *E. coli* by a slightly modified version of a previously reported procedure [12]. SARS-CoV-2 $3CL^{pro}$ (1–306), containing an N-terminal 10-histidine tag followed by a thrombin cleavage site, was cloned into pET-SUMOpro Amp (Nacalai Tesque) vectors (6His-SUMO-COVPR-SUMOpro). To generate the mutant $3CL^{pro}$ construct, appropriate mutations were introduced into 6His-SUMO-COVPR-SUMOpro using primers via a PCR protocol that amplifies the entire plasmid template. The primers are shown in Supplemental Table 1.

Plasmids were transformed into *E. coli* DH5-alpha (Takara Bio or Toyobo) competent cells and bacterial cultures overexpressing the target proteins were grown in LB (Luria-Bertani, Shiotani M.S.) medium containing 100 μg/mL ampicillin at 37 °C. Expression of the target protein was induced at an optical density (A_{600}) of over 1.0 by the addition of isopropyl β-d-1-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM, followed by incubation at 18 °C overnight. Bacterial cells were harvested by centrifugation (8,000×*g*, 20 min, 4 °C) and resuspended in lysis buffer containing 20 mM Tris (pH 8), 300 mM NaCl, 1 mM DTT, and 20 mM imidazole, after which bacterial cells were lysed by sonication. The lysed cell suspension was clarified by centrifugation and the supernatant was purified using a 0.22 μm filter. The clarified lysate was subjected to HisTrap FF 5 mL (Cytiva) equilibrated with 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1 mM DTT, and 20 mM imidazole, and the proteins were eluted with a linear concentration gradient of imidazole (20–500 mM). Fractions containing SARS-CoV-2 $3CL^{pro}$ were collected and mixed with SUMO protease at 4 °C overnight to remove the N-His-tag. SUMO protease-treated SARS-CoV-2 $3CL^{pro}$ was applied to HisTrap FF 5 mL to remove proteins with uncleaved His-tags. The flow-through fraction was applied to a 10/300 GL Superdex 75 prep grade column (Cytiva) equilibrated with 20 mM HEPES (pH 7.5), 150 mM NaCl, and 1 mM DTT, and the fraction containing the major peak was collected.

2.2. Cell culture and virus

VeroE6 cells expressing transmembrane serine protease 2 (VeroE6T) from the National Institutes of Biomedical Innovation were used [12]. The following SARS-CoV-2 clinical isolates were obtained from the National Institute of Infectious Diseases: hCoV-19/Japan/TY28-444/2021 (Theta, P.3), hCoV-19/Japan/TY33-456/2021 (Lambda, C.37), hCoV-19/Japan/TY38-871/2021 (Omicron, B.1.1), hCoV-19/Japan/TY26-717/2021 (Mu, B.1.621), hCoV-19/Japan/TY40-385/2022 (Omicron, BA.2), hCoV-19/Japan/TY41-716/2022 (Omicron, BA.2.75), hCoV-19/Japan/TY41-703/2022 (Omicron, BA.4), hCoV-19/Japan/TY41-702/2022 (Omicron, BA.5), hCoV-19/Japan/TY41-796/2022 (Omicron, BQ.1.1), hCoV-19/Japan/TY41-795/2022 (Omicron, XBB.1), and hCoV-19/Japan/TY41-686/2022 (Omicron, XE). All SARS-CoV-2 strains were propagated in VeroE6T and infectious titers were determined based on the standard 50% tissue culture infectious dose ($TCID_{50}$) in VeroE6T.

2.3. Antiviral assays

Antiviral activity was conducted as described previously [12]. Briefly, VeroE6T cells (1.5×10^4/well) were seeded into 96-well plates suspended in minimum essential medium (MEM) (Thermo Fisher Scientific) with heat-inactivated 2% FBS and diluted compounds in each well. Cells were infected with each SARS-CoV-2 at 300–3000 $TCID_{50}$/well and cultured at 37 °C with 5% CO_2 for 3 or 4 days. Cell viability was assessed using a CellTiter-Glo 2.0 assay (Promega). EC_{50} values were determined by plotting the compound concentration versus inhibition and fitting data with a four-parameter logistical fit (Model 205, XL fit).

2.4. SARS-CoV-2 genomes and $3CL^{pro}$ annotation pipeline

Genome sequences and patient metadata for ~4.9 million isolates were obtained from the GISAID [13] EpiCoV database (www.epicov.org) through February 10, 2022. GISAID data meeting the following criteria were included in the analysis: (i) the amino acid sequence of nsp5 did not include any ambiguous codes; (ii) the amino acid sequence of nsp5 was longer than 299 amino acids; and (iii) isolates were from humans. A total of 7,404,490 amino acid sequences met these criteria.

The prevalence of polymorphisms of SARS-CoV-2 $3CL^{pro}$ in each amino acid was calculated as the ratio of each amino acid to the total number of sequences; the prevalent mutations (>1% mutation frequency) were identified. This analysis was also carried out for each individual variant of concern (VOC), variant of interest (VOI), and variant under monitoring (VUM), as designated by the World Health Organization (WHO) as of 31 January 2022. There were five SARS-CoV-2 lineages as VOC (Alpha, Beta, Gamma, Delta, and Omicron variants), two lineages as VOI (Lambda and Mu variants), and three lineages as VUM (B.1.1.318, C.1.2, and B.1.640, including all descendant lineages). SARS-CoV-2 WIV04 (hCoV-19/Wuhan/

WIV04/2019, GISAID_ID: EPI_ISL_402124) was employed as a reference sequence.

2.5. Kinetic measurements

For measurement of the K_m/V_{max} of SARS-CoV-2 $3CL^{pro}$ mutants, proteolytic reactions were carried out with continuous fluorescence resonance energy transfer (FRET) assay, as previously described [14,15]. Briefly, purified SARS-CoV-2 $3CL^{pro}$ mutants were used as an enzyme source and the synthesized peptide between SARS-CoV-2 polypeptide NSP4 and NSP5 was used as a substrate peptide. The peptide sequence was DABCYL-KTSAVLQSGFRKME-EDANS (Peptide Institute, Inc.). The assay reaction buffer contained 20 mM Tris−HCl (pH 7.5), 1 mM EDTA, 10 mM DTT, and 0.01% BSA. To measure the initial enzyme reaction velocity, 3–6 nM SARS-CoV-2 $3CL^{pro}$ mutants were added to 384-well plates, and the enzyme reaction was initiated by adding ~200 μM substrate. The fluorescence of the cleaved EDANS peptide (excitation 340 nm/emission 490 nm) was measured using a fluorescence intensity protocol on a PHERAstar FSX microplate reader (BMG Labtech). Initial velocity was plotted against substrate concentration and fitted to the Michaelis−Menten equation to obtain values of K_m and maximum velocity of enzymes (V_{max}). V_{max} in relative fluorescent unit/s was converted to M/s and k_{cat} factor was calculated from V_{max} using the following formula: $k_{cat} = V_{max}$/enzyme concentration.

2.6. SARS-CoV-2 $3CL^{pro}$ inhibition assay

SARS-CoV-2 $3CL^{pro}$ inhibition studies were conducted as described previously [12]. Briefly, substance solution was diluted to 250 μM, followed by stepwise threefold dilution with DMSO. Finally, the solutions were mixed with 20 mM Tris-HCl (pH 7.5) as a compound solution. Next, 10 μL of compound solution was added manually to each well, and then 5 μL of substrate in inhibition buffer (2 mM EDTA, 20 mM DTT, 0.02% BSA, and 20 mM Tris-HCl, pH 7.5) was added. The final substrate concentration was 2 μM for T21I, T24I, K88R, L89F, P108S, A193V, and A255V and 4 μM for G15S, K90R, P132H, and H246Y. The reaction was initiated by adding 5 μL of $3CL^{pro}$ in an inhibition buffer, followed by incubation at room temperature for 2–3 h. The final enzyme concentration was 2 nM for T24I, K88R, 193V, and A255V; 3 nM for G15S, K90R, and P132H; and 6 nM for T21I, L89F, and P108. After incubation, the reaction was stopped by adding 45 μL of water solution containing 0.1% formic acid, 10% acetonitrile, and 0.05 μM internal standard (IS) peptide [DABCYL-KTSAVLeu (13C6,15 N)-Q, custom-synthesized by Peptide Institute, Inc.]. The reactions were analyzed by mass spectrometry (MS) using a RapidFire 360 high-throughput sampling robot (Agilent Technologies) connected to an iFunnel Agilent 6550 accurate mass quadrupole time-of-flight mass spectrometer using electrospray. Peak areas were acquired and analyzed using a RapidFire Integrator (Agilent Technologies). Reaction product peak areas were acquired from m/z 499.27; IS peak areas were acquired from m/z 502.78. IC_{50} values were determined by plotting the compound concentration versus inhibition and fitting data with a four-parameter logistical fit (Model 205, XLfit, IDBS).

3. Results

The antiviral activities of ensitrelvir were assessed using VeroE6T against eleven SARS-CoV-2 variants, including eight omicron variants, which have continuously emerged and circulated globally: omicron (BA1.1, BA.2, BA.2.75, BA.4, BA.5, BQ.1.1, XBB.1, and XE), mu (B.1.621), lambda (C.37), and theta (P.3) [16]. Ensitrelvir exhibited similar antiviral activities against all tested SARS-CoV-2 variants and EC_{50}s were 0.22–0.52 μM (Table 1).

Genetic surveillance was conducted against SARS-CoV-2 variants designated as VOC, VOI, and VUM by the WHO, using GISAID data as of 10 February 2022. The prevalence of polymorphisms in each amino acid of $3CL^{pro}$ was calculated as the proportion of each amino acid relative to the total number of sequences; the prevalent mutations were identified. In this survey, $3CL^{pro}$ mutations detected at rates over 1% were defined as prevalent mutations. The survey was conducted against all SARS-CoV-2 reports as a whole and individual VOI/VOC/VUM. As for genetic surveillance against all SARS-CoV-2 reports, we identified $3CL^{pro}$ L89F, K90R, and P132H as prevalent mutations with frequencies of 1.82%, 1.94%, and 12.98%, respectively. As for genetic surveillance against individual VOI/VOC/VUM, we identified $3CL^{pro}$ G15S (Lambda, C.1.2), T21I (B.1.1.318), T24I (C.1.2), K88R (Lambda), K90R (Alpha, Beta, Gamma, Delta, Lambda), P108S (C.1.2), P132H (Omicron B1.1.529), A193V (Beta), H246Y (Gamma), and A255V (B.1.1.318) as prevalent mutations with frequencies of 99.00%, 89.03%, 51.71%, 1.85%, 1.44%–99.78%, 1.28%, 99.69%, 9.53%, 1.13%, and 8.74%, respectively (Table 2).

The identified SARS-CoV-2 $3CL^{pro}$ mutants were purified from *E. coli*, as described in Materials and Methods. Specific activities of these mutant $3CL^{pro}$s were assessed by estimating k_{cat}, K_m, and k_{cat}/K_m. An established FRET-based cleavage assay was used to determine enzyme catalytic activity [17,18]. The catalytic efficacy (k_{cat}/K_m) of these mutant $3CL^{pro}$s was comparable to that of the WT and the k_{cat}/K_m of WT, G15S, T21I, T24I, K88R L89F, K90R, P108S, P132H, A193V, H246Y, and A255V were 46,825, 44,969, 93,073, 55,682, 76,632, 82,268, 63,681, 80,628, 52,264, 113,845, 37,532, and 83,272 $M^{-1}s^{-1}$, respectively (Table 3). To assess whether ensitrelvir has inhibitory effects against the identified $3CL^{pro}$ mutants, enzyme inhibitory assays were conducted against these mutants. The inhibitory effects of ensitrelvir against these mutants were comparable to those against the WT [IC_{50} = 13.2 nM [12]]. Specifically, the IC_{50}s of G15S, T21I, T24I, K88R L89F, K90R, P108S, P132H, A193V, H246Y, and A255V were 8.0, 14.3, 14.0, 12.1, 15.0, 9.7, 13.2, 14.4, 10.2, 12.5, and 10.1 nM, respectively (Table 4).

4. Discussion

Ensitrelvir showed antiviral activity against various SARS-CoV-2 variants that have been circulating globally: omicron (BA1.1, BA1.18, BA.2, BA.2.75, BA.4, BA.5, BQ.1.1, XBB.1, and XE), alpha (B.1.1.7), beta (B.1.351), gamma (P.1), delta (B.1.617.2), mu (B.1.621), lambda (C.37), and theta (P.3). Although some of these variants carry mutation in $3CL^{pro}$ (e.g., omicron variants carry P132H and lambda variants carry G15S), the EC_{50}s of ensitrelvir against these variants were

Table 1
The antiviral activity of Ensitrelvir against SARS-CoV-2 omicron variants.

WHO label	PANGO lineage	$EC_{50} \pm SD$[a] (μM)	Fold Change[b] (vs WK-521)
	WK-521	0.37 ± 0.060[c]	–
Lambda	C.37	0.27 ± 0.048	0.73
Theta	P.3	0.29 ± 0.028	0.78
Mu	B.1.621	0.43 ± 0.069	1.16
Omicron	BA.1.1	0.36 ± 0.077	0.97
Omicron	BA.2	0.52 ± 0.091	1.41
Omicron	BA.2.75	0.30 ± 0.030	0.81
Omicron	BA.4	0.22 ± 0.072	0.59
Omicron	BA.5	0.40 ± 0.082	1.08
Omicron	BQ.1.1	0.48 ± 0.042	1.30
Omicron	XBB.1	0.33 ± 0.098	0.89
Omicron	XE	0.44 ± 0.037	1.19

[a] Values are the mean ± standard deviation (SD) of the results from 3 independent experiments.
[b] Fold change was calculated from mean EC50 of each SARS-CoV-2 strain.
[c] EC50 was reported previously [12].

Table 2
SARS-CoV-2 3CL protease mutants frequently identified in clinical.

3CL protease residue position	Reference amino acid[a]	Substituted amino acid	WHO label	PANGO linage	Prevalence
15	Glycine	Serine	Lambda	C.37	99.00%
			–	C.1.2	97.01%
21	Threonine	Isoleucine	–	B.1.1.318	89.03%
24	Threonine	Isoleucine	–	C.1.2	51.71%
88	Lysine	Arginine	Lambda	C.37	1.85%
89	Leucine	Phenylalanine	–	–	1.82%[b]
90	Lysine	Arginine	–	–	1.94%[b]
			Beta	B.1.351	99.78%
			Gamma	P.1	1.96%
			Delta	B.1.617.2	1.56%
			Lambda	C.37	1.50%
			Alpha	B.1.1.7	1.44%
108	Proline	Serine	–	C.1.2	1.28%
132	Proline	Histidine	–	–	12.98%[b]
			Omicron	B.1.1.529	99.69%
193	Alanine	Valine	Beta	B.1.351	9.53%
246	Histidine	Tyrosine	Gamma	P.1	1.13%
255	Alanine	Valine	–	B.1.1.318	8.74%

[a] Reference sequence was SARS-CoV-2 WIV04 (hCoV-19/Wuhan/WIV04/2019, GISAID_ID: EPI_ISL_402124).
[b] The prevalence was calculated in whole SARS-CoV-2 reports because the 3CLpro mutations were frequently detected in whole SARS-CoV-2 reports.

Table 3
Enzymatic activity of SARS-CoV-2 3CL protease mutants.

3CL protease residue position	k_{cat} (sec^{-1})[a]	Km (μM)[a]	k_{cat}/Km ($M^{-1}sec^{-1}$)[a]	Fold Change (vs WT)[b]
WT[c]	0.70	14.92	46825	–
G15S	0.75	16.75	44969	0.96
T21I	1.19	12.81	93073	1.99
T24I	0.99	17.28	55682	1.19
K88R	1.08	14.05	76632	1.64
L89F	0.97	11.77	82268	1.76
K90R	0.86	13.52	63681	1.36
P108S	0.89	11.11	80628	1.72
P132H	0.89	17.06	52264	1.12
A193V	1.33	11.53	113845	2.43
H246Y	0.89	23.42	37532	0.80
A255V	1.31	15.45	83272	1.78

[a] Values are the mean of the results from 2 independent experiments.
[b] Fold change was calculated from k_{cat}/Km of each SARS-CoV-2 strain.
[c] SARS-CoV-2 WIV04 3CLpro amino acid sequence was defined as Wild type (WT).

Table 4
The IC_{50} of ensitrelvir against SARS-CoV-2 3CL protease mutants.

3CL protease Amino acid position	IC_{50} of Ensitrelvir (nM)[a]	Fold change (vs WT)[b]
WT[c]	13.2 ± 1.1	1.0
G15S	8.0 ± 1.1	0.60 ± 0.08
T21I	14.3 ± 0.8	1.08 ± 0.06
T24I	14.0 ± 2.7	1.06 ± 0.20
K88R	12.1 ± 1.1	0.91 ± 0.08
L89F	15.0 ± 1.2	1.13 ± 0.09
K90R	9.7 ± 1.1	0.73 ± 0.09
P108S	13.2 ± 1.0	1.00 ± 0.07
P132H	14.4 ± 2.2	1.09 ± 0.16
A193V	10.2 ± 0.8	0.77 ± 0.06
H246Y	12.5 ± 1.0	0.95 ± 0.08
A255V	10.1 ± 0.4	0.77 ± 0.04

[a] Values are the mean ± standard deviation (SD) of the results from 3 independent experiments.
[b] Fold change was calculated for each experiment, and a mean fold change ± SD was calculated with these values.
[c] SARS-CoV-2 WIV04 3CLpro amino acid sequence was defined as Wild type (WT).

comparable to those of the WT (Supplementary Table 2). Genetic surveillance of SARS-CoV-2 $3CL^{pro}$ revealed 11 major $3CL^{pro}$ mutations, including those mentioned above. The $3CL^{pro}$s carrying these mutations have enzymatic activities comparable to those of the WT. Consistent with a previous report, the k_{cat}/K_m of G15S, K90R, and P132H $3CL^{pro}$ were comparable to those of the WT. Furthermore, this study revealed that the k_{cat}/K_m of T21I, T24I, K88R, L89F, P108S, A193V, H246Y, and A255V $3CL^{pro}$ were also comparable to those of the WT [19]. Because the specific activities of these enzymes were comparable to those of theWT, novel SARS-CoV-2 variants carrying the identified $3CL^{pro}$ mutations could emerge and circulate around the world.

The crystal structure of ensitrelvir bound to wild-type $3CL^{pro}$ was reported [12]. Each of the identified amino acid mutants was plotted and superimposed on the structure and these mutations were found not to interact with ensitrelvir directly (Supplementary Figs. 1a and b). This analysis is reasonable given the result that $3CL^{pro}$ mutant activities were inhibited by ensitrelvir with inhibitory effects comparable to those of the WT.

The frequency of amino acid substitutions is indicated to differ among SARS-CoV-2 proteins. For example, SARS-CoV-2 S protein is a mutational hotspot because the host immune system recognizes this protein [6]. Some COVID-19 treatment agents, especially monoclonal antibodies, have lost their efficacy against SARS-CoV-2 variants because of SARS-CoV-2 S protein mutations, which restricted their clinical value [2–4]. In contrast, SARS-CoV-2 $3CL^{pro}$ has been considered to have a low mutation rate. Because the

antiviral activities of ensitrelvir against SARS-CoV-2 variants carrying G15S or P132H in $3CL^{pro}$ were unchanged, ensitrelvir could be effective against SARS-CoV-2 variants carrying the identified mutations in $3CL^{pro}$. These findings indicate that ensitrelvir should be a beneficial COVID-19 treatment agent, potentially being effective against not only currently circulating SARS-CoV-2 variants but also newly emerging ones.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: All authors are employees of Shionogi & Co., Ltd. and a subsidiary company. Kae Inoue reports a relationship with Shionogi and Co Ltd that includes: equity or stocks. Haruaki Nobori reports a relationship with Shionogi and Co Ltd that includes: equity or stocks. Masatomo Rokushima reports a relationship with Shionogi and Co Ltd that includes: equity or stocks. Teruhisa Kato reports a relationship with Shionogi and Co Ltd that includes: equity or stocks. Haruaki Nobori has patent issued to Licensee. Yuki Tachibana has patent issued to Licensee.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.01.040.

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