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**Basic research contributing to the development of
treatments for viral infections and antimicrobial
resistant bacterial infections**

(ウイルス感染症および薬剤耐性細菌感染症の
治療法の確立に資する基盤研究)

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Abbreviations

293T/ACE2:	293T cells stably expressing ACE2
293T/DPP4:	293T cells stably expressing DPP4
293T/ACE2/TMPRSS2:	293T cells stably expressing ACE2 and TMPRSS2
<i>A. baumannii</i> :	<i>Acinetobacter baumannii</i>
ACE2:	angiotensin-converting enzyme 2
ACTIN:	β -actin
ALT:	alanine aminotransferase
BHK-21:	baby hamster kidney 21
bid:	<i>bis in die</i>
BSL:	biosafety level
BSR-T7/5:	BHK-21 cells stably expressing T7 RNA polymerase
BXA:	baloxavir acid
BXM:	baloxavir marboxil
ca.:	<i>circa</i>
CC ₅₀ :	50% cytotoxicity
CCCP:	carbonylcyanide-m-chlorophenylhydrazine
cDNA:	complementary deoxyribonucleic acid
CEN(s):	cap-dependent endonuclease(s)
CENi(s):	CEN inhibitor(s)
CI:	combination index
COVID-19:	coronavirus disease 2019
CPE:	cytopathic effect
Ctrl:	control
DA:	the EC ₅₀ of substance A alone
DA/A + B:	the concentration of substance A; given a 50% inhibition combined with substance B
DABCYL:	4-[(4'-(N,N-dimethylamino)phenyl)azo]benzoic acid
DB:	the EC ₅₀ of substance B alone
DB/A + B:	the concentration of substance B; given a 50% inhibition combined with substance A.
DMEM:	Dulbecco's modified Eagle's medium
DMF:	N,N-dimethylformamide
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid

dpi:	day(s) post-infection
DPP4:	dipeptidyl peptidase-4
DTT:	dithiothreitol
E41G:	glutamic acid-to-glycine at amino acid position 41
E41V:	glutamic acid-to-valine at amino acid position 41
<i>E. coli</i> :	<i>Escherichia coli</i>
EC ₃₀ :	30% effective concentration
EC ₅₀ :	50% effective concentration
EC ₉₀ :	90% effective concentration
EDANS:	5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid
EDTA:	ethylenediaminetetraacetic acid
FBS:	fetal bovine serum
FC:	fold change
FRET:	Förster resonance energy transfer
H&E:	hematoxylin and eosin
HIV:	human immunodeficiency virus
hpi:	hours post-infection
HRP:	horseradish peroxidase
I38T:	isoleucine-to-threonine at amino acid position 38
I52M:	isoleucine-to-methionine at amino acid position 52
IC ₅₀ :	concentration achieving 50% inhibition
IFN:	interferon
IFV(s):	influenza virus(es)
IHC:	immunohistochemistry
IMP(s):	inner membrane protein(s)
JUNV:	Junin virus
K44T:	lysine-to-threonine at amino acid position 44
K67R:	lysine-to-arginine at amino acid position 67
<i>K. pneumoniae</i> :	<i>Klebsiella pneumoniae</i>
L:	Luria
L40S:	leucine-to-serine at amino acid position 40
LACV:	La Crosse virus
LASV:	Lassa virus
LCMV:	lymphocytic choriomeningitis virus
MA104-TP2:	MA104 cells stably expressing type II transmembrane serine protease

MDBK:	Madin-Darby bovine kidney
MDCK:	Madin-Darby canine kidney
MDR:	multidrug-resistant
MEM:	minimum essential medium
MERS-CoV:	Middle East respiratory syndrome coronavirus
MES:	2-(N-morpholino)ethanesulfonic acid
MFP:	membrane fusion protein
MIC(s):	minimum inhibitory concentration(s)
MOI:	multiplicity of infection
MTT:	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MW:	molecular weight
NGS:	next-generation sequencing
NHC:	N-hydroxycytidine
OMP(s):	outer membrane protein(s)
ORF(s):	open reading frame(s)
PA:	polymerase acidic subunit
<i>P. aeruginosa</i> :	<i>Pseudomonas aeruginosa</i>
PBS:	phosphate-buffered saline
PDB:	Protein Data Bank
PFU(s):	plaque-forming unit(s)
PPB:	potassium phosphate buffer
P/S:	penicillin-streptomycin
QD:	<i>quaque die</i>
RBV:	ribavirin
RNA:	ribonucleic acid
RND:	resistance-nodulation-cell division superfamily
RT-qPCR:	real-time reverse-transcriptase quantitative polymerase chain reaction
RVFV:	Rift Valley fever virus
S:	spike
SARS-CoV-2:	severe acute respiratory syndrome coronavirus-2
SARS1:	severe acute respiratory syndrome coronavirus
SD:	standard deviation
SDS:	sodium dodecyl sulfate
<i>S. marcescens</i> :	<i>Serratia marcescens</i>

SFTSV:	severe fever with thrombocytopenia syndrome virus
SI:	selectivity index
TCID ₅₀ :	50% tissue culture infectious dose
TMPRSS2:	type II transmembrane serine protease
TNF:	tumor necrosis factor
TPMV:	Thottapalayam virus
TPP:	tetraphenylphosphonium chloride
V ₀ :	initial velocities
<i>V. cholerae</i> :	<i>Vibrio cholerae</i>
<i>V. parahaemolyticus</i> :	<i>Vibrio parahaemolyticus</i>
vRNP(s):	viral ribonucleoprotein(s)
WT:	wild type

Notes

Contents of this thesis were published in the following articles.

1. **Toba S*, Sato A*, Kawai M, Taoda Y, Unoh Y, Kusakabe S, Nobori H, Uehara S, Uemura K, Taniguchi K, Kobayashi M, Noshi T, Yoshida R, Naito A, Shishido T, Maruyama J, Paessler S, Carr MJ, Hall WW, Yoshimatsu K, Arikawa J, Matsuno K, Sakoda Y, Sasaki M, Orba Y, Sawa H, Kida H.** Identification of cap-dependent endonuclease inhibitors with broad-spectrum activity against bunyaviruses. *Proc Natl Acad Sci U S A.*, 119(36), e2206104119, 2022.

*Both authors equally contributed to this work.

2. **Toba S*, Uemura K*, Sanaki T, Kusakabe S, Konishi K, Miki S, Maruyama Y, Iimuro A, Shishido T, Sasaki M, Orba Y, Hall WW, Sawa H, Sato A.** Synergistic antiviral activity of a cathepsin B/L inhibitor and a TMPRSS2 inhibitor against SARS-CoV-2 *in vitro* and *in vivo*. *Virology.*, 612, 110661, 2025.

*Both authors equally contributed to this work.

3. **Toba S*, Minato Y*, Kondo Y, Hoshikawa K, Minagawa S, Komaki S, Kumagai T, Matoba Y, Morita D, Ogawa W, Gotoh N, Tsuchiya T, Kuroda T.** Comprehensive analysis of resistance-nodulation-cell division superfamily (RND) efflux pumps from *Serratia marcescens*, Db10. *Sci Rep.*, 9(1), 4854, 2019.

*Both authors equally contributed to this work.

General introduction

Infectious diseases affecting humankind are caused by a wide range of pathogens, including viruses and bacteria. For viral infections, effective therapeutic agents remain unavailable for several diseases. Lassa fever, which has repeatedly caused localized outbreaks in humans in recent years, is one such disease for which no safe and effective treatment has been established (1). Severe Lassa fever can cause life-threatening complications such as gastrointestinal hemorrhage, encephalopathy, and shock, underscoring the urgency of drug development. Similarly, for emerging infectious diseases such as coronavirus disease 2019 (COVID-19), which has become a global concern since 2019, effective therapeutics are typically lacking in the early stage of an outbreak (2, 3). During pandemics, the rapid development of effective drugs becomes critically important. In addition to conventional drug discovery approaches, drug repositioning, repurposing compounds that have already undergone development and approval processes with well-established pharmacological properties and safety profiles is gaining significant attention as a practical strategy (4).

Meanwhile, bacterial infections are becoming increasingly complex due to the emergence of multidrug-resistant (MDR) strains, which are major factors in hospital-acquired opportunistic infections (5). Opportunistic infections pose a significant risk to immunocompromised patients, making the development of therapeutics to overcome MDR bacteria an urgent priority. While multiple mechanisms underlie the acquisition of multidrug resistance, efflux pumps play a crucial role by actively expelling antimicrobial agents from bacterial cells, thereby conferring simultaneous resistance to multiple drugs (6, 7). Research on efflux pumps holds the potential to restore drug susceptibility and offers a novel therapeutic approach distinct from traditional antibiotic development. Recent advances in structural analysis of efflux pumps and high-throughput screening of inhibitors have further highlighted their potential as promising drug targets.

In this study, compound library screening, a conventional approach in drug discovery, was employed to identify candidate therapeutic compounds for viral infections lacking established approved treatments. Furthermore, the feasibility of drug repositioning for viral diseases requiring rapid therapeutic interventions during pandemics was evaluated, with a focus on compounds possessing established pharmacological profiles and prior regulatory approval. Finally, multidrug efflux pumps were characterized as potential targets for the development of novel antimicrobial strategies to address bacterial infections complicated by multidrug resistance.

Chapter I

Identification of cap-dependent endonuclease inhibitors with broad-spectrum activity against bunyaviruses

Summary

Viral hemorrhagic fevers caused by members of the class *Bunyaviricetes* represent both endemic and emerging infections of major public health concern. Despite the severity of these diseases, therapeutic options remain limited, underscoring the urgent need for effective antiviral agents to reduce disease burden. Similar to influenza viruses (IFVs), bunyaviruses encode a cap-dependent endonuclease (CEN) that mediates the critical cap-snatching step required for viral RNA transcription. In this study, compounds from a CEN inhibitor (CENi) library were screened, and specific structural analogs were identified that exhibited 100- to 1,000-fold greater *in vitro* activity against bunyaviruses, including Lassa virus (LASV), lymphocytic choriomeningitis virus (LCMV), and Junin virus (JUNV), compared with ribavirin (RBV). To elucidate the inhibitory mechanism of action, drug-resistant viral mutants were generated in cell culture under selective pressure. Whole-genome sequencing revealed amino acid substitutions within the CEN region of resistant viruses, located at positions analogous to those observed in the CEN α 3-helix loop of IFVs under drug selection. These findings suggest that CENi compounds inhibit replication of both bunyaviruses and IFVs through a mechanistically similar process. Structural analysis further demonstrated that the carboxyl group side chain at the seventh position of the compound's core structure is essential for potent antiviral activity against bunyaviruses. In LCMV-infected mice, treatment with these compounds significantly reduced blood viral load, alleviated clinical manifestations such as thrombocytopenia and hepatic dysfunction, and improved survival rates. Collectively, these results indicate the potential broad-spectrum clinical utility of CENi compounds for the treatment of severe influenza and hemorrhagic diseases caused by bunyaviruses.

Introduction

Infections in humans caused by certain members of the class *Bunyaviricetes* are associated with severe hemorrhagic fever syndromes, constituting a major public health concern in endemic regions (8). The LASV is recognized as the etiological agent of Lassa fever, with incidence rates in African countries such as Nigeria exhibiting a significant increase since 2016 (1). Likewise, JUNV is the causative pathogen of Argentine hemorrhagic fever, imposing substantial morbidity and mortality burdens across affected areas of South America. Currently, most bunyaviruses lack efficacious antiviral therapeutics and licensed vaccines, thereby highlighting the imperative for the development of potent pharmacological interventions to address these viral diseases as a critical global health priority (9).

CENs constitute a compelling target for antiviral drug development, as these enzymes are virus-specific and absent from the human genome. The catalytic activity of CENs depends on the presence of divalent metal ions (Mg^{2+} or Mn^{2+}), prompting the screening of metal-chelating compounds as potential inhibitors of viral metalloenzymes. In previous studies, several IFV CENis were identified and demonstrated potent suppression of viral replication (10, 11). Notably, the active sites of CENs across diverse viral families, including IFV, Hantaan virus, LASV, and La Crosse virus (LACV), exhibit significant structural homology, thereby providing a strong rationale for the development of broad-spectrum antiviral agents targeting these enzymes (12). Furthermore, recent reports have documented the antiviral efficacy of the CENi baloxavir marboxil (BXM) against hantavirus (13).

An extensive library comprising approximately 40,000 metal-chelating compounds, including the human immunodeficiency virus (HIV) integrase inhibitors dolutegravir (14) and cabotegravir (15), as well as the IFV CENi baloxavir acid (BXA) (16), was assembled. These antiviral candidates were systematically screened against multiple viruses, encompassing members of the class *Bunyaviricetes*, LCMV, JUNV, LACV, severe fever with thrombocytopenia syndrome virus (SFTSV), Rift Valley fever virus (RVFV), and Thottapalayam virus (TPMV). Screening of two-metal chelating compounds identified highly potent anti-bunyavirus agents that are structurally analogous to IFV CENis, but distinct from HIV integrase inhibitors. The CEN catalytic center exhibits strong conservation across diverse viral families, implying that amino acid substitutions may incur significant fitness costs (17). Here, preclinical pharmacological data are presented characterizing the antiviral activity of CENis against bunyaviruses in both *in vitro* and *in vivo* models. Collectively, these findings highlight the potential of CENis as selective, broad-spectrum antivirals for

the treatment of severe influenza and hemorrhagic diseases associated with bunyavirus infections.

Materials and methods

Cell culture and viruses

Baby hamster kidney 21 (BHK-21) cells, human A549 cells, Madin–Darby canine kidney (MDCK) cells, Madin–Darby bovine kidney (MDBK) cells, and African green monkey VeroE6 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C under 5% CO₂. HEK293T and KB cells were maintained in minimum essential medium (MEM) supplemented with 5% FBS. For IFV A/Anhui/1/2013 (A/H7N9), the cleavage of hemagglutinin by host cell proteases is essential for virus infectivity and propagation. MA104 cells stably expressing the type II transmembrane serine protease (TMPRSS2) (18, 19) (MA104-TP2 cells) were used for the propagation of A/H7N9. MA104-TP2 was maintained in high-glucose DMEM supplemented with 10% FBS. LCMV Armstrong and WE strains [National Institute of Infectious Diseases (Tokyo, Japan)], JUNV Candid 1 strain (National Institute of Infectious Diseases), LACV ATCC VR-1834 strain [American Type Culture Collection (Manassas, VA, USA)], and TPMV VRC-66412 strain [Hokkaido University (Hokkaido, Japan)] were grown in BHK-21 cells. IFV A/H1N1 A/WSN/33 strain [Osaka Prefectural Institute of Public Health (Osaka, Japan)] and SFTSV ArtLN/2017 strain [Hokkaido University; (20)] were grown in MDCK cells. IFV A/H5N1 A/Hong Kong/483/97 strain [The University of Hong Kong (Hong Kong, HKSAR, China)] and A/H7N9 A/Anhui/1/2013 strain (National Institute of Infectious Diseases) were propagated in embryonated chicken eggs and harvested from virus-containing allantoic fluids. Recombinant RVFV MP-12 strain was rescued from the plasmids [The University of Texas Medical Branch (Galveston, TX, USA); (21)] using BSR-T7/5 cells which stably expresses T7 RNA polymerase [Max von Pettenkofer Institute (Munich, BY, Germany); (22)] and grown in BHK-21 cells. LASV Josiah strain [The University of Texas Medical Branch; (23)] was grown in VeroE6 cells.

Compounds

All screening compounds were supplied by Shionogi & Co., Ltd. (Osaka, Japan). Ribavirin (RBV) was purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). Favipiravir (T-705) was purchased from PharmaBlock Sciences (Nanjing, China). For *in vitro* experiments, all compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted in 2% FBS-MEM for antiviral assays. For *in vivo* experiments, compounds were dissolved in phosphate-buffered saline (PBS) with 5% DMSO to prepare 3 mg/mL solutions and each solution was diluted with the same vehicle for

dosing.

Inhibition of *in vitro* cap-dependent endonuclease activity

The methods for the inhibition of CEN activity were previously described (10, 11). In brief, viral ribonucleoproteins (vRNPs) of the A/WSN/33 (H1N1) strain were purified from viruses propagated in embryonated chicken eggs. The allantoic fluids were recovered, and virus particles were purified by ultracentrifugation using 20% sucrose and solubilized using Triton X-100 and lysolecithin. The vRNP fractions were collected by ultracentrifugation and used as sources of CEN activity.

The oligonucleotide RNA (sequence: 5'-cap-8AA UA5 GCA UCA CUA GUA AGC UUU GCU CUA-BHQ2-3') was used as a substrate, where "8" indicates 2'-O-methyl-G, and "5" indicates cyanine 3'-labeled U. The enzymatic reaction was performed in reaction buffer (1 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol (DTT), 0.05% Tween 20, 3 nM capped RNA substrate, and 50 mM Tris HCl, [pH 7.8]). Each reaction mixture was incubated at 37 °C for 1 h followed by adding excess ethylenediaminetetraacetic acid (EDTA) to stop the reaction. The products were examined using the 3130xL Genetic Analyzer and relatively quantitated using GeneMapper version 4.1 software [Life Technologies (Carlsbad, CA, USA)]. The drug concentration achieving 50% inhibition of the enzymatic production (IC₅₀) was calculated by dividing the peak area of the Cy3-labeled enzymatic product by the peak area of the internal standard (15-nt-sized marker in GeneScan-120 LIZ).

***In vitro* antiviral activity and cell viability**

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to evaluate cell viability following viral infection according to methods previously described (24). For experiments evaluating drug inhibition of viral replication, each sample was serially diluted in two-fold increments in duplicates and plated on 96-well microplates. Cells used for each virus were infected with 4 to 10 50% tissue culture infectious dose (TCID₅₀) and added to the plates. Plates were incubated for 3–4 days, and the cytopathic effect (CPE) was determined by calculation of 50% end points using MTT. The detailed assay conditions for each virus are shown in Figure 1. The 50% effective concentration (EC₅₀) was defined in GraphPad Prism version 8.4.3 [GraphPad Software (Boston, MA, USA)] with a variable slope (four parameters). Noninfected cells were used as a control for 100% inhibition whereas, for infected cells, DMSO-treated cells were used as a control for 0% inhibition. The concentration achieving 50% cytotoxicity (CC₅₀) was also measured for each cell line using the same method. Cell-free

samples were used as a 100% cytotoxicity control, and DMSO-treated cells were used as a 0% cytotoxicity control. The selectivity index (SI) was calculated as mean EC₅₀/mean CC₅₀.

Virus yield reduction assay

The real-time reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) assay was performed to calculate the levels of viral RNA in culture supernatants following viral infection. KB cells were infected with LCMV and HEK293T cells were infected with JUNV in 96-well plates with 10 TCID₅₀ per well. The infected cells were treated with serial five-fold dilutions of inhibitors and cultured at 37 °C in a 5% CO₂ incubator. At 72 h after infection, the culture supernatant was collected, and RNA was extracted. The expression levels of vRNA from CEN regions were quantified as described. RT-qPCR was performed using the Brilliant III Ultra-Fast SYBR Green QRT PCR Master Mix, 1-Step Kit [Agilent Technologies (Santa Clara, CA, USA)]. The primers for the RT-qPCR are listed in Table 1. The EC₉₀ value was defined in GraphPad Prism version 8.4.3 (GraphPad Software) with a variable slope (Find ECanything, F = 90). For LASV, the plaque assay was performed to calculate the viral titer in culture supernatants following viral infection. VeroE6 cells were infected with LASV in 24-well plates at an MOI of 0.01, and incubated for 1 h at 37 °C. Followed by washing with DMEM containing 2% FBS three times, infected cells were cultured in DMEM, 2% FBS and various doses of the compound at 37 °C. Samples were collected at 24 and 48 h after infection and titrated by the plaque assay to calculate plaque-forming units (PFUs) as described elsewhere (25).

Virus antigen reduction assay

For immunofluorescence staining, KB cells were infected with LCMV in 24-well plates with 10 TCID₅₀ per well. The infected cells were treated with serial 10-fold dilutions of inhibitors and cultured in a 5% CO₂ incubator. At 72 h after infection, infected cells were fixed with 100% methanol at -20 °C and stained with anti-LCMV nucleoprotein rabbit monoclonal antibody [Bio X Cell (Lebanon, NH, USA)] in 25% Block Ace [KAC (Osaka, Japan)] in PBS for 1 h. Alexa Fluor 555-conjugated anti-rabbit immunoglobulin G antibody [Invitrogen, Thermo Fisher Scientific (Carlsbad, CA, USA)] was used as the secondary antibody. Nuclei were stained with Hoechst 33342 (Invitrogen, Thermo Fisher Scientific). Fluorescent images were captured using an IX73 fluorescence microscope [Olympus (Tokyo, Japan)].

Time-of-addition assay

LCMV Armstrong strain was incubated in the presence or absence of compound #B (100 nM) or RBV (100 μ M) for 1 h at 37 °C, and then KB cells were inoculated with LCMV incubated with or without inhibitors at an MOI of 0.1 for 1 h. After washing, KB cells were cultured with fresh media for 24 or 72 h. As a post-treatment condition, KB cells were infected with LCMV without pretreatment with inhibitors and were cultured in the presence of inhibitors (compound #B [100 nM] or RBV [100 μ M]) for 24 or 72 h after cell washing. Culture supernatants were collected at 24 or 72 hours post-infection (hpi), and infectious viral titers in supernatants were measured by TCID₅₀ assay using MDCK cells. In brief, collected culture supernatants including LCMV were serially diluted 10-fold with MEM supplemented with 2% FBS. MDCK cells were inoculated with serially diluted supernatants in a 96-well plates and cultured with MEM supplemented with 2% FBS for 72 h. At 72 hpi, viral titers were determined by microscopic observation of CPE.

Isolation and characterization of drug-resistant mutants

To isolate drug-resistant LCMV mutants, LCMV-infected and noninfected KB cells were seeded into the same 12-well plate in the presence of the compounds. Three drug concentrations, specifically $2 \times EC_{50}$, $6 \times EC_{50}$, and $20 \times EC_{50}$, were used. Infected cells were passaged every 2–3 days. When a CPE was observed by microscopy, the LCMV-infected culture supernatant was transferred to noninfected KB cells in the presence of the compounds. After continuous culture of the viruses for 26–30 days for LCMV, the viral genome was then examined by sequencing the CEN region. To isolate drug-resistant JUNV mutants, JUNV-infected and non-infected HEK293T cells were seeded into the same 12-well plates in the presence of the compounds, and passage was performed as described for the isolation of drug-escape LCMV variants. To isolate drug-escape LACV mutants, BHK-21 cells were infected with LACV, passaged twice (7 days), used to infect NIH 3T3 cells, and passaged six times every other day (12 and 19 days in total).

A/H5N1-infected BHK-21 cells and A/H7N9-infected MA104-TP2 cells were also used to isolate drug-resistant variants. Three drug concentrations, namely $10 \times EC_{50}$, $30 \times EC_{50}$, and $100 \times EC_{50}$, were used. In dose-escalating and dose-constant conditions, the drug-escape mutants were detected following 3 weeks in culture. However, the replication of A/H5N1 and A/H7N9 was rapid and suppressing viral replication using drugs was difficult. Taking advantage of the fact that CENs have strong ability to suppress viruses, a method to isolate drug-escape viruses by

changing the drug concentration in two steps was performed, namely in the virus growth phase in the presence of drugs and in the virus selection phase. Modified isolation methods (two-step approach) were employed, and CEN variants carrying the isoleucine-to-threonine at amino acid position 38 (I38T) substitution in the PA were isolated within approximately 7–8 days.

Genome sequence analysis

To analyze mutations, viral RNA was extracted from culture supernatant or infected cells using a PureLink Viral RNA/DNA Mini Kit [Thermo Fisher Scientific (Waltham, MA, USA)], Wizard SV 96 Binding Plates (Promega), or Direct-zol RNA Mini Prep Kit [Zymo Research (Irvine, California, USA)]. The CEN region of each virus was amplified by RT-qPCR using a PrimeScript II High Fidelity One Step RT-PCR Kit [Takara Bio (Shiga, Japan)] and specific primers (Table 1). The products were sequenced using a 3500xL Genetic Analyzer (Life Technologies). The nucleotide sequence of the CEN region was compared with that of the WT virus, and amino acid substitutions were identified. Whole-genome sequencing was performed using the Ion Torrent PGM System (Life Technologies) as previously described (26). Briefly, viral RNA was extracted using a PureLink Viral RNA/DNA Mini Kit, and double-stranded complementary DNA (cDNA) was synthesized using a PrimeScript Double Strand cDNA Synthesis Kit (Takara Bio). Double-stranded cDNA was fragmented using a Covaris S2 focused ultrasonicator and used to prepare a 400-base-read library using an Ion Plus Fragment Library Kit (Life Technologies). After emulsion PCR, sequencing was performed using an Ion PGM sequencer with an Ion PGM Sequencing 400 Kit and Ion 318 Chip V2 (Life Technologies). Data analysis was performed using CLC Genomics Workbench version 7.5.1 software [CLC bio, Qiagen (Aarhus, Denmark)].

Pharmacokinetic parameters of compounds in mouse plasma

The animal study protocol was approved by the director of the institute after review by the Institutional Animal Care and Use Committee of Shionogi & Co., Ltd. relative to the 3R (replacement/reduction/refinement) principle. (approval no. S18013D2).

For intramuscular injection of compound #B, five female ICR mice (6-week-old) were purchased from CLEA Japan (Tokyo, Japan). Compound #B was dissolved in PBS with 5% DMSO to prepare 0.3, 1, or 3 mg/mL solutions. The solutions were intramuscularly administered under non-fasting conditions. Blood samples were collected with disposable capillaries containing heparin at 0.5, 1, 3, 6,

24, 48, and 72 h after administration. Blood samples were centrifuged ($13,000 \times g$, $4\text{ }^{\circ}\text{C}$, 2 min) to obtain plasma samples, which were transferred to individual tubes and stored at $-20\text{ }^{\circ}\text{C}$ until analysis. Plasma concentrations were determined and the pharmacokinetic parameters of compounds in plasma were calculated by the same methodology as described above.

Ethical statement

All experiments with LASV was performed in the biosafety level-4 (BSL-4) facility of the Galveston National Laboratory at the University of Texas Medical Branch in accordance with institutional guidelines. The use of the virus in this study was approved by the Institutional Biosafety Committee of the University of Texas Medical Branch in Notification of Use #2015106. All other experiments were authorized by the Biosafety Management Committee on Pathogens and Other Hazardous Agents and the Safety Committee on Genetic Recombination Experiments, Hokkaido University. All animal experiments were performed in accordance with the National University Corporation, Hokkaido University Regulations on Animal Experimentation. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Hokkaido University (approval no. 16-0032, 22-0067).

***In vivo* efficacy of CENis in a lethal murine model of LCMV Infection**

Six-week-old specific pathogen-free female ICR mice (CLEA Japan) were used for all experiments. To compare the *in vivo* efficacy of compound #B with RBV, mice were intraperitoneally inoculated with 3.0×10^3 TCID₅₀ of the LCMV WE strain. Immediately after infection, mice were intramuscularly administered compound #B (3, 10, or 30 mg/kg), RBV (3, 10, or 30 mg/kg), or control (Ctrl, vehicle) *quaque die* (QD) for 1 day. Survival was monitored daily up to 21 days after infection. For delayed treatment, mice were intramuscularly administered compound #B (30 mg/kg), RBV, or only the empty vehicle QD for 1 or 5 days starting 24 hpi. Survival was monitored daily up to 21 days after infection.

Viral load in blood

To quantify viral RNA levels in blood, mice were intraperitoneally inoculated with 3.0×10^3 TCID₅₀ of the LCMV WE strain. Immediately after infection, mice were intramuscularly administered compound #B (3, 10, or 30 mg/kg), RBV (3, 10, or 30 mg/kg), or vehicle QD for 1 day. At 1 day after infection, whole blood was collected from each mouse, mixed with TRIzol (Invitrogen,

Thermo Fisher Scientific), and subjected to RNA extraction using a Direct-zol RNA Mini Prep Kit. The viral RNA levels were analyzed by RT-qPCR using Brilliant III Ultra-Fast qRT-PCR Master Mix. RT-qPCR was performed using a StepOne Plus qPCR System [Applied Biosystems (Waltham, MA, USA)].

Blood biochemistry profile

To investigate the blood biochemistry profiles, mice were intraperitoneally inoculated with 3.0×10^3 TCID₅₀ of the LCMV WE strain. Immediately after infection, mice were intramuscularly administered compound #B (30 mg/kg) or vehicle QD for 1 day. At 5 days after infection, whole blood was collected from each mouse and analyzed for platelet counts and ALT levels using a Celltac Alpha MEK-6450 [Nihon Kohden (Tokyo, Japan)] and VETSCAN VS2 [Abaxis (Union City, CA, USA)], respectively.

Cytokine quantification

To evaluate the cytokine levels in serum, mice were intraperitoneally inoculated with 3.0×10^3 TCID₅₀ of the LCMV WE strain. Immediately after infection, mice were intramuscularly administered compound #B (30 mg/kg), RBV (30 mg/kg), or vehicle QD for 1 day. At 5 days after infection, serum was collected from each mouse, and cytokine levels were quantified using a Quantikine ELISA Kit [R&D Systems (Minneapolis, MN, USA)].

Histopathological examination

Liver tissues at 6 days after infection were fixed in 3.7% formaldehyde in PBS. Histopathological analysis was performed on snap-frozen tissue sections stained with rat monoclonal antibodies targeting LCMV nucleoprotein (clone VL4, Bio X Cell) using Histofine Simple Stain Mouse Max-PO (Rat) and a diaminobenzidine substrate kit [Nichirei Biosciences (Tokyo, Japan)].

Molecular modeling

Models of compound #B bound to CEN of LASV, LCMV, and IFVA were constructed via superimposition on a crystal structure of BXA and CEN of IFVA complex (Protein Data Bank [PDB] ID code 6fs6). First, the CEN structures for LASV (PDB ID code 4miw) and LCMV (PDB ID code 5t2t) were aligned to that of IFVA using three active-site atoms, including two atoms involved in metal chelation and a backbone Ca atom on the same position of the α 3-helix (residues H41, S47, and S46 for IFVA, LASV, and LCMV, respectively). Because the sequence similarity

of CENs between bunyaviruses and IFVA was relatively low (22%), the superposition of two-metal chelators and the α 3-helix resulted in more feasible active-site alignment than the protein backbone alignment. Second, a three-dimensional structure of compound #B was built using the LigPrep program and superimposed on BXA using the Flex align program of Schrödinger suite 2019-1 [Schrödinger (New York, NY, USA)]. Consequently, three binding models consistent with the experimental results were obtained (described in the Results section). Molecular docking of compound #B to the CENs was also attempted; however, no plausible binding mode could be identified for LASV or LCMV. It was considered that minor conformational adjustments around the active sites of CENs may be required to reveal the actual binding modes.

Results

Selection of candidate compounds for broad-spectrum antiviral activities

To facilitate horizontal expansion and identification of anti-bunyavirus compounds from the antiviral library, both LCMV, genetically related to the risk group 4 pathogens LASV, and JUNV were employed as a representative South American hemorrhagic fever virus and used as the screening target. To identify effective candidates, an MTT-based screening system was established, which had previously been utilized for the identification of dolutegravir (14) and BXA (16) (Figure 1). From the metal-chelating library (~40,000 compounds), 6,077 candidates were selected. Among these, 185 and 42 hits (corresponding to hit rates of 3.04% and 0.69%) were identified by the LCMV and JUNV assays, respectively. Each hit exhibited EC₅₀ values lower than 2.5 μ M, and notably, their chemical structures were related to influenza CENi two-metal chelate compounds. Based on structural similarity, antiviral activity was further assessed for approximately 1,000 compounds from the CENi library. According to EC₅₀ values against LCMV and JUNV, four structurally distinct compounds designated #A through #D (Figure 2A) were selected. Antiviral activity against LCMV and JUNV was evaluated using the MTT assay, with RBV serving as a positive control. Compounds #A–#D demonstrated EC₅₀ values that were >500- to 1,000-fold lower than those of RBV against LCMV (Figure 2B) and JUNV (Figure 2C). To further assess inhibitory activity, viral replication assays were performed using LCMV-infected KB cells and JUNV-infected HEK293T cells, and viral RNA in supernatants was quantified by RT-qPCR. The 90% inhibitory concentration (EC₉₀) values of the four compounds were within the sub-nanomolar range for both LCMV (Figure 2D) and JUNV (Figure 2E). Antiviral data are summarized in Table 2. Moreover, immunofluorescence analysis revealed that LCMV nucleoprotein expression was undetectable at compound #B concentrations ranging from 1 to 1,000 nM, whereas RBV exhibited inhibition only at 1,000 nM (Figure 2F).

Next, anti-LASV activity was evaluated using compound #B under BSL-4 conditions. Viral replication in LASV-infected cells and viral infectivity in culture supernatants were assessed by plaque assay. At 24 hpi, compound #B achieved a 3 log₁₀ reduction in viral titers at a concentration of 1 μ M, whereas RBV produced only a 2 log₁₀ reduction at 100 μ M (Figure 2G). At 48 hpi, compound #B resulted in a 2.5 log₁₀ reduction at 1 μ M, while RBV exhibited a 2 log₁₀ reduction at 100 μ M (Figure 2H). These findings indicate that compound #B possesses potent antiviral activity not only against LCMV and JUNV but also against LASV, with an efficacy approximately 100- to 1,000-fold greater than that of RBV. Time-of-addition assays for compound

#B and RBV were conducted to elucidate their effects on the viral life cycle of LCMV. RBV is known to inhibit viral replication at the post-entry stage by targeting the viral polymerase, thereby suppressing viral propagation. Pretreatment with either RBV or compound #B resulted in a reduction of infectious viral particles in culture supernatants compared with the untreated control at 24 hpi; however, viral titers in supernatants increased at 72 hpi following pretreatment with these compounds. In contrast, posttreatment with RBV or compound #B significantly reduced viral propagation at both 24 and 72 hpi. These findings suggest that compound #B mechanistically inhibits post-entry steps of viral propagation in a step comparable to RBV (Figure 2I).

To investigate the broad-spectrum inhibitory potential of these compounds, antiviral activity of compounds #A through #D against LACV, SFTSV, RVFV, TPMV, and IFV (A/H5N1 and A/H7N9) was assessed using the MTT assay (Figure 3A). RBV and favipiravir were utilized as positive controls for antiviral activity, while BXA served as a reference compound for anti-IFV activity. Compounds #A through #D exhibited antiviral activities more than 200-fold greater against LCMV, JUNV, and LACV compared with RBV and favipiravir. Furthermore, compounds #A, #B, and #D demonstrated inhibitory activity against LACV, SFTSV, and IFVs A/H5N1 and A/H7N9, whereas no detectable activity was observed against RVFV and TPMV *in vitro*. BXA displayed strong antiviral efficacy against IFVs (H5N1 and H7N9) and weak activity against LACV but was ineffective (>1,000 nM) against SFTSV, RVFV, and TPMV.

Compound #D consisted of a racemic mixture of isomers #E and #F, which were obtained through chromatographic resolution (Figure 3A). Isomer #E exhibited greater antiviral potency than isomer #F and was active against LCMV, JUNV, LACV, SFTSV, and IFV A/H5N1 and A/H7N9. Compounds #G and #H shared the same benzhydryl side chain but differed in the position of the R1 substituent; compound #H, with the R1 group at the second position, demonstrated reduced activity against IFV A/H5N1. Compound #I lacked a carboxylic acid moiety at the seventh position and displayed diminished activity against LCMV and JUNV, while showing enhanced activity against IFVs. Only compounds #F through #H exhibited inhibitory activity against TPMV, a member of the hantavirus genus. All compounds, except #E and #F that were not tested, demonstrated inhibitory activity against the influenza CEN enzyme.

The cytotoxicity of these compounds was subsequently evaluated, and compounds #A through #D were found to exhibit minimal cytotoxic effects, with a high selectivity index (SI), which reflects the ratio between antiviral efficacy and

cellular toxicity. Specifically, compound #B demonstrated an SI of 1,000 or greater in the LCMV and JUNV assays, and no cytotoxicity was detected in VeroE6 cells, used in LASV experiments, even at concentrations up to 10,000 nM (Figure 3B).

Identification of the action mechanism of the compounds by selection of drug-resistant mutants

Notably, all identified hit compounds were analogs of IFV CENi; however, their mechanism of action against bunyaviruses remained unclear. To investigate this, drug-resistant mutants of LCMV and JUNV were generated by propagating the viruses in cultured cells under selective pressure with anti-bunyavirus compounds #A through #D and #G through #I. Whole-genome sequences of the resulting mutants were analyzed using next-generation sequencing (Table 3). A summary of the drug-resistant mutant data related to CEN is presented in Figure 4A. Adjacent mutations, E41G, E41V, K44T, and I52M in LCMV, and L40S and K67R in JUNV, were detected within the CEN α 3-helix in the virus RNA polymerase (L protein) following extended passage under drug selection.

To confirm the drug susceptibility of the drug-resistant mutants, viral replication kinetics of selected LCMV clones were evaluated. All mutants exhibited reduced replicative capacity, with the exception of LCMV clone 1 (E41G), which maintained sufficient replication for assessment by the MTT assay. Figure 4B presents the EC₅₀ values of compounds against LCMV clone 1 (E41G) compared with the wild type (WT) virus (Figure 4B). The E41G mutant displayed approximately 10-fold or greater resistance to compounds #B through #D relative to the parental WT strain. These findings indicate that the E41G substitution within the CEN α 3-helix in the virus protein is a critical residue associated with the development of CENi drug resistance (Table 4). To further characterize the virological properties of the E41G mutant, growth curves of LCMV WT and the E41G variant were compared. In KB cells, propagation of the E41G mutant was markedly reduced compared with WT, and this reduction was commonly observed in the different viral inoculum (multiplicity of infection [MOI] 0.1 or 0.01) (Figure 4C). These results suggest that the LCMV mutant harboring the E41G substitution in the L protein exhibits a replication deficiency compared with WT virus, supporting the concept that antiviral strategies targeting to the virally encoded CEN activity of bunyaviruses may provide therapeutic benefit.

Several compounds exhibited antiviral activity against LACV, IFV A/H5N1, and A/H7N9, and drug-resistant mutants were generated by propagating these viruses in cultured cells under selective pressure. For IFV A/H5N1 and A/H7N9 mutants, amino acid substitutions, including I38T within the CEN α 3-helix of the polymerase

acidic (PA) subunit, were detected in all tested compounds (#A, #B, and #I). These observations are consistent with previous reports indicating that IFV A/H1N1 and A/H3N2 CENi-resistant mutants harbor the adjacent I38T substitution in the CEN α 3-helix of PA (27). For LACV, the M31T mutation was identified in the CEN α 2-helix following selection for resistance to compound #A. Figure 4D illustrates amino acid sequence alignments of the α 3- and α 2-regions of CEN, based on prior research (12). Catalytic residues essential for CEN activity are highlighted in red columns, whereas yellow columns indicate amino acid substitutions observed in drug-resistant mutants. Notably, these substitutions occurred at approximately the same positions across all examined viruses. Table 4 presents the EC₅₀ values of compounds against the A/H5N1 clone (I38T) compared with the WT virus. The IFV A/H5N1 mutant carrying the I38T substitution exhibited approximately 10-fold or greater resistance to compounds #A through #D relative to the WT strain. These findings suggest that the I38T substitution within the CEN α 3-helix of IFV A/H5N1 represents a key residue responsible for the development of CENi drug resistance.

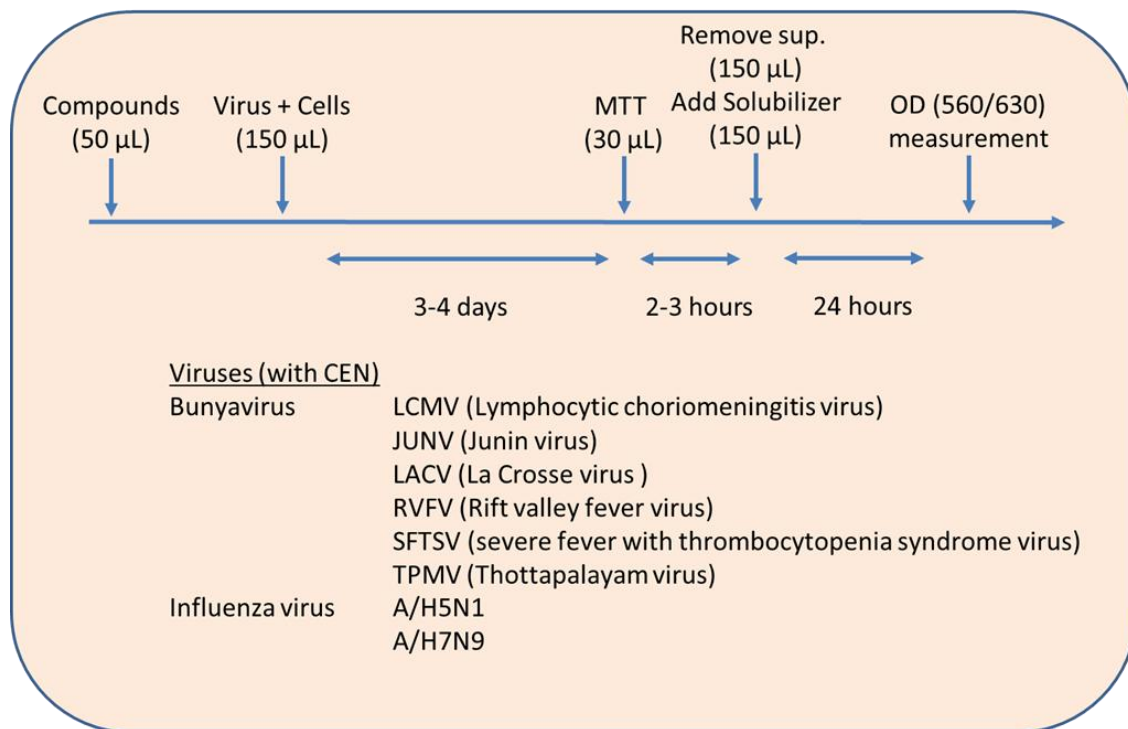
Effect of CENi chelate compounds on survival in a murine model of LCMV

To determine whether the compound exhibits potent *in vivo* efficacy, a mouse model of lethal LCMV infection was established. Compound #B was selected for evaluation in this model, as it demonstrated the most potent antiviral activity. Plasma concentrations of compound #B following intramuscular administration were measured (Figure 5A). In the absence of treatment, the survival rate of ICR mice infected with the LCMV WE strain was 0% at 14 days post-infection (dpi). Using this lethal mouse model, the *in vivo* efficacy of compound #B was assessed (Figure 5B). Following intraperitoneal infection with 3.0×10^3 TCID₅₀ of LCMV, groups of mice (n = 5) received QD intramuscular administrations of compound #B at doses of 3, 10, or 30 mg/kg, RBV at 30 mg/kg, or vehicle, beginning on the day of viral challenge. Treatment with compound #B at 10 mg/kg or higher dose and 3 mg/kg resulted in survival rates of 100% and 80%, respectively, whereas administration of 30 mg/kg RBV yielded a survival rate of only 20%. Furthermore, compound #B significantly reduced viral load in blood compared with vehicle-treated controls at 1 dpi in a dose-dependent manner (Figure 5C). Mice treated with compound #B maintained platelet counts and alanine aminotransferase (ALT) levels comparable to those of uninfected controls (Figure 5D and E).

Additionally, pathological and immunohistochemical (IHC) analyses were performed. In vehicle-treated and RBV-treated mice, inflammatory foci were observed, whereas such lesions were absent in both uninfected and compound #B-treated mice.

Furthermore, IHC revealed that viral antigens were nearly undetectable in compound #B-treated mice, in contrast to their presence within hepatocytes of vehicle- and RBV-treated animals (Figure 5F). Subsequently, serum levels of tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) were quantified. In compound #B-treated mice, IFN- γ concentrations were approximately five-fold lower than those in vehicle-treated controls, and TNF- α levels were comparable to those of uninfected mice. Conversely, in RBV-treated mice, IFN- γ levels were similar to those in vehicle-treated animals, while TNF- α concentrations exceeded those observed in compound #B-treated mice (Figure 5G and H). Collectively, these findings demonstrate that compound #B exhibits substantially greater anti-LCMV efficacy *in vivo* than RBV.

Finally, the delayed-treatment efficacy of compound #B was assessed. LCMV-infected mice ($n = 3$) received once-daily intramuscular administrations of compound #B, RBV, or vehicle at a dose of 30 mg/kg for either 1 or 5 days, initiated at 24 or 48 hpi. At 24 hpi, compound #B-treated mice in both the 1- and 5-day treatment groups exhibited a 100% survival rate (Figure 5I). At 48 hpi, survival rates were 80% and 100% for the 1- and 5-day compound #B treatment groups, respectively (Figure 5J). These findings indicate that compound #B retains therapeutic efficacy even when treatment is initiated after viral propagation *in vivo*.



Viruses	Cells	Cell number/96well	Medium	Assay period
LCMV	KB	20,000	2%FBS-MEM	4
JUNV	HEK293T	20,000	5%FBS-MEM	4
LACV	MDBK	25,000	5%FBS-MEM	3
RVFV	BHK-21	25,000	10%FBS-RPMI	3
SFTSV	MDCK	20,000	2%FBS-MEM	4
TPMV	HEK293T	15,000	5%FBS-MEM	4
A/H5N1	A549	20,000	5%FBS-MEM	3
A/H7N9	MA104-TP2	20,000	5%FBS-MEM	3

Figure 1. Outline of MTT assay and viruses used in the assay.

Screening summary of MTT assay used in this study is shown. An MTT assay system was constructed for 6 bunyaviruses and 2 IFVs. The assay conditions (cells and assay medium) for each virus were described.

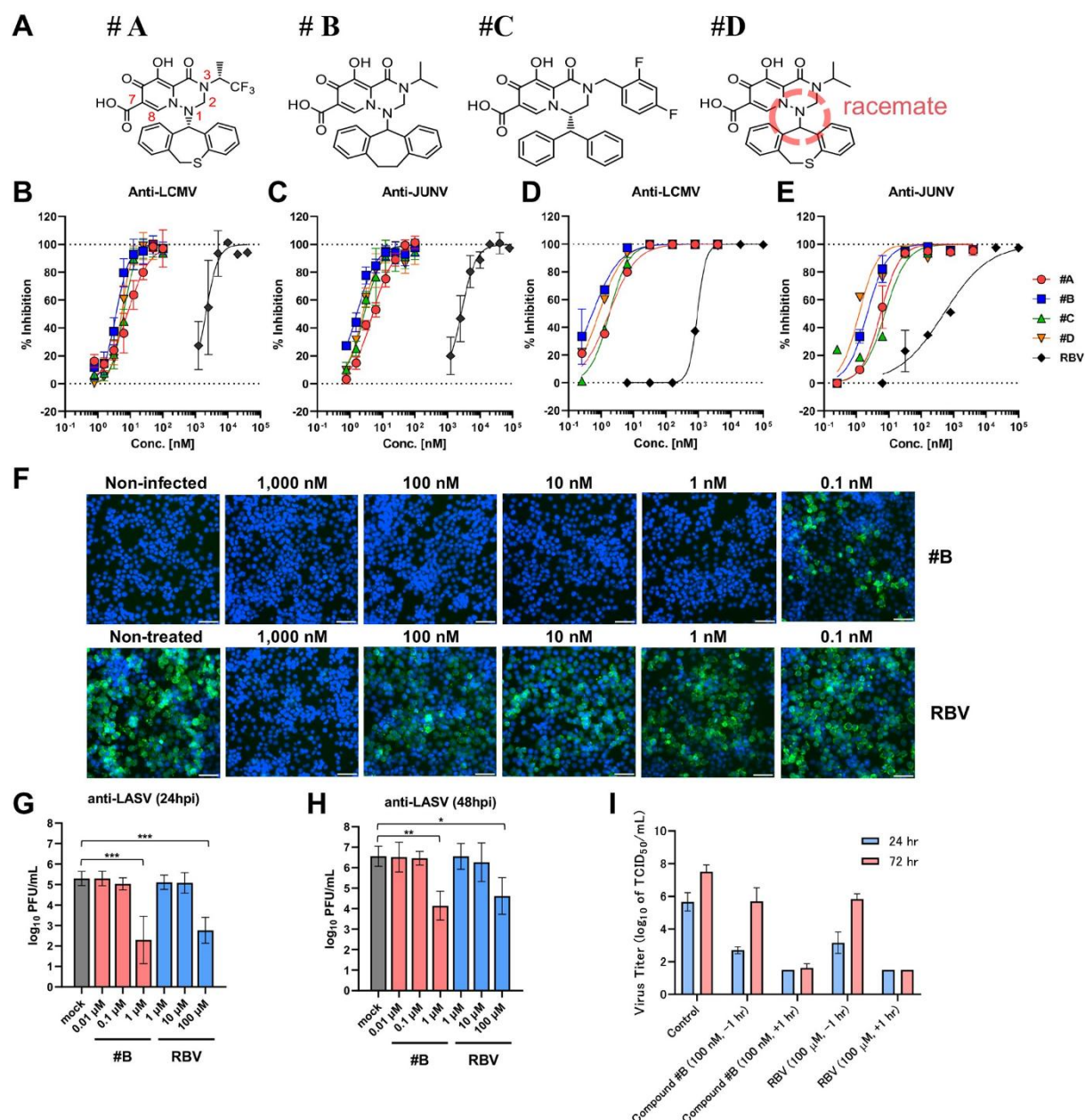
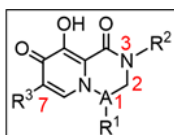


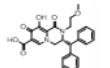
Figure 2. Influenza CENi inhibits bunyavirus infection.

(A) Chemical structures of compounds employed in the present studies. The red numbers in compound #A showed the position numbers of the main chelate structure. Compound #D was a racemic mixture of the compound isomers #E and #F prepared by chromatographic resolution (Figure 3A). (B) Inhibitory effect of compounds #A–#D and RBV in LCMV-infected KB cells by MTT assay. (C) Inhibitory effect of compounds #A–#D and RBV in JUNV-infected HEK293T cells by MTT assay. (D) Inhibitory effect of compounds #A–#D and RBV in LCMV-infected KB cells by RT-qPCR assay. (E) Inhibitory effect of compounds #A–#D and RBV in JUNV-infected HEK293T cells by RT-qPCR assay. (F) Immunofluorescence staining of LCMV-infected cells. KB cells were infected with LCMV and then cultured in the presence of compound #B for 72 h.

Cells were stained with anti-LCMV antibody (green) and DAPI (magenta). Scale bars, 100 μ m. Viral infectivity (PFU) at (G) 24 and (H) 48 hpi in the cell culture supernatant of LASV-infected VeroE6 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, 1-way ANOVA (Tukey). (I) Time of addition assay for compound #B.

A



Number	Chemical structure				Bunyavirales						Articulavirales		
					Arenaviridae		Peribunyaviridae	Phenuiviridae		Hantaviridae	Orthomyxoviridae		Influenza virus
	Scaffold	R1	R2	R3	LCMV	JUNV	LACV	SFTSV	RVFV	TPMV	A/H5N1	A/H7N9	CEN Enzyme inhibition
					EC ₅₀ (nM) Mean (SD)								IC ₅₀ nM
#A					12 (4)	7.3 (1.0)	110 (29)	700 (410)	>1,000	>1,000	20 (9)	78 (38)	39
#B					4.5 (0.6)	2.4 (0.3)	25 (10)	180 (60)	>1,000	>1,000	38 (7)	250 (120)	69
#C					6.2 (0.7)	3.4 (0.6)	93 (69)	370 (300)	>1,000	>1,000	230 (7)	>1,000	65
#D					8.0 (1.0)	3.6 (0.8)	120 (70)	670 (510)	>1,000	>1,000	23 (3)	65 (27)	121
#E					3.4 (0.9)	4.7 (1.1)	61 (13)	770 (140)	>1,000	>1,000	33 (19)	67 (9)	NT*
#F					110 (40)	73 (41)	530 (240)	>1,000	>1,000	410 (70)	>1,000	>1,000	NT*
#G					33 (6)	66 (37)	800 (210)	250 (40)	>1,000	350 (60)	210 (100)	>1,000	55
#H					34 (2)	55 (27)	>1,000	340 (180)	>1,000	650 (250)	>1,000	>1,000	86
#I					290 (150)	250 (110)	180 (40)	840	>1,000	>1,000	24 (10)	230 (160)	63
BXA					>1,000	>1,000	240 (40)	>1,000	>1,000	>1,000	0.89 (0.2)	11 (3)	2.5
RBV					3,500 (1,500)	2,500 (400)	38,000 (15,000)	7,900 (2,200)	16,000 (3,000)	13,000 (5,900)	29,000 (3,900)	7,800 (4,300)	NT*
Favipiravir					64,000 (15,000)	19,000 (5,000)	31,000 (9,400)	11,000 (9,000)	53,000 (3,000)	27,000 (3,900)	15,000 (10,000)	13,000 (5,100)	NT*

*NT: Not tested

B

Number	Chemical structure				Cell toxicity								
					KB cell	HEK293T cell	MDCK cell	BHK-21 cell	MDCK cell	A549 cell	MA104-TP2 cell	VeroE6 cell	VeroE6 cell (pre-sheated)
					CC ₅₀ nM Mean (SD)								
	Scaffold	R1	R2	R3									
#A					3,400 (1,200)	5,000 (2,200)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
#B					2,900 (1,500)	4,100 (600)	>10,000	4,300 (100)	3,500 (100)	>10,000	>10,000	>10,000	>10,000
#C					5,200 (2,300)	6,600 (300)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
#D					1,200 (600)	1,900 (200)	>10,000	8,400 (500)	7,000 (800)	>10,000	>10,000	>10,000	>10,000
BXA					7,700 (200)	5,900 (500)	5,700 (400)	5,300 (100)	7,100 (1,100)	>10,000	>10,000	>10,000	>10,000
RBV					110,000 (30,000)	200,000 (20,000)	8,000 (300)	340,000 (34,000)	360,000 (29,000)	330,000 (62,000)	>400,000	>400,000	>400,000
Favipiravir					310,000 (10,000)	>400,000	310,000 (16,000)	>400,000	>400,000	>400,000	>400,000	310,000 (53,000)	>400,000

Figure 3. Chemical structures and antiviral activity (nM) and cytotoxicity of compounds employed in the present study.

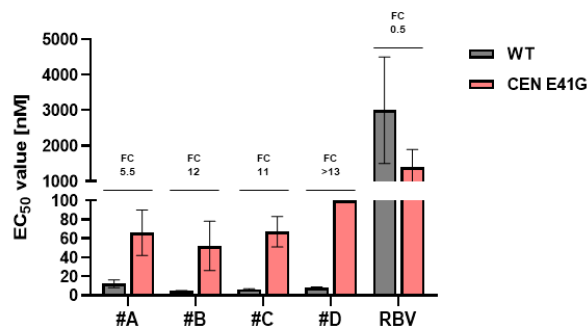
(A) Chemical structures of compounds and EC₅₀ and (B) CC₅₀ values (nM) determined by MTT assay against LCMV, JUNV, LACV, SFTSV, RVFV, TPMV, IFVs A/H5N1, and A/H7N9.

A/WSN/33(H1N1) strain was used for determination of IC₅₀ of CEN enzyme activity. Compound #D was a racemic mixture of the compound isomers #E and #F prepared by chromatographic resolution. Compound #I represent modifications of a carboxylic acid moiety at the seventh position. Compounds #G and #H possessed a same side chain with a different position of the R1 or R2 side chain. Data were presented as the mean and standard deviation (SD) determined using at least three experiments performed in duplicate. CEN enzyme inhibition was determined by one experiment data.

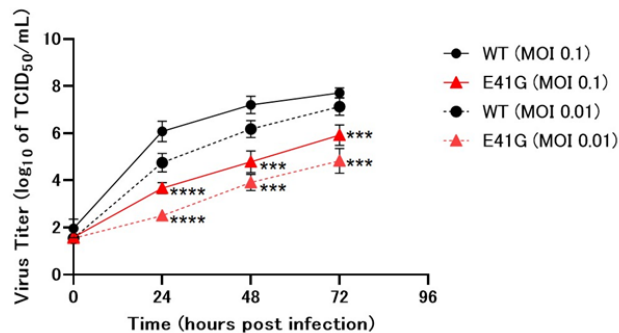
A

Virus	LCMV	JUNV	LACV	A/H5N1	A/H7N9
CEN Helix	α -3	α -3	α -2	α -3	α -3
#A	E41G D66N	K67R	M31T	I38T G81R	K19N I38T
#B	K44T I52M H62Y	L40S	ND	E119D	I38T
#C	E41G A156V N174D	ND	NT	NT	NT
#D	I52M H62Y	ND	NT	NT	NT
#G	ND	L40S	NT	NT	NT
#H	E41G E41V	L40S	NT	NT	NT
#I	E41G	ND	NT	I38T	I38T

B



C



D

IFV α 3 a.a.	a.a number	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
	H1N1	K	I	E	T	N	K	F	A	A	I $\Rightarrow$ T	C	T	H	L	E	V	C	F	M	Y	M	D	F	H
	H3N2	K	I	E	T	N	K	F	A	A	I $\Rightarrow$ T	C	T	H	L	E	V	C	F	M	Y	M	D	F	H
	H5N1	K	I	E	T	N	K	F	A	A	I $\Rightarrow$ T	C	T	H	L	E	V	C	F	M	Y	M	D	F	H
	H7N9	K	I	E	T	N	K	F	A	S	I $\Rightarrow$ T	C	T	H	L	E	V	C	F	M	Y	S	D	F	H
LACV α 2 a.a.	a.a number	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
	LACV	A	K	D	I	D	V	D	L	L	M $\Rightarrow$ T	A	R	H	D	Y	F	G	R	E	L	C	K	S	L
Arenavirus α 3 a.a.	a.a number	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58
	LCMV	P	R	M	V	L	I	E $\Rightarrow$ G,V	G	L	K $\Rightarrow$ T	L	L	S	R	C	I	E	I $\Rightarrow$ M	D	S	A	D	K	S
	JUNV	P	S	S	L	L	L $\Rightarrow$ S	E	G	F	K	L	L	S	S	L	V	E	L	E	S	C	E	A	H
	LASV	P	R	M	L	L	M	E	G	L	K	L	L	S	L	C	I	E	V	D	S	C	N	A	N

Figure 4. Identification of target viral proteins of CENi compounds by isolation of drug-escape mutants.

(A) Amino acid substitutions of drug-escape mutants of LCMV, JUNV, LACV, IFVs A/H5N1, and A/H7N9. Red letters were corresponding mutations in the CEN α -3 region in the target virus protein of LCMV, JUNV, IFVs A/H5N1, and A/H7N9, or α -2 region of LACV. ND: not detected,

NT: not tested. (B) Inhibitory effect of compounds #A–#D in LCMV infected KB cells by MTT assay. Fold change (FC) is the EC_{50} of LCMV E41G mutant divided by the that of WT virus. Raw data is shown in Table 4. (C) Growth kinetics of the LCMV WT and E41 mutant. LCMV Armstrong strain (WT) and E41G mutant were inoculated and the viral titer in the culture supernatants was measured. The growth curves of the viruses inoculated are shown. *** $p < 0.001$, **** $p < 0.0001$, compared with WT virus (t -test). (D) Amino acid sequence alignment of α -3 or α -2 region of CEN in the virus protein. The alignment was based on prior research (6). Yellow columns are the amino acid substitution in drug-resistant mutants. Red columns are catalytic sites essential for CEN activity.

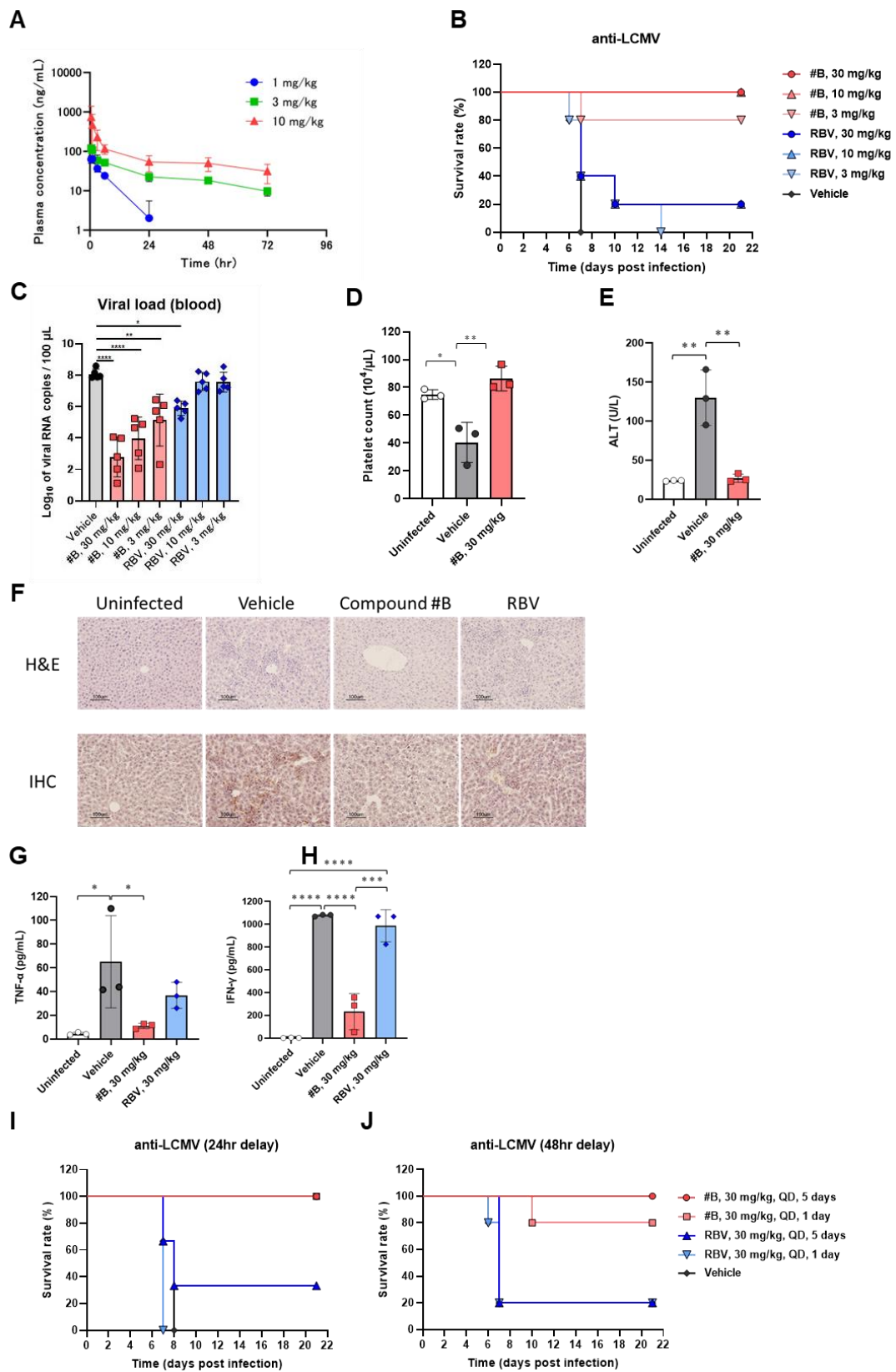


Figure 5. Compound #B improved survival rates in a lethal murine model with LCMV infection.

(A) Concentrations of compound #B in plasma after a single intramuscular administration in mice. (B) Survival rates in lethal murine model with LCMV infection (n = 5 per group). Compound #B (3–30 mg/kg), RBV (3–30 mg/kg) or vehicle control (0 mg/kg) was administered intramuscularly for 1 day. Dose-dependent effect of Compound #B and RBV were compared on survival rates. Dosing regimens are shown at right. * $p < 0.05$, ** $p < 0.01$, compared with virus control (log-rank test). (C) Viral load in blood of compound #B and RBV-treated group was compared to vehicle-treated groups at 1 day after infection. (D) The platelet counts and (E) alanine aminotransferase (ALT) levels in blood of compound #B-treated group were compared to those in the vehicle-treated group at 5 days after infection. (F) Histopathological findings in the livers of LCMV infected mice that were administered compound #B and RBV. The mice were infected with LCMV and sacrificed at 6 days after infection for histopathological examinations. Compound #B (30 mg/kg), RBV (30 mg/kg) or vehicle control (0 mg/kg) was administered intramuscularly QD for 1 day. Representative histopathological images for the lung sections obtained from the animals given antivirals indicated as indicated above each panel (n = 3). Upper panels, hematoxylin and eosin (H&E) staining. Lower panels, IHC targeting the nucleoprotein of LCMV. Scale bars in each panel, 100 μ m. (G) The serum TNF- α and (H) IFN- γ levels in compound #B and RBV-treated group were compared to those in vehicle-treated group at 5 days after infection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, 1-way ANOVA (Tukey). Survival rates in a lethal murine model of LCMV infection with delayed treatment. Compound #B (30 mg/kg), RBV (30 mg/kg) or vehicle control (0 mg/kg) was administered intramuscularly at (I) 24 or (J) 48 hpi. The survival rates of Compound #B (treatment for 1 or 5 days) and RBV (treatment for 1 or 5 days) were compared to that in vehicle-treated group. Dosing regimens are shown at right.

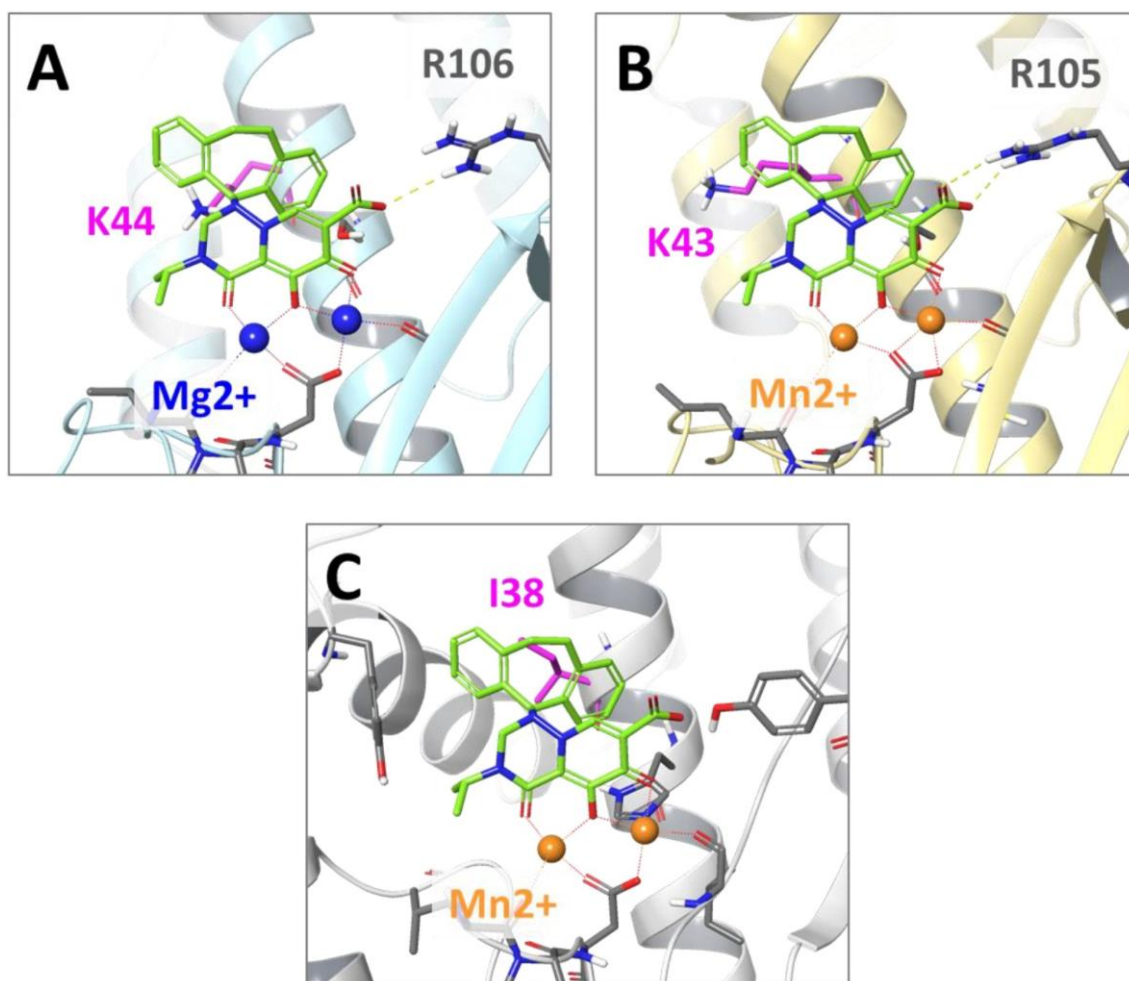
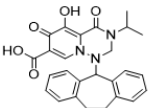
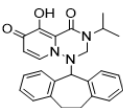
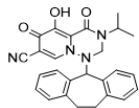
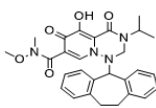
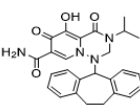


Figure 6. Binding models of compound #B to LASV, LCMV and IFVA.

Superimposed structures of compound #B bound to the endonuclease of (A) LASV (pdb-id: 4miw), (B) LCMV (pdb-id 5t2t) and (C) IFVA (pdb-id: 6fs6). Residues where amino acids substitutions in the CEN active center were identified following drug selection and the predicted location of the divalent metal cation are indicated. The 44th amino acid in the CEN region of the LCMV Armstrong strain was K44, and the genetic information of LCMV used in this model was K43.

Number		#B	#J	#K	#L	#M
7th position		carboxy group	No	cyano group	N-methoxy-N-methylcarbamoyl group	carbamoyl group
Chemical structure						
EC ₅₀ (nM) Mean (SD)	LCMV	4.5 (0.6)	>1,000	190 (20)	88 (28)	630 (180)
	JUNV	2.4 (0.3)	>1,000	380 (140)	64 (38)	340 (110)
	A/H5N1	38 (7)	25 (4)	130 (20)	820 (110)	290 (100)
	A/H7N9	250 (120)	370 (110)	>1,000	>1,000	>1,000

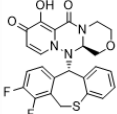
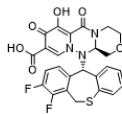
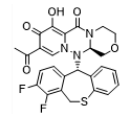
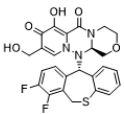
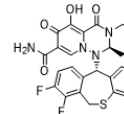
Number		Baloxavir	#N	#O	#P	#Q
7th position		No	carboxy group	acetyl group	hydroxymethyl group	carbamoyl group
Chemical structure						
EC ₅₀ (nM) Mean (SD)	LCMV	>1,000	170 (4)	>1,000	>1,000	>1,000
	JUNV	>1,000	88 (10)	>1,000	>1,000	>1,000
	A/H5N1	0.89 (0.2)	79 (7)	16 (2)	15 (1)	360 (80)
	A/H7N9	11 (3)	170 (14)	130 (30)	57 (3)	>1,000

Figure 7. Antiviral activity of the 7-position transformants of compound #B and BXA.

Chemical structures of the 7-position transformants of compound #B and BXA and EC₅₀ values (nM) determined by MTT assay against LCMV, JUNV, A/H5N1, and A/H7N9. Data were presented as the mean and SD determined using at least three experiments performed in duplicate. Antiviral activities of the compounds with the carboxylic acid moiety at the 7-position against LCMV and JUNV are highlighted in yellow.

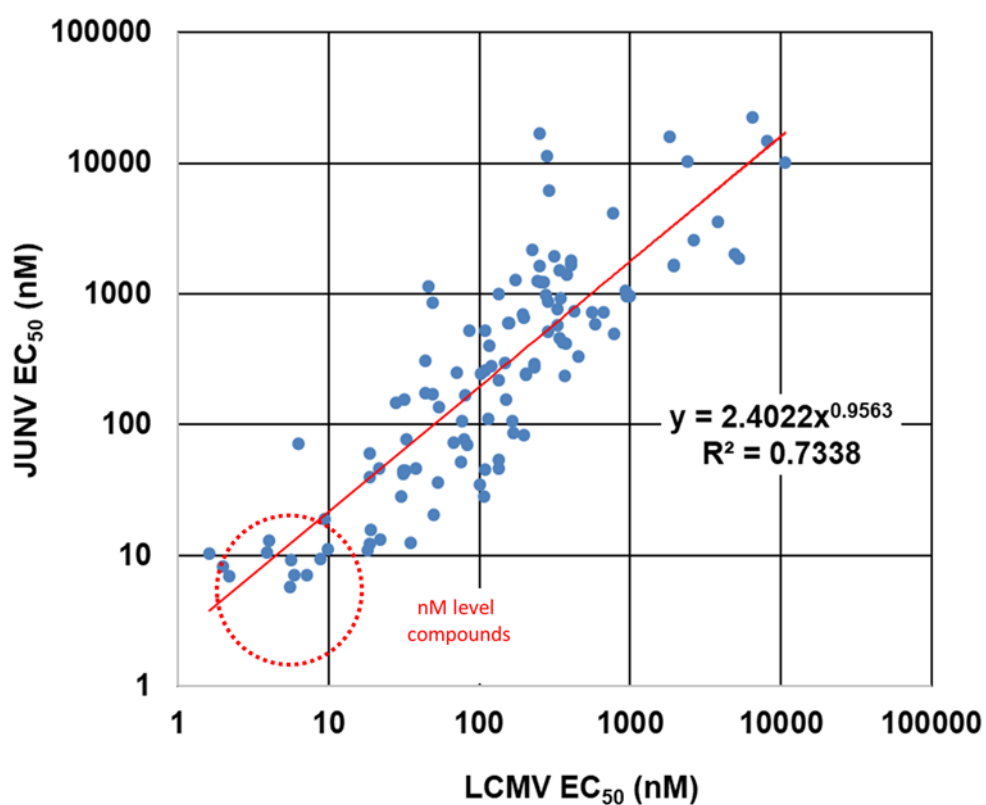


Figure 8. Correlation of the EC₅₀ values (nM) for CENi compounds for LCMV and JUNV.

The 116 compounds from the influenza CENi library were plotted with the EC₅₀ values of LCMV and JUNV. EC₅₀ of LCMV (x-axis) was compared with that of JUNV (y-axis) with a correlation curve. The top 20 compounds with nanomolar-level activity had a carboxylic acid moiety at the seventh position of the main structure.

Table 1. The primer list of 5 viruses for PCR amplification and sequencing.

Primers for PCR

Target	name	Sequence
LCMV	Fw	CGACTGCCTCCCTGCTTAATGTTAA
(for Armstrong)	Rv	ATGGATGAAATCATCTCAGAATTGAGAGAG
LCMV	Fw	CAAGATCCATGCCGTG
(for WE)	Rv	GAACCTACGGACCAGA
JUNV	Fw	CCCAACTGCTTGTCAAAAATCTCCTTC
	Rv	ATGGAGGAATCAGTAAATGAAATAAAAAGCC
LACV	Fw	TCCTATCTACAAAACCTACAGAAAATTCA
	Rv	AAATGGGGTGTTTCCAAAATTCAGG
H5N1	Fw	ATGGAAGACTTTGTGCGACAATGC
	Rv	TGAGAGGACGTGGTGTTCCTTCAG
H7N9	Fw	ATGGAAGACTTTGTGCGACAGTGC
	Rv	TCCATCAGCAAGAATTCGACCGCT

Primers for sequencing

Target	name	Sequence
LCMV	seq_Fw	GAACTAATGTGACACCCGCAAC
	seq_Rv	ATTGAGAGAGTTATGTTTAAAC
JUNV	seq_Fw	GCTGTAGCTAGATCTGCCATCAACC
	seq_Rv	AGCATTTTCCAGAAAGACAGGA
LACV	seq_Fw	TGCATAATGGACTATCAAGAGTATCA
	seq_Rv	AGGAATTCTTCATCATCACCGAA
H5N1	seq_Fw	GACTTTGTGCGACAATGCTTCAATCCA
	seq_Rv	AGTGGCCATCTCTTCTCCAGTGA
H7N9	seq_Fw	AGACTTTGTGCGACAGTGCTTCA
	seq_Rv	TGGCCATCTCTTCTCCAGTGAATG

Table 2. Anti-LCMV and JUNV activity of compounds #A–#D.

Inhibitory effect of compounds #A–#D and RBV in LCMV infected KB cell and JUNV infected HEK293T cells. EC₅₀ values of compounds #A–#D and RBV were determined by MTT assay and RT-qPCR assay. Data were presented as the mean and SD were determined by at least three experiments performed in duplicate.

LCMV	MTT EC ₅₀ (nM)	RT-qPCR EC ₉₀ (nM)
Compounds	Mean ± SD	Mean
#A	8.3 ± 2.8	15
#B	3.8 ± 0.9	4.7
#C	6.2 ± 1.4	8.0
#D	5.4 ± 0.4	5.6
RBV	2,375 ± 1,123	1,832

JUNV	MTT EC ₅₀ (nM)	RT-qPCR EC ₉₀ (nM)
Compounds	Mean ± SD	Mean
#A	5.1 ± 0.7	25
#B	1.7 ± 0.3	10
#C	3.0 ± 1.0	42
#D	2.6 ± 0.5	5.1
RBV	2,720 ± 907	17,216

Table 3. Mutations identified in the genome of LCMV and JUNV mutants.

Amino acid substitutions in the CEN for drug escape mutants of LCMV and JUNV determined by NGS. The sequence variants with >5% frequency were listed. LCMV clones 1–2 were isolated from #H- and #I-escape mutants, respectively. JUNV clones 1–2 were isolated from #G-escape mutants, whereas clone 3 was isolated from #H-escape mutant.

LCMV Gene	amino acid position	wild type	Clone-1	Clone-2
L	41	E(100%)	G(79%)/E(21%)	V(59%)/G(32%)/E(9%)
L	156	A(75%)/V(35%)	A(100%)	V(90%)/A(10%)
L	631	S(70%)/G(30%)	G(96%)/S(4%)	S(100%)
L	1505	L(100%)	S(94%)/L(6%)	L(100%)
L	1523	L(100%)	L(100%)	I(62%)/L(38%)
L	1626	E(79%)/G(21%)	E(100%)	E(100%)
L	1710	L(78%)/P(22%)	L(100%)	L(100%)
L	2014	A(85%)/K(15%)	A(100%)	A(100%)
GPC	526	N(96%)/D(4%)	N(96%)/D(4%)	N(95%)/D(5%)
GPC	754	L(82%)/F(18%)	L(83%)/F(17%)	L(78%)/F(22%)
GPC	778	F(100%)	F(100%)	F(58%)/L(42%)
GPC	1028	F(93%)/Y(7%)	F(97%)/Y(3%)	F(87%)/Y(13%)
NP	254	K(100%)	K(100%)	K(77%)/R(23%)

(Table 3 continued)

JUNV Gene	amino acid position	wild type	Clone-1	Clone-2	Clone-3
L	40	L(100%)	S(92%)/L(8%)	S(83%)/L(17%)	S(86%)/L(14%)
L	1331	V(100%)	V(100%)	V(100%)	V(100%)
L	1618	I(100%)	I(100%)	I(100%)	I(100%)
L	1886	N(100%)	N(100%)	N(100%)	N(91%)/D(9%)
L	1887	K(100%)	K(100%)	K(100%)	K(92%)/R(8%)
L	1894	V(100%)	V(100%)	V(100%)	V(100%)
L	1958	V(100%)	V(94%)/I(6%)	V(100%)	V(100%)
GPC	146	Q(100%)	Q(94%)/K(6%)	Q(100%)	Q(100%)
NP	127	N(100%)	N(100%)	N(78%)/D(22%)	N(100%)
Z	81	T(100%)	T(100%)	T(94%)/A(6%)	T(100%)
Z	93	P(100%)	P(100%)	P(100%)	P(95%)/L(5%)

Table 4. Anti-LCMV and IFV activities of compounds #A–#D against CENi mutants.

Inhibitory effects of compounds #A–#D, RBV, favipiravir in LCMV-infected KB cells and IFV A/H5N1-infected A549 cells. The EC₅₀ values of the compounds were determined by MTT assay. The fold change was EC₅₀ of the mutants divided by that of WT virus. Data were presented as the mean and standard deviation determined using at least three experiments performed in duplicate.

Compounds	Anti-LCMV activity		Fold Resistance	Anti-IFV activity		Fold Resistance
	WT	CEN E41G		WT	CEN I38T	
	EC ₅₀ (nM) Mean (SD)	EC ₅₀ (nM) Mean (SD)		EC ₅₀ (nM) Mean (SD)	EC ₅₀ (nM) Mean (SD)	
#A	12 (4)	66 (24)	5.5	20 (9)	>1,000	>50
#B	4.5 (0.6)	52 (26)	12	38 (7)	>1,000	>26
#C	6.2 (0.7)	67 (16)	11	230 (7)	>1,000	>4
#D	8.0 (1)	>100	>13	23 (3)	>1,000	>43
#H	34 (2)	>1,000	>29	0.53 (0.3)	29 (3)	55
#I	290 (150)	>1,000	>3	1.4 (0.5)	61 (13)	44
RBV	3,000 (1,500)	1,400 (500)	0.5	15,000 (3,000)	7,100 (3,200)	0.5
T-705	64,000 (15,000)	12,000 (5,000)	0.2	11,000 (6,000)	9,300 (7,000)	0.8

Discussion

The primary objective of this drug discovery effort is the identification of compounds with activity against a broad spectrum of viral families, with a current focus on agents effective against members of the class *Bunyaviricetes*. These viral infections are associated with high mortality rates, and no licensed antivirals or vaccines have been demonstrated to provide effective treatment for the corresponding viral hemorrhagic diseases. In the present study, an MTT-based assay system was established for multiple bunyaviruses to facilitate the identification of antiviral candidate compounds from an influenza CENi library. Broad-spectrum antiviral compounds exhibiting inhibitory effects both *in vitro* and *in vivo* against several members of the family *Arenaviridae* within the class *Bunyaviricetes* were identified.

Arenavirus infections represent significant zoonotic diseases transmitted from rodents and are characterized by high morbidity and mortality. These viruses are classified into two groups, Old World and New World arenaviruses, with at least eight recognized pathogens known to cause hemorrhagic fevers (28). Consequently, the development of effective antiviral therapies and prophylactic vaccines remains a critical unmet global public health need that could substantially reduce disease burden. LCMV was the first described arenavirus and is associated with aseptic meningitis, a severe neurological disease involving inflammation of the brain and spinal cord, whereas JUNV, LASV, Guanarito virus, Lujo virus, Machupo virus, Sabia virus, and Whitewater Arroyo virus are etiological agents of viral hemorrhagic fever syndromes (9). Among these, LASV, a member of the family *Arenaviridae*, has been ranked as the highest-risk virus for animal-to-human spillover among newly identified pathogens (29). In this study, a compound exhibiting nanomolar-level antiviral activity against LCMV and JUNV was successfully identified and demonstrated to possess *in vivo* efficacy, as evidenced by reduced clinical symptoms and suppressed lethality in an LCMV mouse model. Notably, this compound also displayed CEN enzyme-inhibitory activity against IFVs, and findings from arenavirus drug selection experiments suggest that its mechanism of action involves inhibition of the virally encoded CEN enzyme. Given that the compound also exhibited antiviral activity against LASV, LACV, and IFV, it is reasonable to conclude that this agent may be broadly effective against arenaviruses and represents a promising candidate for the long-sought broad-spectrum antiviral therapy for arenavirus infections.

CEN is conserved across the viral family *Orthomyxoviridae* and the class *Bunyaviricetes* and mediates cap-snatching during viral RNA transcription initiation, a

process essential for viral replication. Importantly, CEN enzymes are absent in humans and therefore represent ideal targets for broad-spectrum antiviral drug development. Based on findings from drug-resistant mutants selected under antiviral pressure, exposure to the examined compounds were associated with clusters of nonsynonymous mutations within the CEN α 3-region of both IFVs and bunyaviruses. CEN coordinates two divalent cations, absolutely required for enzymatic function, through catalytic amino acids that constitute the active site. Cocrystal structures of IFV A and B CENs complexed with BXA have been resolved, revealing that the I38T substitution within the α -chain alters interactions with the compound's hydrophobic aromatic ring (10). Based on crystal structures of LCMV and LASV, it is hypothesized that compound #B possesses an active center capable of tightly binding to the CEN active site (Figure 6). The structural model further suggests that the carboxylic acid moiety at the seventh position of the compound is critical for antiviral activity against bunyaviruses when interacting with CEN residue R105 (or R106).

To evaluate this hypothesis, antiviral activities of 7-position derivatives of compound #B and BXA were assessed. For LCMV and JUNV, compounds containing the 7-position carboxylic acid exhibited strong antiviral activity, whereas, in contrast, in IFV assays, derivatives lacking the 7-position modification also demonstrated potent antiviral effects. Furthermore, additional substitutions at the 7-position were examined, and the carboxylic acid moiety was identified as the most active group against both LCMV and JUNV (Figure 7).

Strikingly, a carboxylic acid moiety at the seventh position of the main structure was necessary for the antiviral activity. Approximately 1,000 compounds were screened and the correlation between the chemical structures and antiviral activities against LCMV and JUNV was investigated. The EC₅₀ values of the 116 compounds against LCMV and JUNV were plotted, and a significant correlation was observed between the LCMV and JUNV EC₅₀ values (Figure 8), confirming the requirement of a carboxylic acid at the seventh position of the main structure for nanomolar level anti-LCMV and anti-JUNV activity.

As two-metal chelators bind divalent cations that are essential for CEN catalytic activity, highly active hit compounds can be identified during the early stages of drug discovery. In this study, these compounds were demonstrated to exhibit nanomolar-level activity against LCMV, JUNV, LASV, LACV, and IFV, while showing minimal or no activity against SFTSV, RVFV, and TPMV. Recently, Wang *et al.* (30) reported that BXA inhibited the replication of bunyaviruses (SFTSV and Heartland virus) with an EC₅₀ value of 263 nM for SFTSV; however, BXA did not inhibit SFTSV

replication at 1,000 nM in the present experiments. This discrepancy may be likely due to differences in assay methodology. Previous studies have reported mechanistic variations among cap-snatching endonucleases from segmented negative-strand RNA viruses, including LCMV, RVFV, and hantaviruses (31, 32). It is speculated that structural differences in the target region of these compounds may exist, particularly for RVFV and TPMV. Therefore, future screening of new chemical libraries will be necessary to expand the repertoire of potential antiviral candidates. Additionally, diverse chemical side chains can be introduced onto various two-metal chelate scaffolds, enabling the design of compounds with desirable physicochemical properties (e.g., solubility, pharmacokinetics) in combination with a second pharmacophore. This approach could facilitate the development of inhibitors tailored to specific pathogen targets or agents with broader activity against multiple viral families. Collectively, these findings underscore the potential of two-metal chelators to inhibit replication across a wide range of viral families and highlight their promise for advancing therapeutic strategies against viral hemorrhagic fevers.

Chapter II

Synergistic antiviral activity of cathepsin B/L and TMPRSS2 inhibitors against SARS-CoV-2 *in vitro* and *in vivo*

Summary

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike (S) protein binds to a host cell receptor, which determines the viral entry pathway. SARS-CoV-2 employs two distinct entry mechanisms mediated by host proteases: type II transmembrane serine protease (TMPRSS2) and cathepsins. These proteases cleave the viral S protein and initiate membrane fusion, enabling viral infection. Previously, a SARS-CoV-2 mutant lacking the furin cleavage site in the S gene (del2) was isolated, revealing differences in cell tropism between WT and del2 viruses. In this study, the antiviral activities of cellular protease inhibitors against SARS-CoV-2 WT and del2 viruses were evaluated using multiple cell lines. The TMPRSS2 inhibitor camostat exhibited strong antiviral activity against WT virus but not against del2, whereas the cathepsin B/L inhibitor K11777 demonstrated potent antiviral activity against the del2 virus. K11777-escape mutants of SARS-CoV-2 and SARS-CoV were isolated, and these mutations were shown to facilitate S protein cleavage at the S2' site mediated by cathepsin L. Furthermore, combination treatment with K11777 and camostat potently inhibited SARS-CoV-2 WT infection both *in vitro* and *in vivo*, underscoring the potential of combination therapeutics targeting host TMPRSS2 and cathepsin proteases for coronavirus infections. In summary, this study characterizes K11777 as an inhibitor of S2' cleavage by cathepsins and highlights the critical role of the S2' site in SARS-CoV-2 cellular entry. These findings provide important insights into the viral entry process and have significant implications for the development of therapeutic strategies against SARS-CoV-2 infection.

Introduction

COVID-19 has profoundly impacted global health and economies. Since SARS-CoV-2 was identified as the etiological agent of COVID-19 in December 2019, this virus has rapidly spread worldwide, resulting in millions of infections and deaths (2, 3). Numerous studies have focused on drug repurposing and the development of novel therapeutics for COVID-19. Several small-molecule agents, including remdesivir, molnupiravir, nirmatrelvir, ensitrelvir, simnotrelvir, and leritrelvir, have been evaluated in clinical trials for the treatment of COVID-19 (33–38).

SARS-CoV-2 cellular entry is mediated by interaction between its S glycoprotein and the host receptor angiotensin-converting enzyme 2 (ACE2), followed by proteolytic cleavage of the full-length S protein by host proteases, including furin, TMPRSS2, and cathepsin L. TMPRSS2 has been confirmed to be essential for S protein priming, suggesting that TMPRSS2 inhibitors, such as camostat mesylate may suppress SARS-CoV-2 infection (39). Furthermore, cathepsin L has been shown to provide an alternative cellular entry pathway, underscoring the importance of lysosome-mediated viral entry (40). Consequently, ACE2, TMPRSS2, and cathepsins are considered critical host targets for inhibiting SARS-CoV-2 entry (41, 42). K11777 (also referred to as K777, S-001, or SLV213) is a potent, irreversible covalent inhibitor of mammalian cathepsins B and L, with demonstrated efficacy against various viruses and parasites (43). Clinical trials evaluating K11777 as a COVID-19 treatment are also underway (44).

SARS-CoV-2 variants harboring mutations at the S protein cleavage site were previously isolated, among which the del2 strain exhibited a distinct cellular tropism compared with WT SARS-CoV-2 (45). Furthermore, it was demonstrated that camostat, a TMPRSS2 inhibitor, specifically inhibited the entry of the WT virus, whereas E64d, an inhibitor of cathepsins B and L, markedly suppressed the cellular entry of the del2 variant (45). These findings indicate that SARS-CoV-2 utilizes at least two distinct entry pathways: the TMPRSS2-dependent pathway and the cathepsin-mediated pathway.

This study evaluated the antiviral activities of host protease inhibitors camostat, nafamostat (a TMPRSS2 inhibitor), K11777, and E64d against SARS-CoV-2 and SARS-CoV. In addition, K11777-resistant mutations within the S proteins of SARS-CoV-2 and SARS-CoV were identified, and their impact on cleavage efficiency by cathepsin L was evaluated using a fluorogenic substrate assay. Finally, the efficacy of combination therapy with the cathepsin inhibitor K11777 and the TMPRSS2 inhibitor camostat was examined *in vitro* and *in vivo*. Collectively, these findings demonstrate

that combination therapy using different host protease inhibitors represents a promising therapeutic strategy targeting viral entry pathways in coronavirus infections.

Materials and methods

Cell lines

VeroE6 (American Type Culture Collection), 293T [RIKEN BRC (Ibaraki, Japan)] cells were maintained in high-glucose Dulbecco's modified Eagle's medium [Gibco, Thermo Fisher Scientific (Waltham, MA, USA)] supplemented with 10% fetal bovine serum [FBS; (Gibco, Thermo Fisher Scientific)] and penicillin-streptomycin [P/S; (Wako Pure Chemical Co., Ltd.)] at 37 °C.

Generation of TMPRSS2- and ACE2-expressing cells

VeroE6 cells stably expressing human TMPRSS2 (VeroE6/TMPRSS2) and 293T cells stably expressing human ACE2 (293T/ACE2), a receptor for SARS-CoV2 infection of cells, or human dipeptidyl peptidase-4 (DPP4) (293T/DPP4), a receptor for MERS-CoV infection of cells, were generated by lentiviral transduction as described in a previous report (45). Then, human TMPRSS2 was lentivirally transduced into 293T/ACE2 cells to generate 293T/ACE2 cells stably expressing TMPRSS2 (293T/ACE2/TMPRSS2).

Viruses

SARS-CoV-2 strain WK-521 (WT) (Ancestral, Pango Lineage: A, GISAID:302 EPI_ISL_408667), QK002 (Alpha, Pango Lineage: B.1.1.7, GISAID: EPI_ISL_768526), TY8-612 (Beta, Pango Lineage: B.1.351, GISAID: EPI_ISL_1123289), TY7-501 (Gamma, Pango Lineage: P.1, GISAID: EPI_ISL_833366), TY11-927 (Delta, Pango Lineage: B.1.617.2, GISAID: EPI_ISL_2158617), TY38-873 (Omicron, Pango Lineage: 306 BA.1, GISAID: EPI_ISL_7418017), a clinical isolate from a patient with COVID-19, were kindly provided by the National Institute of Infectious Diseases (National Institute of Infectious Diseases)] and amplified in VeroE6/TMPRSS2 cells. The S gene mutant virus clone of WK-521 (del2) was isolated as previously described and propagated in VeroE6 cells (45). SARS-CoV (SARS1) strain Hanoi was kindly provided by Dr. Morita [Nagasaki University (Nagasaki, Japan)] and was amplified in VeroE6 cells (46). MERS-CoV strain EMC2012 (47) was kindly provided by Dr. Haagmans [Erasmus University Medical Center (Rotterdam, ZH, Netherlands)] and amplified on VeroE6/TMPRSS2 cells. SARS-CoV-2 (WT and del2), SARS-CoV, MERS-CoV were handled in a BSL-3 laboratory.

Antiviral compounds

Camostat mesylate, nafamostat mesylate, and E64d were supplied by Wako Pure Chemical Co., Ltd. K11777 (Figure 9A) was synthesized at Shionogi & Co., Ltd. N-hydroxycytidine (NHC, active form of molnupiravir) was purchased from Angene International Limited (Nanjing, China). All compounds were solubilized in 100% DMSO [Sigma-Aldrich (St. Louis, MO, USA)] for *in vitro* studies and in 0.5% methylcellulose solution or saline for *in vivo* studies.

CPE-based antiviral and cytotoxicity assays

The MTT assay was carried out as previously described (45), with the following modifications. VeroE6/TMPRSS2 and 293T/ACE2/TMPRSS2 cells were infected with WT SARS-CoV-2. VeroE6 and 293T/ACE2 cells were infected with del2 SARS-CoV-2. VeroE6/TMPRSS2, 293T/ACE2/TMPRSS2, and 293T/ACE2 cells were infected with SARS-CoV. VeroE6/TMPRSS2, 293T/ACE2/TMPRSS2, and 293T/DPP4 cells were infected with MERS-CoV. All cells were infected with viruses at 4–10 TCID₅₀. Camostat or nafamostat was mixed with cells and incubated at 37 °C for 1 h before the virus infection. The inhibition rate was assessed using an MTT assay at 2–3 dpi. The EC₅₀ or EC₃₀ value was defined in GraphPad Prism version 8.4.3 (GraphPad Software) with a variable slope (four parameters). Non-infected cells were used as a control for 100% inhibition, whereas for infected cells, DMSO alone was used as a control for 0% inhibition.

Selection of K11777 escape mutants and viral genome sequencing

Drug escape mutants were selected by SARS-CoV and SARS-CoV-2 (del2) using 293T/ACE2 cells. The cells at 2–3 dpi were suspended and inoculated onto naïve 293T/ACE2 cells in 12-well plates in the presence of K11777 at concentration 16, 80, or 400 nM. Following 2–3 days incubation at 37 °C, the virus-infected cells with CPE were suspended and passaged for the next round of selection in dose-escalating conditions. After several rounds of passage for the selection of escape mutants, the viruses in the supernatants were infected into cells in the presence of diluted drugs. The viruses in CPE-positive wells were sequenced to identify amino acid substitutions by next-generation sequencing (NGS) as described previously (45).

Immunoblotting

VeroE6/TMPRSS2 cells or VeroE6 cells were infected with WT or del2 viruses, respectively, at an MOI of 0.1 in the presence of K11777 at 5,000, 1,000, or 200 nM. After 24 hpi, cells were lysed in RIPA buffer [Nacalai tesque (Kyoto, Japan)] supplemented

with Complete ULTRA protease inhibitor cocktail [Roche Diagnostics (Basel, BS, Switzerland)], and then boiled in the SDS (sodium dodecyl sulfate) sample buffer. The proteins were resolved by SDS-PAGE, and transferred onto nitrocellulose membranes (iBlot2, Thermo Fisher Scientific). The membranes were incubated with anti-SARS-CoV-2 S primary antibody [GTX632604, GeneTex (Irvine, CA, USA)] and horseradish peroxidase (HRP)-conjugated secondary antibody. HRP-conjugated anti- β -actin antibody [P-053-7, MBL (Aichi, Japan)] was used to detect the internal control. The immune complexes were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) by Image Quant 800 [Cytiva (Marlborough, MA, USA)].

***In vitro* cleavage assay with fluorogenic peptides and cathepsin L**

Fluorogenic peptides derived from SARS-CoV and SARS-CoV-2 S protein (S2' site) and harboring the 4-[(4'-(N,N-dimethylamino)phenyl)azo]benzoic acid /5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (DABCYL/EDANS) FRET pair were synthesized by GenScript (Nanjing, China) and solubilized in 100% N,N-dimethylformamide (DMF; Sigma-Aldrich). Recombinant cathepsin L was purchased from R&D Systems.

For each fluorogenic peptide, reactions were performed in 40 μ L volumes in 384-well plates with an assay buffer composed of 25 mM 2-(N-morpholino)ethanesulfonic acid (MES), 5 mM DTT, 1 mM EDTA, 0.005% (w/v) Brij-35, pH 6.0 for cathepsin L (0.1 ng/ μ L) and the serially diluted fluorogenic peptides. Proteolytic reactions were performed at room temperature in quadruplicate, and fluorescence signals were measured every minute using EnVision Microplate Reader [PerkinElmer, Inc. (Waltham, MA, USA)] with λ_{ex} 340 nm and λ_{em} 485 nm wavelength settings. The initial velocities (V_0) from each reaction were determined from the linear portion of the progress curve. Data were fitted by non-linear regression to the Michaelis-Menten equation to derive the K_m and V_{max} values. No significant hydrolysis of the peptide substrates was observed in the absence of cathepsin.

Determination of combination antiviral activity *in vitro*

The MTT assay using K11777 and camostat was performed using VeroE6/TMPRSS2 cells as described above. The EC_{50} values for inhibition of SARS-CoV-2 (WT) infection of each substance alone and at a fixed concentration of the other were determined. Data were analyzed according to the method of previous report (48, 49). The combination effect was determined according to the following criteria: combination index (CI) values ≤ 0.8 , synergy; $0.8 < CI < 1.2$, additive; $1.2 \leq CI$, antagonism.

Ethical statement

All experiments were authorized by the Biosafety Management Committee on Pathogens and Other Hazardous Agents and the Safety Committee on Genetic Recombination Experiments, Hokkaido University. All animal experiments were performed in accordance with the National University Corporation, Hokkaido University Regulations on Animal Experimentation. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Hokkaido University (approval no. 20–0060).

Determination of combined antiviral activity *in vivo*

Specific-pathogen-free, 6-week-old female Syrian hamsters (Japan SLC) were inoculated intranasally with 200 μ L of 2×10^2 TCID₅₀/hamster of SARS-CoV-2 (WT) under anesthesia (n =5/group). After inoculation, hamsters were treated with camostat and K11777 using oral and subcutaneous administration, respectively, twice daily at 12 h intervals (bid). NHC (oral administration, bid) served as a positive control. At 1 dpi, the infected hamsters were euthanized under deep anesthesia by isoflurane inhalation, and whole lung tissues were harvested and homogenized in PBS with TissueRuptor (Qiagen). The homogenates were centrifuged for 10 min at $2,000 \times g$ to pellet tissue debris and the supernatants were subjected to TCID₅₀ assays using VeroE6/TMPRSS2 cells for virus titration.

Data and statistical analysis

All bar graphs in this study were presented as mean $\pm$ SD. One-way ANOVA followed by Dunnett's or Tukey's multiple comparisons test was also performed to determine the statistical significance using GraphPad Prism version 8.4.3 (GraphPad Software).

Results

Different susceptibilities of SARS-CoV-2, SARS-CoV, and MERS-CoV to protease inhibitors

In this study, four compounds with established anti-SARS-CoV-2 activity were utilized: camostat and nafamostat as TMPRSS2 inhibitors, and K11777 and E64d as cathepsin B/L inhibitors. Previous studies have demonstrated that, in contrast to the WT virus, the del2 mutant utilizes a cathepsin-dependent endocytosis pathway rather than TMPRSS2-mediated membrane fusion (45). Accordingly, VeroE6/TMPRSS2 and 293T/ACE2/TMPRSS2 cell lines were employed for WT virus infection, whereas VeroE6 and 293T/ACE2 cells were used for del2 virus infection. In WT virus infection, antiviral activities were observed for camostat, nafamostat, and K11777, with mean EC_{50} values of 3,173, 10,302, and 9,973 nM in VeroE6/TMPRSS2 cells, and 746, 445, and >20,000 nM in 293T/ACE2/TMPRSS2 cells, respectively (Figure 9B). In contrast, E64d exhibited no detectable antiviral effect (EC_{50} >50,000 nM) (Figure 9B and Table 5).

In the case of del2 virus infection, K11777 and E64d demonstrated strong antiviral activities, with mean EC_{50} values of 0.26 and 184 nM in 293T/ACE2 cells, respectively. In contrast, camostat and nafamostat showed no measurable antiviral effect (EC_{50} >50,000 nM) (Figure 9C and Table 5). NHC, active form of molnupiravir, employed as a positive control, exhibited antiviral activity against both WT and del2 strains. These findings indicate that camostat and nafamostat act by inhibiting TMPRSS2-mediated SARS-CoV-2 entry, whereas K11777 and E64d target cathepsin-dependent endocytosis, thereby suggesting their contribution to the inhibition of viral entry through a TMPRSS2-independent pathway.

The antiviral activity of K11777 and other inhibitors against SARS-CoV and MERS-CoV was further evaluated. Antiviral activities of the inhibitors were assessed against SARS-CoV in VeroE6/TMPRSS2, 293T/ACE2/TMPRSS2, and 293T/ACE2 cells, and against MERS-CoV in VeroE6/TMPRSS2, 293T/ACE2/TMPRSS2, and 293T/DPP4 cells (Table 5). In VeroE6/TMPRSS2 cells, K11777 displayed antiviral activity with mean EC_{50} values of 2,228 and 4,877 nM against SARS-CoV and MERS-CoV, respectively. Conversely, in 293T/ACE2 or 293T/DPP4 cells, K11777 exhibited potent antiviral effects, with mean EC_{50} values of 1.45 and 3.24 nM against SARS-CoV and MERS-CoV, respectively (Table 5). In VeroE6/TMPRSS2 and 293T/ACE2/TMPRSS2 cells, camostat and nafamostat demonstrated strong antiviral activity, whereas no inhibitory effect was observed in 293T/ACE2 and 293T/DPP4 cells (EC_{50} >50,000 nM). These observations are consistent with previous reports regarding host protease dependency in SARS-CoV

and MERS-CoV infection (50, 51). In 293T/ACE2 and 293T/DPP4 cells, the findings support that SARS-CoV and MERS-CoV utilize a cathepsin-dependent pathway for viral entry.

Antiviral activities of K11777 against SARS-CoV-2 variants

During the pandemic, the emergence and global dissemination of SARS-CoV-2 variants have been documented (52). These variants accumulate mutations within the S protein, thereby altering its proteolytic properties and cell tropism. The virus proliferation and antiviral effects of K11777 on multiple SARS-CoV-2 variants were evaluated (Figure 10 and Table 6). K11777 exhibited strong antiviral activity, with EC_{50} values ranging from 0.21 to 0.55 nM against all tested virus strains in the MTT assays using 293T/ACE2 cells (Table 6). In VeroE6 cells, the EC_{50} values of K11777 were 2.5- to 42-fold lower than those observed in VeroE6/TMPRSS2 cells for all variants. To determine the influence of TMPRSS2 expression on the antiviral activity of cathepsin inhibitors against SARS-CoV-2, an MTT assay was performed using 293T/ACE2/TMPRSS2 cells. As expected, the antiviral potency of K11777 was reduced in 293T/ACE2/TMPRSS2 cells compared to 293T/ACE2 cells. These findings indicate that the antiviral activity of K11777 against SARS-CoV-2 is dependent on the cell type and TMPRSS2 expression in both VeroE6 and 293T/ACE2 cells (Table 6).

Additionally, the potential impact of K11777 on the expression level of the S protein was investigated. WT and del2 strains were propagated in VeroE6/TMPRSS2 and VeroE6 cells in the presence of K11777 at concentrations of 5,000, 1,000, and 200 nM (Figure 11). As noted above, K11777 can inhibit cathepsin-dependent processing of the S protein, whereas it does not interfere with TMPRSS2-mediated cellular entry pathways. In VeroE6 cells infected with the del2 virus, the amount of S protein was reduced by K11777 treatment in a concentration-dependent manner (Figure 11). In contrast, in VeroE6/TMPRSS2 cells infected with WT virus, K11777 treatment did not result in a noticeable reduction of full-length S protein or cleaved S2 protein (Figure 11). These findings indicate that infection by the del2 virus was suppressed by K11777, leading to decreased S protein levels.

Selection of K11777-escape mutants of SARS-CoV-2 and SARS-CoV

An attempt was made to generate variants with mutations conferring resistance to K11777 in the S proteins of SARS-CoV-2 and SARS-CoV. K11777-susceptible SARS-CoV-2 (del2) and SARS-CoV were serially passaged in 293T/ACE2 cells with progressively increasing concentrations of K11777, reaching up to 400 nM. After 13

passages, pronounced CPE was observed even in the presence of K11777 at 400 nM (Figure 12A). However, NGS analysis of the passaged viruses revealed multiple mutations within the S protein (Tables 7 and 8).

Through limiting dilution culture of the passaged viruses and subsequent NGS analysis, several viral clones carrying mutations near the S2' cleavage site of the S protein were obtained: SARS-CoV T795N, P794S/T795N, and Δ 794–795 mutants, as well as SARS-CoV-2 K811N and K811N/P812L mutants (Figure 12B). The T795N and K811N substitutions reduced the susceptibility of SARS-CoV and SARS-CoV-2 to K11777 in 293T/ACE2 cells, respectively (Table 9). In contrast, the P794S/T795N, K811N/P812L, and Δ 794–795 mutations conferred a higher level of resistance to K11777 (Table 9), suggesting that the S2' cleavage process plays a critical role in the antiviral activity of K11777 during coronavirus infection.

K11777-escape mutations enhance S2' cleavage by cathepsin L

It was hypothesized that mutations in the S2' site alter the proteolytic cleavage susceptibility of the S protein by cathepsin, thereby enabling more efficient priming in the presence of protease inhibitors. Based on a previous study (53), Förster resonance energy transfer (FRET) peptides SARS1_WT and SARS2_WT, representing the S2' site of SARS-CoV and SARS-CoV-2, respectively, were prepared with DABCYL as a quencher at the N-terminus and EDANS as a fluorophore at the C-terminus (Figure 13A). In addition, FRET peptides carrying mutations identified in K11777 escape clones of SARS-CoV (SARS1_794/795) and SARS-CoV-2 (SARS2_811/812) were also synthesized (Figure 13A). Proteolytic cleavage and FRET signal development were assessed using recombinant cathepsin L. Treatment with cathepsin L resulted in a time-dependent increase in fluorescence, indicating cleavage of all four peptides (Figure 13B). The K_m values of cathepsin L demonstrated that SARS2_811/812 was cleaved more efficiently than SARS2_WT (Table 10). Similarly, SARS1_794/795 exhibited enhanced cleavage by cathepsin L (Table 10). These findings suggest that cathepsin L targets the S2' site of the viral S protein and that K11777-escape mutations enhance cleavage efficiency by cathepsin L.

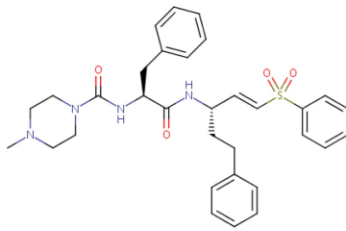
Synergistic antiviral activity of K11777 and camostat against SARS-CoV-2 *in vitro* and *in vivo*

Combining protease inhibitors targeting both the primary entry routes for SARS-CoV-2, the TMPRSS2-dependent direct fusion pathway and the cathepsin-dependent endocytic pathway, was expected to yield potent inhibitory effects against viral infection.

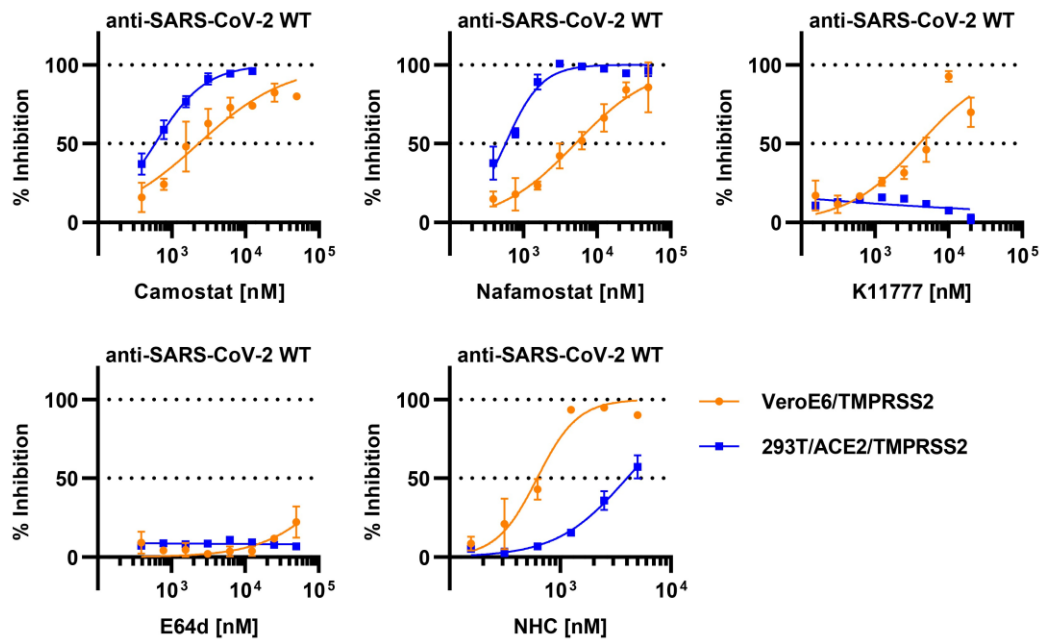
To evaluate the additive or synergistic effects, combinations of the cathepsin inhibitor K11777 and the TMPRSS2 inhibitor camostat were tested in SARS-CoV-2 infection using VeroE6/TMPRSS2 cells. In a cell-based assay, the combination of K11777 with camostat yielded a CI value of 0.55, indicating that the cathepsin inhibitor exerted a synergistic effect on SARS-CoV-2 infection when combined with the TMPRSS2 inhibitor *in vitro* (Figure 14A).

Combination therapy with K11777 and camostat was further evaluated in a hamster model infected with SARS-CoV-2. Animals were intranasally inoculated with SARS-CoV-2 and administered camostat, K11777 monotherapy, or combination therapy at the simultaneous time points following viral inoculation. NHC, a nucleotide analogue known to inhibit SARS-CoV-2 replication *in vitro* and *in vivo* (54), was included as a comparator. Camostat monotherapy (50 mg/kg, bid) reduced viral titers in the lungs, whereas K11777 monotherapy (50 mg/kg, bid) had no measurable impact on viral titers (Figure 14B). Combination therapy with multiple dosing regimens of camostat (10 or 30 mg/kg) and K11777 (15 or 50 mg/kg) was also tested. Notably, co-treatment with higher doses of camostat and K11777 resulted in approximately a 3-log reduction in lung viral titers, comparable to the effect observed with NHC monotherapy (300 mg/kg) (Figure 14B). These findings indicate that combination treatment with TMPRSS2 and cathepsin B/L inhibitors exerts a synergistic antiviral effect both *in vitro* and *in vivo*.

A



B



C

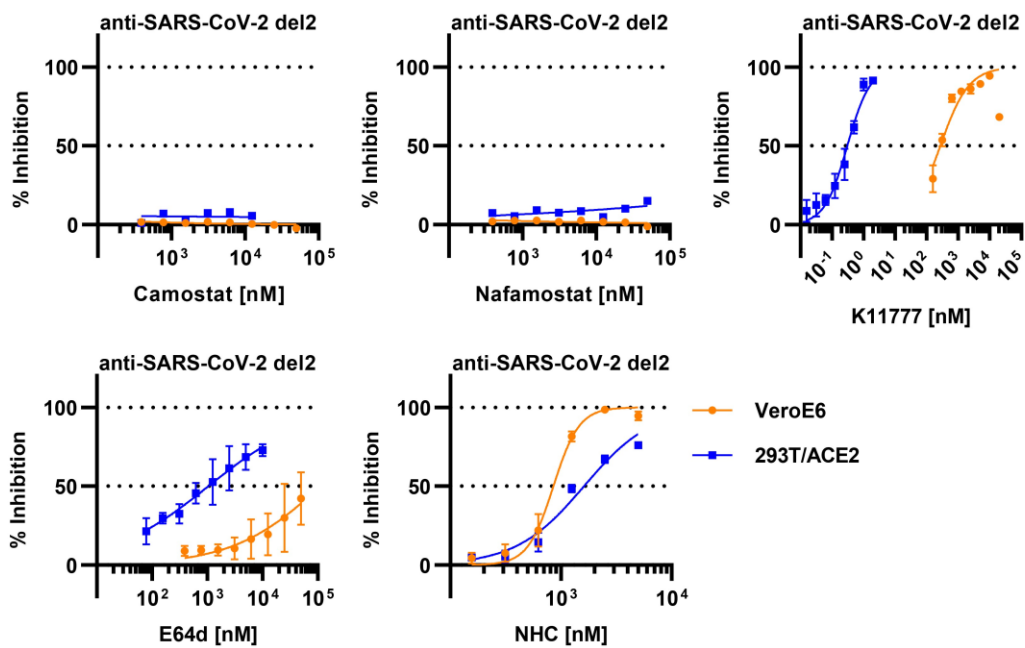


Figure 9. Antiviral activities of camostat, nafamostat, E64d, and K11777 against WT and del2 of SARS-CoV-2.

(A) The Chemical structure of K-11777. The cells were treated with serially diluted compounds (camostat, nafamostat, K11777, E64d, and NHC) and infected with the WT (B) and del2 (C) of SARS-CoV-2. The inhibition rate was assessed using an MTT assay at 2–3 dpi as described in the Material and Methods section. These data are representative of three independent experiments performed in duplicate.

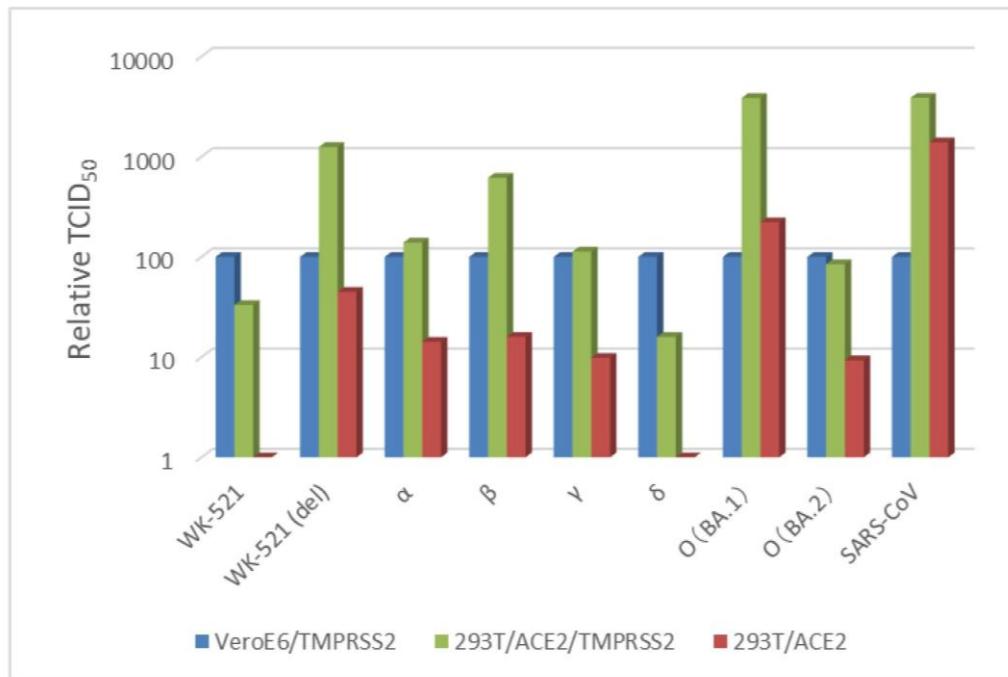


Figure 10. Comparison of virus proliferation.

Cells were inoculated with SARS-CoV-2 strains and SARS-CoV at an MOI of 0.1. Culture supernatants were harvested at 72 h after inoculation. Virus titration was performed by TCID₅₀ by MTT methods. The titer of each virus was expressed as a relative percentage divide by the TCID₅₀ value of VeroE6/TMPRSS2.

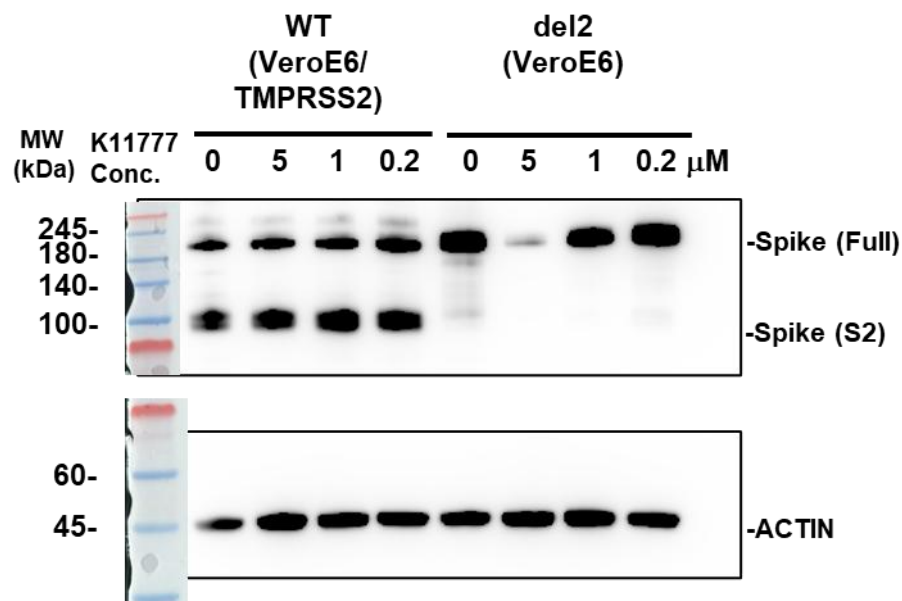


Figure 11. The effect of K11777 antiviral activity on the expression level of the S protein.

VeroE6/TMPRSS2 or VeroE6 cells were infected with WT or del2 mutant, respectively and incubated for 24 h in the presence of K11777. Expression of viral S proteins in VeroE6/TMPRSS2 or VeroE6 cells were detected by Western Blotting. The positions of molecular weight (MW) markers are indicated on the sides of the blots and are expressed in kilodaltons. The full-length S (ca. 180 kDa) and cleaved S2 proteins (ca. 100 kDa) are indicated. β -actin (ACTIN) was used as a loading control to ensure equal protein loading across lanes.

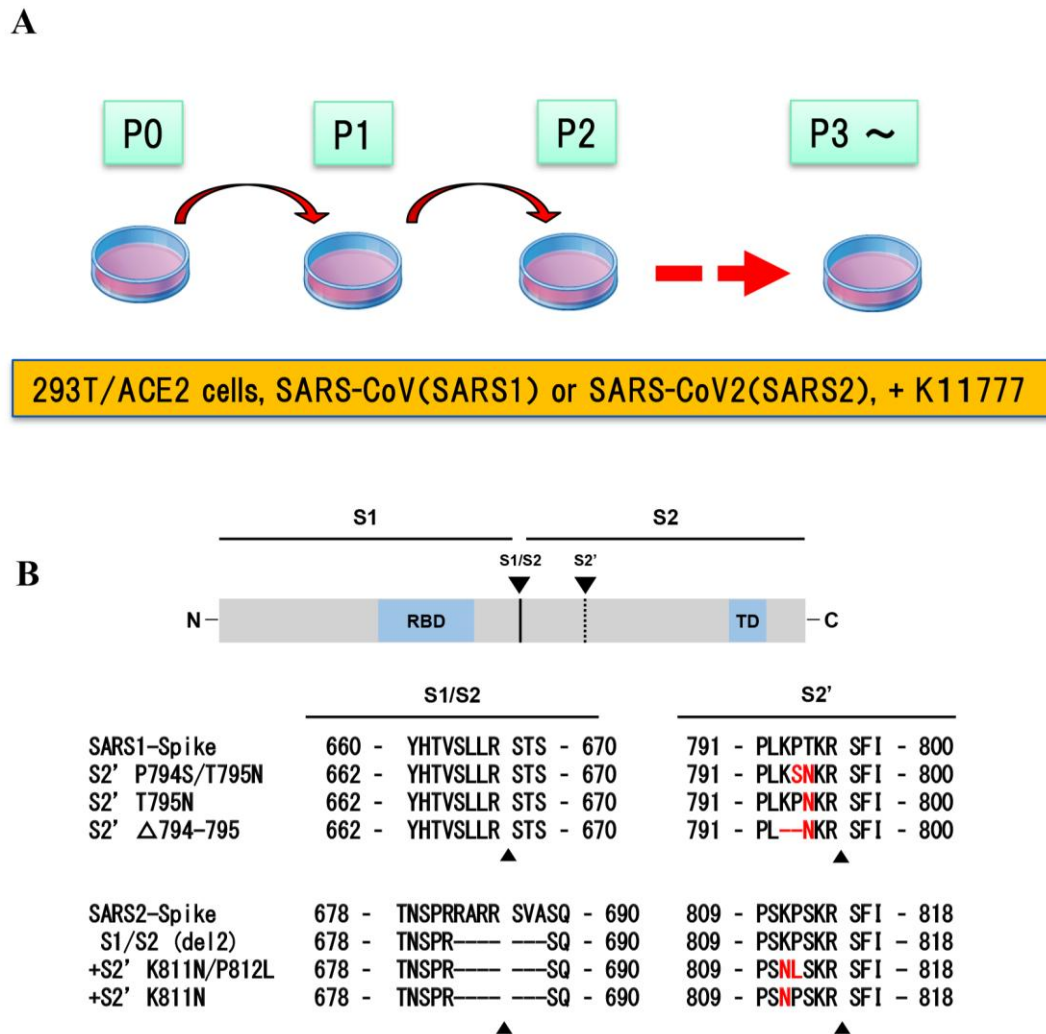
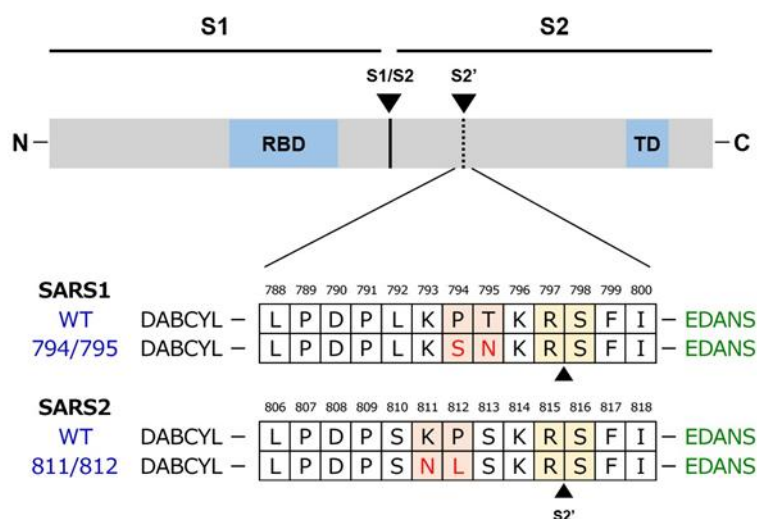


Figure 12. Isolation of K11777 escape viruses in SARS-CoV-2 (del) and SARS-CoV.

(A) Schematic representation of the experimental design of selection for K11777-resistant viruses. Viruses were propagated in 293T/ACE2 cells, and the supernatant-cell mixture was added to 293T/ACE2 cells treated with K11777 (passage 1: P1). Viruses were passaged every 2-3 days with K11777; after 31 days-culture, RNA from the supernatant was extracted for analysis of viral genome mutations using NGS. (B) Amino acid substitutions observed in each clone after cloning. The red letters indicate the amino acid mutations observed in the spike region.

A



B

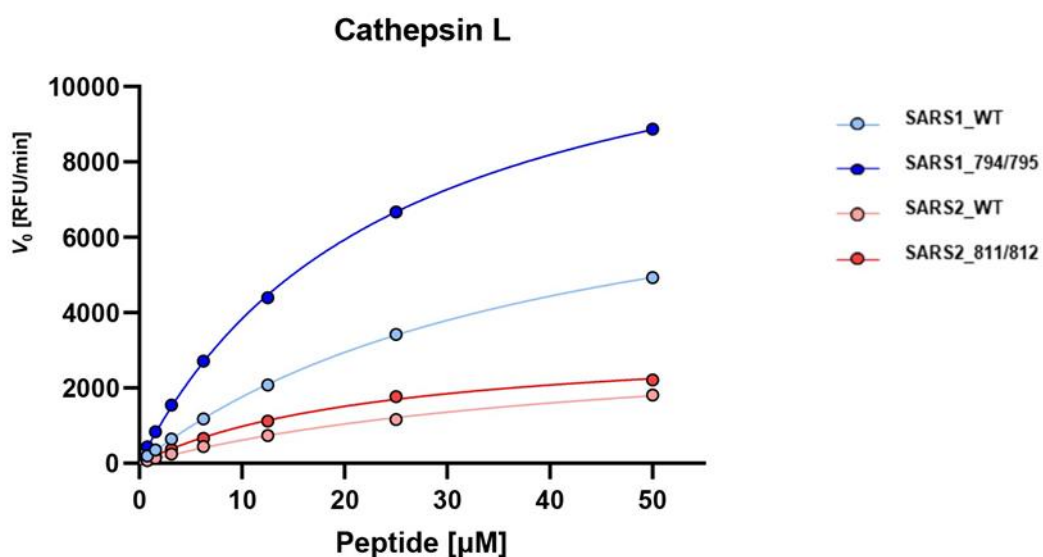


Figure 13. *In vitro* cleavage assay with fluorogenic peptides and cathepsin L.

(A) Schematic illustration of SARS-CoV and SARS-CoV-2 S protein including functional domains (RBD, receptor binding domain; TD, transmembrane domain) and two proteolytic cleavage sites (S1/S2, S2'). Arrow heads indicate the cleavage site. Amino acid substitution of K11777 mutants is represented in red color.

(B) Fluorogenic peptides mimicking the S2' cleavage sites of SARS-CoV (SARS1) and SARS-CoV-2 (SARS2) were incubated with recombinant cathepsin L. The fluorescence signal from peptides was measured using fluorometer and the V₀ values of each cleavage reaction were used to calculate the K_m values. The assays were performed two independent experiments. Data represent the average of four replicates from a single experiment.

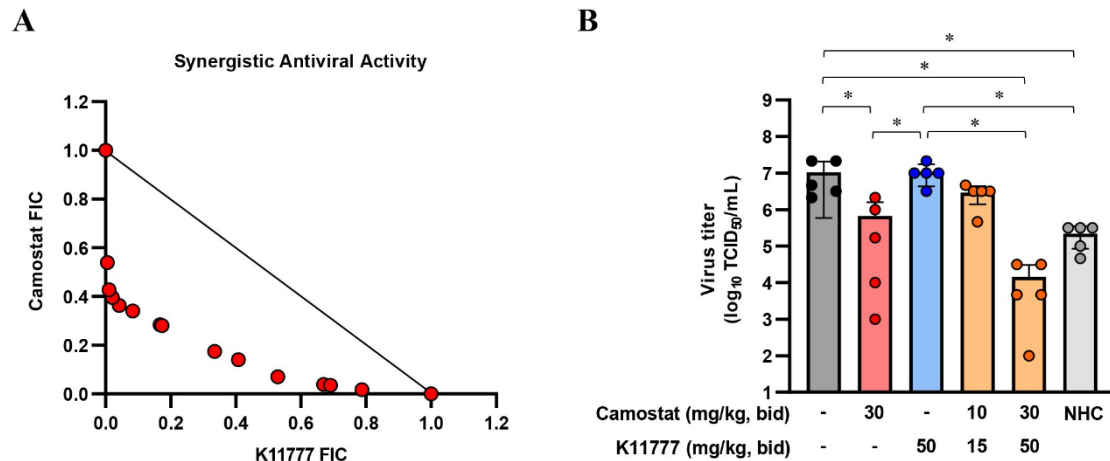


Figure 14. Synergistic antiviral activity of K11777 and camostat against SARS-CoV-2 *in vitro* and *in vivo*.

(A) *In vitro* combination assays with K11777 and camostat. The isobologram plot of K11777 combined with camostat, the EC₅₀ values of each substance alone and at a fixed concentration were determined. (DA/A + B)/DA and (DB/A + B)/DB were plotted on the x- and y-axes, respectively. DA is the EC₅₀ of substance A alone, DB is the EC₅₀ of substance B alone, DA/A + B is the concentration of substance A; given a 50% inhibition combined with substance B, DB/A + B is the concentration of substance B; given a 50% inhibition combined with substance A. These data are representative of three independent experiments performed in duplicate.

(B) *In vivo* combination assay with K11777 and camostat. The hamsters were intranasally inoculated with SARS-CoV-2 and treated with camostat, K11777 monotherapy or their combination at a three simultaneous time points from viral inoculation. At 1 dpi, whole lung tissues were harvested and homogenized in PBS and the supernatants were subjected to TCID₅₀ assays using VeroE6/TMPRSS2 cells for virus titration (n = 5/group). The values shown are means ± SD. The log-rank test was performed for comparing the survival times between each group (*p < 0.05).

Table 5. EC₅₀ (EC₃₀) values of protease inhibitors in SARS-CoV-2-, SARS-CoV-, MERS-CoV-infected cells.

Each value represents as the mean $\pm$ SD from three independent experiments. NC indicates the EC₅₀ could not be calculated.

Virus	Cell	EC ₅₀ (EC ₃₀) [nM]														
		Camostat			Nafamostat			K11777			E64d			NHC		
SARS-CoV-2 (WT)	VeroE6/TMPRSS2	3,173	±	1,318	10,302	±	3,167	9,973	±	1,109	> 50,000			497	±	56
	293T/ACE2/TMPRSS2	746	±	303	445	±	72	> 20,000			> 50,000			3,547	±	152
SARS-CoV-2 (del2)	VeroE6	> 50,000			> 50,000			216	±	23	> 50,000			871	±	138
	293T/ACE2	> 50,000			> 50,000			0.26	±	0.02	184	±	34	1,299	±	285
SARS-CoV	VeroE6/TMPRSS2	NC (4,841 ± 1,272)			NC (36,576 ± 11,303)			2,228	±	604	> 50,000			690	±	39
	293T/ACE2/TMPRSS2	NC (3,609 ± 524)			NC (2,797 ± 2,022)			> 20,000			> 50,000			3,546	±	401
	293T/ACE2	> 50,000			> 50,000			1.45	±	0.08	2,675	±	198	3,629	±	235
MERS-CoV	VeroE6/TMPRSS2	39,428	±	11,939	26,526	±	3,798	4,877	±	3,179	> 50,000			449	±	24
	293T/ACE2/TMPRSS2	8,286	±	3,711	4,201	±	2,462	> 20,000			> 50,000			5,194	±	1,024
	293T/DPP4	> 50,000			> 50,000			3.24	±	0.25	2,166	±	1,006	799	±	400

Table 6. EC₅₀ values of K11777 in SARS-CoV-2 variants.

Each value represents as the mean ± SD from three independent experiments. NT indicates not tested.

Virus	Strain	EC ₅₀ [nM]								
		VeroE6/TMPRSS2			VeroE6			293T/ACE2/TMPRSS2		293T/ACE2
SARS-CoV-2	WK-521 (WT)	9,973	±	1,109	NT			> 10,000		NT
	WK-521 (del2)	NT			393	±	335	> 10,000		0.25 ± 0.11
	α	9,037	±	82	758	±	64	> 10,000		0.30 ± 0.14
	β	9,528	±	102	225	±	1	> 10,000		0.21 ± 0.10
	γ	2,401	±	87	260	±	29	> 10,000		0.29 ± 0.12
	Δ	6,099	±	1,344	NT			> 10,000		NT
	O (BA-1)	3,030	±	1,893	817	±	242	> 10,000		0.42 ± 0.17
	O (BA-2)	2,493	±	734	999	±	127	> 10,000		0.55 ± 0.25

Table 7. Mutations identified in the genome of SARS-CoV-2.

Amino acid substitutions for drug escape mutants of SARS-CoV-2 del2 strain were determined by next generation sequencing. The Sequence variants with >20% frequency are listed and highlighted in a red gradient, with color intensity increasing proportionally to their frequency. Substitutions that reduce the susceptibility of SARS-CoV-2 to K11777 are highlighted in yellow. SNV indicates single nucleotide variant.

2019-nCoV_Japan_TY_WK-521_2020_LC522975								4962 escape viruses												WT(del2)	
Region	Mutation Type	Reference	Allele	ORF	Amino acid mutations	nsp	Amino acid mutations	1	2	3	4	5	6	7	8	9	10	11	12		
654	SNV	C	T	ORF1ab	A131V	nsp1	A131V					36	39								
3648	SNV	A	C	ORF1ab	Q1129P	nsp3	Q311P											35			
6891	SNV	G	T	ORF1ab	C2210F	nsp3	C1392F			29											
6924	SNV	C	T	ORF1ab	P2221L	nsp3	P1403L								24						
7345	SNV	T	G	ORF1ab	N2361K	nsp3	N1543K					23									
7372	SNV	T	G	ORF1ab	N2370K	nsp3	N1552K							44	37						
7445	SNV	A	C	ORF1ab	K2395Q	nsp3	K1577Q											64			
8067	SNV	T	A	ORF1ab	F2602Y	nsp3	F1784Y	86													
8241	SNV	A	C	ORF1ab	Q2660P	nsp3	Q1842P			45	32										
8869	SNV	G	T	ORF1ab	L2869F	nsp4	L106F				51	30	24								
9518	SNV	C	A	ORF1ab	L3086I	nsp4	L323I	100	100	100	100	100	100	100	100	100	100	100	100		
9690	SNV	C	T	ORF1ab	A3143V	nsp4	A380V	22													
11376	SNV	C	T	ORF1ab	A3705V	nsp6	A136V	24	24			37	39								
11457	SNV	C	T	ORF1ab	S3732F	nsp6	S163F	30	29												
11508	SNV	C	T	ORF1ab	T3749I	nsp6	T180I												75		
11519	SNV	T	G	ORF1ab	F3753V	nsp6	F184V	28	29			34	33								
12289	SNV	G	A	ORF1ab	M4009I	nsp8	M67I	24													
12834	SNV	T	C	ORF1ab	L4191S	nsp9	L51S							41	33						
16655	SNV	C	T	ORF1b	T1064I	nsp13	T141I												75		
20177	SNV	A	T	ORF1b	Q2238L	nsp15	Q187L			33											
21749	SNV	T	C	spike	W64R	spike	W64R												42		
21762	SNV	T	G	spike	I68R	spike	I68R												92		
21765	SNV	A	G	spike	H69R	spike	H69R												28		
23992	SNV	A	C	spike	K811N	spike	K811N	98	98	98	96	96	96	95	99	100	99				
23994	SNV	C	T	spike	P812L	spike	P812L	98	97		57	96	93								
24460	SNV	C	A	spike	S967R	spike	S967R								36						
26014	SNV	T	C	ORF3a	S209P	ORF3a	S209P							34							
26835	Deletion	A	-	membrane	Deletion	membrane	Deletion											97			
27906	SNV	T	A	ORF8	F6I	ORF8	F6I					2									
28251	Deletion	A	-	ORF8	Deletion	ORF8	Deletion	68	45		76	50	40	68	37	50	71	69	43		
23606..23626	Deletion	GGGACAGGTATTGTACCTAGT	-	spike	Deletion	spike	Deletion	25				29				34		26			
23607..23626	Deletion	GGGACAGGTATTGTACCTAGT	-	spike	Deletion	spike	Deletion		68	84	92		85	82	86		100		90		
27894..27907	Deletion	AAATTCTCTGTTT	-	spike	Deletion	spike	Deletion					65	74								

Table 8. Mutations identified in the genome of SARS-CoV.

Amino acid substitutions for drug escape mutants of SARS-CoV were determined by next generation sequencing. The Sequence variants with >20% frequency are listed and highlighted in a red gradient, with color intensity increasing proportionally to their frequency. Substitutions that reduce the susceptibility of SARS-CoV to K11777 are highlighted in yellow. SNV indicates single nucleotide variant.

SARS coronavirus Tor2, complete genome								4962 escape viruses						WT	
Region	Mutation Type	Reference	Allele	ORF	Amino acid mutations	nsp	Amino acid mutations	1	2	3	4	5	6	7	8
814	SNV	C	T	ORF1ab	R184C	nsp2	R4C	66	57	71	77	68	93	49	46
3350	SNV	G	A	ORF1ab	S1029N	nsp3	S211N						50		
3411	Deletion	A	-	ORF1ab	V1049G	nsp3	V231G								59
6458	SNV	A	G	ORF1ab	Q2065R	nsp3	Q1247R	41	37	36			74		
7919	SNV	C	T	ORF1ab	A2552V	nsp3	A1734V	99	100	99	100	100	99	99	99
9912	SNV	T	G	ORF1ab	N3216K	nsp4	N476K				55	31			
10000	SNV	A	G	ORF1ab	M3246V	nsp5	M6V				31		69		
10046	SNV	C	T	ORF1ab	T3261I	nsp5	T21I				51	31			
10481	SNV	A	C	ORF1ab	E3406A	nsp5	E166A				42				
11437	SNV	G	A	ORF1ab	V3725I	nsp6	V179I	41	27	35	53	59		84	81
17642	SNV	A	C	ORF1ab	Q5793H	nsp13	Q492H				52				53
17644	SNV	T	A	ORF1ab	I5794K	nsp13	I493K				52				52
20122	SNV	A	T	ORF1ab	E6620V	nsp15	E191V						71		
20709	SNV	A	T	ORF1ab	M6815L	nsp16	M41L								50
20711	SNV	G	C	ORF1ab	M6816I	nsp16	M41I								50
23220	SNV	G	T	spike	A577S	spike	A577S	99	100	100	100	100	100	100	100
23875	SNV	C	A	spike	T795N	spike	T795N	78	95	90	56	27	66		
24344	SNV	T	A	spike	N951K	spike	N951K					58			
24865	SNV	C	T	spike	P1125L	spike	P1125L	23			100			82	69
25177	SNV	C	T	spike	A1229V	spike	A1229V								
25298	SNV	A	G	X1,ORF3a	R11G	X1,ORF3a	R11G	100	100	100	100	100	100	100	100
25652	SNV	C	T	X1,ORF3a	L129F	X1,ORF3a	L129F					22	71		
26165	SNV	G	A	envelope	V17I	envelope	V17I	100	100	96	100	100	99	100	100
26226	SNV	T	G	envelope	L37R	envelope	L37R	100	100	100	99	100	100	98	99
27268	SNV	C	T	gap05	T3M	gap05	T3M		99	73	81	96	94		22
27453	SNV	A	G	X4,ORF7a	T61A	X4,ORF7a	T61A	97	100	85	75	57	99	98	96
27836	SNV	G	T	ORF8a	V20L	ORF8a	V20L							49	32
27875	SNV	G	T	ORF8b	K4N	ORF8b	K4N					37			
29653	SNV	A	C	3'UTR	K90Q	3'UTR	K90Q						28		
29710	SNV	G	A	3'UTR	A109T	3'UTR	A109T							48	63

Table 9. EC₅₀ values of K11777 and NHC in K11777 escape virus clones.

Each value represents as the mean $\pm$ SD from three independent experiments.

Virus	Mutation	EC ₅₀ [nM]					
		K11777			NHC		
SARS-CoV	WT	2.46	$\pm$	0.93	2,008	$\pm$	461
	P794S/T795N	20.35	$\pm$	14.26	1,387	$\pm$	40
	T795N	3.57	$\pm$	0.68	1,238	$\pm$	153
	deletion	249.47	$\pm$	101.13	1,191	$\pm$	56
SARS-CoV-2 (del2)	WT	7.73	$\pm$	1.38	1,081	$\pm$	88
	K811N/P812L	119.49	$\pm$	24.03	1,423	$\pm$	253
	K811N	92.93	$\pm$	7.51	1,570	$\pm$	141

Table 10. K_m values and V_{max} values of cathepsin L against SARS-CoV and SARS-CoV-2 peptides.

Each value represents as the mean $\pm$ SD from two independent experiments.

Cathepsin L		K_m [μ M]			V_{max} [RFU/min ⁻¹]		
SARS-CoV (SARS1)	WT	40.71	$\pm$	0.37	9,389.0	$\pm$	581.2
	794/795	25.22	$\pm$	1.19	14,136.5	$\pm$	1,359.8
SARS-CoV-2 (SARS2)	WT	44.32	$\pm$	2.04	3,370.5	$\pm$	87.0
	811/812	24.28	$\pm$	0.49	3,561.5	$\pm$	337.3

Discussion

The S protein requires processing by host cell proteases for activation, with TMPRSS2, cathepsin L, and furin-like proteases playing critical roles in SARS-CoV-2 entry. The present study demonstrated that K11777, a cathepsin L inhibitor, functions as a potent anti-SARS-CoV-2 agent by targeting pathways involving specific host proteases. Furthermore, cathepsin inhibitors exhibited antiviral activity against both SARS-CoV and MERS-CoV infections. The antiviral effect of K11777 was cathepsin-dependent and was markedly reduced against viruses utilizing a TMPRSS2-dependent entry pathway.

K11777 is a potent cathepsin inhibitor that blocks SARS-CoV and SARS-CoV-2 entry; however, the mechanism underlying its reduced antiviral activity in the presence of TMPRSS2 expression remains unclear (51, 55). In this study, amino acid substitutions within the S2' cleavage site of the S protein were identified in viruses passaged with K11777, suggesting that K11777 inhibits cathepsin-mediated S2' cleavage, thereby significantly impairing viral entry.

The mechanism of action of K11777 involves inhibition of cathepsins. Interestingly, both SARS-CoV-2 and SARS-CoV variants that escaped K11777 treatment harbored mutations within the S2 region of the S protein, which is normally cleaved by cathepsins. Notably, it has been reported that when HIV-1 is cultured in the presence of protease inhibitors, amino acid substitutions occur not only within the active site of the protease but also in the gag peptide region, which serves as the protease substrate. These mutations confer resistance to protease inhibitors during HIV-1 infection (56). Such substrate mutations are thought to enhance proteolytic efficiency by compensating for reduced enzyme activity or facilitating cleavage through alternative enzymes, thereby promoting viral replication. The identification of similar mutations in both SARS-CoV-2 and SARS-CoV supports the hypothesis that these changes improve proteolytic efficiency and contribute to viral fitness under selective pressure.

K11777 exhibited strong inhibitory activity against SARS-CoV-2 in 293T/ACE2 cells but showed reduced efficacy in VeroE6, VeroE6/TMPRSS2, and 293T/ACE2/TMPRSS2 cells. In contrast, camostat was more effective in VeroE6/TMPRSS2 and 293T/ACE2/TMPRSS2 cells, while its activity was limited in VeroE6 and 293T/ACE2 cells. Combination therapy with both compounds is anticipated to enhance antiviral activity across all cell types. In a hamster model, co-administration of K11777 and camostat demonstrated strong *in vivo* efficacy, whereas K11777 monotherapy had no observable effect. This synergistic effect is consistent with the *in vitro* findings.

Although several therapeutic agents for COVID-19 have been developed, SARS-CoV-2 and related coronaviruses continue to pose significant threats to global health and economies, with new variants emerging. These circumstances underscore the importance of developing antiviral compounds that are effective against future SARS-CoV-2 variants and potentially the next coronavirus pandemic. The present study demonstrates that host-targeted drugs, some of which are already in clinical use, exhibit strong antiviral activity. K11777 alone may provide only limited therapeutic benefit in humans, and forthcoming clinical trial results will be critical to clarify its efficacy. Importantly, these findings indicate that combining K11777 with TMPRSS2 inhibitors could yield substantial antiviral effects against SARS-CoV-2 infection. This suggests that combination therapies involving repurposed drugs or inhibitors targeting host factors essential for viral replication may play a pivotal role in combating emerging coronavirus infections. Such strategies will not only validate the therapeutic potential of K11777 but also provide valuable insights for pandemic preparedness.

Chapter III

Comprehensive analysis of resistance-nodulation-cell division superfamily (RND) efflux pumps from *Serratia marcescens*, Db10

Summary

The role of the resistance-nodulation-cell division (RND) efflux system in intrinsic multidrug resistance in *Serratia marcescens* (*S. marcescens*) was investigated. Eight putative RND efflux system genes were identified in the *S. marcescens* Db10 genome, including the previously characterized systems *sdeXY*, *sdeAB*, and *sdeCDE*. Six of these eight genes were found to confer multidrug resistance on KAM32, a drug-hypersensitive strain of *Escherichia coli* (*E. coli*). Five of the eight genes conferred resistance to benzalkonium, suggesting the importance of RND efflux systems in biocide resistance in *S. marcescens*. Energy-dependent efflux activities of five pumps were examined using a rhodamine 6G efflux assay. When the RND efflux system genes were expressed in the *tolC*-deficient strain of *E. coli* (KAM43), which lacks the outer membrane protein TolC required for efflux, none of the genes conferred resistance. However, when *hasF*, encoding the *S. marcescens* TolC ortholog, was co-expressed in KAM43, all genes conferred resistance, indicating that HasF serves as a major outer membrane protein utilized by all RND efflux systems in *S. marcescens*. Additionally, an *sdeXY* deletion mutant was constructed from a derivative of a clinically isolated multidrug-resistant *S. marcescens* strain, and this mutant was found to be sensitive to a broad spectrum of antimicrobial agents.

Introduction

S. marcescens is classified as Gram-negative bacteria and is widely distributed across diverse environmental habitats. Initially regarded as a non-pathogenic organism, it is now associated with sporadic healthcare-associated outbreaks. Clinical isolates exhibiting significant resistance to β -lactams, aminoglycosides, and fluoroquinolones have been identified, making antimicrobial treatment for *S. marcescens* infections an increasingly challenging task (57, 58).

RND efflux systems are recognized as major determinants of multidrug resistance in Gram-negative bacteria (59–65). Each RND-type system consists of an inner membrane protein (IMP), a periplasmic membrane fusion protein (MFP), and an outer membrane protein (OMP). The driving force for drug efflux with RND efflux systems is generally attributed to the proton electrochemical gradient across the cell membrane. Three RND efflux systems, SdeXY (66), SdeAB (67), and SdeCDE (67), have been characterized in *S. marcescens*. SdeXY was the first multidrug efflux pump to be identified and exhibited broad substrate specificity when expressed in *E. coli* (66). Its expression was upregulated in clinical isolates resistant to tigecycline (68), and gene disruption in the environmental strain *S. marcescens* NCTC10211 increased susceptibility to tigecycline, tetracycline, ciprofloxacin, and ceftiofur. SdeAB also displayed broad substrate specificity in *E. coli* (67), and knockout studies demonstrated its contribution to intrinsic multidrug resistance (69). In a cetylpyridinium chloride resistant mutant of *S. marcescens* showed increased *sdeAB* expression and conferred resistance to fluoroquinolones, tetracycline, chloramphenicol, and benzalkonium chloride (70). In contrast, SdeCDE exhibited narrow substrate specificity, conferring resistance only to novobiocin (71).

This study identified additional RND efflux systems in *S. marcescens* that may confer multidrug resistance. The putative RND efflux genes from strain Db10 were examined, and their substrate profiles were evaluated using the hyper-susceptible *E. coli* strain, KAM32 (72). This analysis identified three uncharacterized RND efflux systems with broad substrate specificity. Mutagenesis experiments confirmed that SdeXY plays a role in intrinsic multidrug resistance.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in the present study are listed in Table 11. Unless otherwise noted, bacterial cells were grown in Luria (L) medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, [pH 7.0]) at 37 °C under aerobic conditions. Antibiotics were supplemented when required as follows: ampicillin, 100 µg/ml; chloramphenicol, 20 µg/ml.

Phylogenetic tree of the IMPs

Entire sequences of IMP were obtained from several databases. The phylogenetic tree was obtained using CLUSTALW (<https://clustalw.ddbj.nig.ac.jp>).

Ethical statement

All experiments were authorized by the Biosafety Management Committee on Research Pathogens and Hazardous Agents and the Safety Management Committee on Recombinant DNA Experiments, Okayama University.

Cloning, sequencing, and gene manipulation

Putative genes encoding RND efflux systems in *S. marcescens* were identified using the *S. marcescens* Db11 genomic sequence database (<http://www.sanger.ac.uk/resources/downloads/bacteria/serratia-marcescens.html>). DNA fragments containing the open reading frames (ORFs) of *S. marcescens* RND efflux system genes or the OMP gene *hasF* (SMA3509) were amplified by PCR using chromosomal DNA from *S. marcescens* Db10, the parental strain of Db11 (73), as the template. Primers employed for cloning are listed in Table 12, and each primer incorporated a restriction enzyme recognition site described by underlines.

The PCR products obtained were digested with the specified restriction enzymes, gel-purified, and subsequently ligated into the corresponding restriction sites of the vectors pUC18, pUC19, or pSTV28 (for *hasF*), positioned downstream of the lac promoter. Because the PCR products did not include the promoter region of the genes, expression was driven by the lac promoter. Cloning of *sdeCDE* (SMA2945–2946–2947) was performed in two steps. The 5' half fragment was amplified using two primers (SMA2945-2947F fw EcoRI and SMA2945-2947F re BamHI), and the 3' half fragment was amplified using two primers (SMA2945-2947B fw EcoRI and SMA2945-2947B re BamHI). After digestion with EcoRI and BamHI, each fragment was individually inserted

into pUC18, generating plasmids designated as pURS4F and pURS4B. Following digestion of pURS4B with MluI and BamHI, the fragment was inserted into pURS4F at the same sites, resulting in the plasmid named pURS4. Additionally, the plasmid pURS44, carrying *sdeC* and *sdeD* but lacking *sdeE*, was constructed by digesting pURS4 with NcoI and self-ligating. Similarly, the plasmid pURS45, carrying *sdeC* and *sdeE* but lacking *sdeD*, was generated by digesting pURS4 with Tth111I, blunting the ends, and self-ligating.

To evaluate the function of OmsA, which is located downstream of *sdePQ* (SM1743–1742), two types of plasmids were constructed. A fragment containing *sdePQ-omsA* was amplified by PCR using primers SMA1743-1741 fw XbaI and SMA1743-1741 re EcoRI. Following digestion with XbaI and EcoRI, the fragment was inserted into pUC19 at the corresponding sites, generating the plasmid designated as pURS8. To construct pURS82, which carries an incomplete *omsA*, pURS8 was digested with HpaI and self-ligated.

Because *S. marcescens* exhibited high β -lactam resistance, the plasmid pURS2 carrying *sdeXY* could not be utilized. To complement *sdeXY*, an alternative plasmid, pSMXY, was constructed. PCR was initially performed using two primers, SMA0370-0369 fw EcoRI and SMA0370-0369 re XbaI, with the Db10 genome as the template. A second PCR was then carried out using two different primers, SMA0370-0369 fw EcoRI and SMA0370-0369 re BglII, to introduce a BglII site in place of the XbaI site. The resulting PCR products were digested with EcoRI and BglII, gel-purified, and subsequently ligated into the EcoRI–BamHI sites of the pSTV28 vector.

Minimum inhibitory concentrations (MICs)

The MICs of various antimicrobial agents were assessed in Muller-Hinton broth [Difco (Franklin Lakes, NJ, USA)] using the standard two-fold dilution method as previously described (74).

Construction of the *sdeXY* deletion strain

S. marcescens KS24, a plasmid pSMC2-cured derivative of *S. marcescens* KS3 (75), was used to construct an *sdeXY* deletion strain. The *sdeXY* deletion strain of *S. marcescens* KS24 was generated by homologous recombination using the lambda Red recombinase system (76). Two-step PCR amplification of the gentamicin resistance cassette flanked by long (1,000 nt) homologous extensions of the target gene was performed as previously described (77), employing pBRFRTGM and genomic DNA from *S. marcescens* KS24 as the template, along with primers listed in Table 12. The resulting

PCR product was separated by agarose gel electrophoresis and purified using the GENECLAN II Kit [MP Biomedicals Inc. (Santa Ana, CA, USA)]. Mutant strains lacking *sdeXY* were obtained by electroporation of the purified PCR product into *S. marcescens* KS24/pKD46, as described previously (76). Deletion of *sdeXY* in the mutant strain was confirmed by PCR.

Measurements of rhodamine 6G and ethidium efflux activities

The efflux of rhodamine 6G and ethidium was assessed as described previously (65, 78). For the rhodamine 6G efflux assay, *E. coli* KAM32 strains were cultured in L medium until reaching an optical density of $O.D_{650} = 0.7$. Cells were harvested by centrifugation, washed twice with potassium phosphate buffer (PPB) containing 5 mM $MgSO_4$, and resuspended in the same buffer supplemented with 1 μM rhodamine 6G and 40 μM carbonylcyanide-m-chlorophenylhydrazone (CCCP). The cell suspension was incubated at 37 °C for 1 h to de-energize the cells, washed twice with PPB lacking CCCP, and resuspended in the same buffer. The final suspension was incubated on ice for 2 h and subsequently used for the efflux assay.

For the ethidium efflux assay, *S. marcescens* KS24 and *S. marcescens* KS24 $\Delta sdeXY$ strains were cultured in L medium until reaching an optical density of $O.D_{650} = 0.7$. Cells were harvested by centrifugation ($3,000 \times g$), washed twice with modified Tanaka buffer (34 mM KH_2PO_4 , 64 mM K_2HPO_4 , 20 mM $(NH_4)_2SO_4$, 0.3 mM $MgSO_4$, 1 μM $FeSO_4$, 1 μM $ZnCl_2$, 10 μM $CaCl_2$, and 2 mM $MgSO_4$, [pH 7.0]) (79), and resuspended in the same buffer. The resulting cell suspension was incubated at 37 °C for 5 min in the presence of 20 mM lactate-tetramethylammonium hydroxide (pH 7.0) and subsequently used for the efflux assay.

Results

Cloning of putative RND-type efflux pumps from *S. marcescens*

At the initiation of this study, the *S. marcescens* Db11 genomic sequence database (<http://www.sanger.ac.uk/resources/downloads/bacteria/serratia-marcescens.html>) was the only publicly available resource for the genomic sequence of this bacterium. Using the Db11 genomic sequence, RND-type efflux systems were searched within the *S. marcescens* genome, and eight RND-type efflux systems were identified (Figure 15). Five novel identified putative RND-type efflux systems, designated as shown in Figure 15, were characterized in this study, in addition to three previously described systems: SdeXY (SMA0370–0369) (66), SdeAB (SMA1197–1196) (67), and SdeCDE (SMA2945–2946–2947) (67). The putative OMP gene *omsA* was located adjacent to *sdePQ*, whereas the other RND efflux systems lacked an adjacent OMP gene. All RND efflux system genes contained a periplasmic MFP gene, except for *sdeS*. Notably, SdeCDE included two IMP genes, *sdeD* and *sdeE*.

A dendrogram analysis of the complete sequences of IMPs from *S. marcescens*, *E. coli*, *Vibrio parahaemolyticus* (*V. parahaemolyticus*), *Vibrio cholerae* (*V. cholerae*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Acinetobacter baumannii* (*A. baumannii*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) was performed, revealing that the IMPs from these organisms were classified into five distinct groups (Figure 16). In contrast to *E. coli*, which lacked IMPs in Groups 2 and 3, each group contained at least one IMP from *S. marcescens*, indicating that *S. marcescens* possesses a broad diversity of RND efflux systems.

All *S. marcescens* RND efflux system genes from the Db10 strain, the parental strain of Db11, were cloned and expressed in the drug-hypersensitive *E. coli* strain KAM32 for further characterization.

Substrate specificities of *S. marcescens* RND efflux systems

To assess the substrate specificity of each RND efflux system, the MICs of various antimicrobial agents were determined using *E. coli* KAM32 strains expressing each RND efflux system gene(s) (Table 13).

Many RND efflux systems in Group 1 play a major role in intrinsic multidrug resistance due to their broad substrate specificities (59, 66, 80–88). Group 1 also includes RND efflux system genes that are not organized in an operon with an MFP gene (e.g., *acrD* from *E. coli*) (Figure 16). Such RND efflux systems generally exhibit narrow substrate specificity, and SdeS conferred resistance only to erythromycin, novobiocin,

SDS, and deoxycholate. Consistent with previous findings (66), resistance to a broad spectrum of antimicrobial agents was conferred when the KAM32 strain expressed *sdeXY* (Table 13). Furthermore, when *sdePQ* was expressed in the KAM32 strain together with the adjacent OMP gene *omsA*, multidrug resistance against several antimicrobial agents was observed.

The RND efflux systems classified into Group 2 exhibit relatively broad substrate specificities and generally confer acquired resistance (60, 67, 69, 70, 89–91). SdeB was categorized within this group and conferred multidrug resistance to *E. coli* KAM32; however, the substrate specificity of SdeAB was not as extensive as that of SdePQ-OmsA or SdeXY (Table 13). The amino acid sequence of SdeB showed similarity to that of the *P. aeruginosa* RND efflux pump MexF. Comparable to MexEF (90), fluoroquinolones and chloramphenicol were identified as good substrates for SdeAB. Consistent with previous findings (67), resistance to the quaternary ammonium compound benzalkonium chloride was observed when the KAM32 strain expressed *sdeAB*.

The RND efflux systems classified within Group 3 exhibit relatively broad substrate specificities and are associated with acquired resistance (60, 92–95). Among the RND IMPs of *S. marcescens*, only SdeH has been assigned to this group. Expression of the *sdeGH* operon in *E. coli* KAM32 was found to confer multidrug resistance. The substrate profile of SdeGH was broader than that of SdeAB, yet it did not resemble those of SdeXY or SdePQ-OmsA. Although SdeH shares amino acid sequence similarity with MexI and MexW, the substrate specificity of SdeGH was not comparable to that of MexHI (95) or MexVW (93).

The *sdeCDE* operon contains two inner membrane protein (IMP) genes, and both SdeD and SdeE have been classified into Group 4. As previously reported (71), novobiocin is the sole substrate for SdeCDE (Table 13). To determine whether both IMP genes are essential for this efflux system, plasmids expressing either *sdeCD* or *sdeCE* were constructed. Neither plasmid conferred novobiocin resistance in *E. coli* KAM32 (Table 13), indicating that both SdeD and SdeE are required for novobiocin resistance. This phenotype resembles other Group 4 RND efflux systems, such as MdtABCD from *E. coli* (96) and MuxABC from *P. aeruginosa* (97).

SdeJ and SdeO have been classified into Group 5. Although Group 5 includes numerous *Vibrio* RND efflux systems that exhibit relatively broad substrate specificities (60, 61, 92, 94), expression of *sdeIJ* conferred resistance only to benzalkonium and rhodamine 6G, whereas *sdeNO* expression conferred resistance exclusively to SDS. TriC and MexK from *P. aeruginosa* have been reported to contribute to triclosan resistance (98,

99). However, introduction of *sdeIJ* and *sdeNO* did not confer triclosan resistance to the *E. coli* host.

Efflux of rhodamine 6G was assessed to evaluate the activity of each RND efflux system, as rhodamine 6G serves as an effective substrate for most *S. marcescens* RND systems (SdeXY, SdeAB, SdeGH, SdeIJ, and SdePQ-OmsA) (Figure 17). All five systems exhibited enhanced rhodamine 6G efflux when lactate was supplied as the energy source, demonstrating that rhodamine 6G efflux is energy-dependent.

Requirement of OMP

With the exception of *sdePQ-omsA*, the remaining eight *S. marcescens* RND efflux systems lack an adjacent OMP gene. Consequently, these systems must utilize *E. coli* OMP(s) when expressed in *E. coli*. TolC is the principal OMP in *E. coli*, and all native *E. coli* RND efflux systems require TolC for their function (100). To determine whether *S. marcescens* RND efflux systems utilize TolC when expressed in *E. coli*, the corresponding genes were introduced into *E. coli* KAM43, a *tolC*-deficient strain, and antimicrobial susceptibility was assessed by measuring MICs. With the exception of *sdePQ-omsA*, none of the *S. marcescens* RND efflux systems exhibited increased MICs in KAM43, indicating that these systems require TolC for activity when expressed in *E. coli* (Table 14).

To determine whether SdePQ employs TolC as its outer membrane component, *sdePQ* was subcloned and expressed in *E. coli* KAM32 and KAM43. When expressed in KAM32, *sdePQ* increased MICs for several antimicrobial agents, similar to KAM32 expressing *sdePQ-omsA*, except for SDS (Table 13). In contrast, expression in KAM43 did not result in any MIC elevation. These findings indicate that TolC can be utilized by SdePQ in the absence of OmsA; however, this interaction appears weaker than that observed with OmsA (Table 14).

S. marcescens is known to possess HasF, a functional ortholog of TolC. Previous studies have demonstrated that SdeAB and SdeXY utilize HasF as their outer membrane component (68, 69). Since TolC may be employed by all *S. marcescens* RND efflux systems, it was hypothesized that the remaining systems also utilize HasF as the OMP. To test this hypothesis, the *hasF* gene was cloned and co-expressed with *S. marcescens* RND efflux systems in the KAM43 strain. Consistent with previous findings, *sdeXY* and *sdeAB* both conferred increased MICs when expressed with HasF in *E. coli* KAM43 (Table 14). When co-expressed with HasF, all *S. marcescens* RND efflux systems exhibited elevated MICs, indicating that HasF can serve as the OMP for these systems. Comparison of Table 13 with Table 14 revealed that SdeAB-HasF and SdeNO-HasF conferred higher MICs

than SdeAB-TolC and SdeNO-TolC, whereas SdeIJ-HasF conferred lower MICs than SdeIJ-TolC. These findings suggest that compatibility between IMP and/or MFP and OMP is a critical determinant of efflux efficiency.

Introduction of *sdeS* into KAM43 or KAM43/pSOS2 did not result in increased MICs for any of the tested antimicrobial agents (Table 14). In contrast, expression in KAM32 led to elevated MICs for novobiocin, SDS, and deoxycholate (Table 13). Since no MIC increase was observed in KAM33, an *acrA* disruptant of KAM32, it is suggested that SdeS utilizes AcrA as the MFP in *E. coli* (Table 15).

The *sdeXY* deletion mutant of *S. marcescens* is susceptible to a broad range of antimicrobial agents

Since the present findings indicated that SdeXY possesses the broadest substrate specificity among the characterized *S. marcescens* efflux pumps (66), an *sdeXY* mutant strain was constructed from *S. marcescens*. An attempt was made to generate the deletion strain from Db10; however, this was unsuccessful for unknown reasons. Consequently, another strain, KS24, a derivative of the clinically isolated strain SM39 (101), was utilized. The *sdeXY* deletion strain of KS24 exhibited increased susceptibility to a broad spectrum of antimicrobial agents compared with the parental strain (Table 16). Furthermore, the mutant strain demonstrated reduced energy-dependent efflux of ethidium (Figure 18). These observations indicate that SdeXY functions as a major RND efflux pump conferring intrinsic resistance to *S. marcescens* against multiple antimicrobial agents.

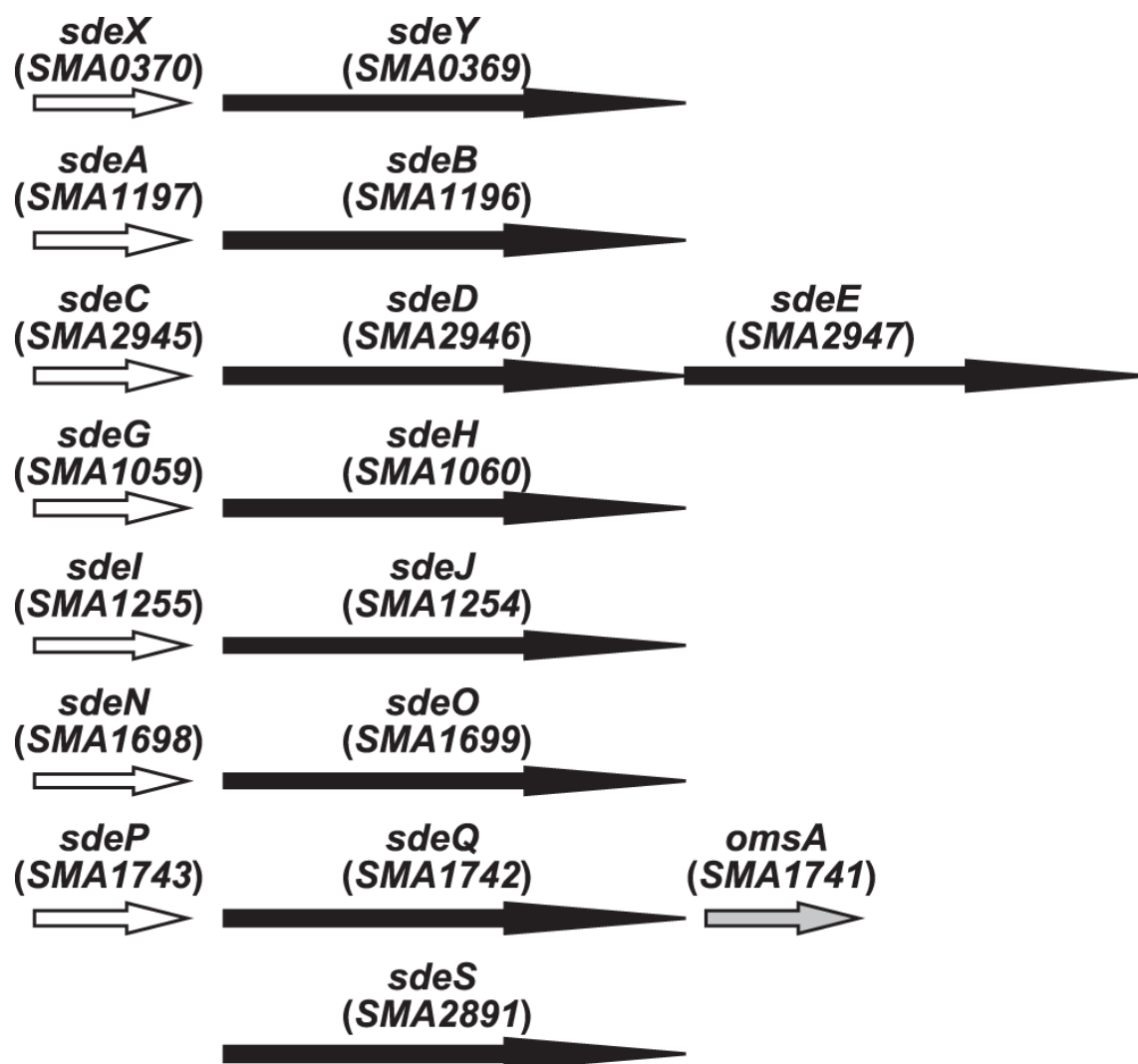


Figure 15. Putative *S. marcescens* RND efflux systems in Db10.

White arrow; gene for the periplasmic membrane fusion protein, Black arrow; gene for the inner membrane protein, Gray arrow; gene for the outer membrane protein.

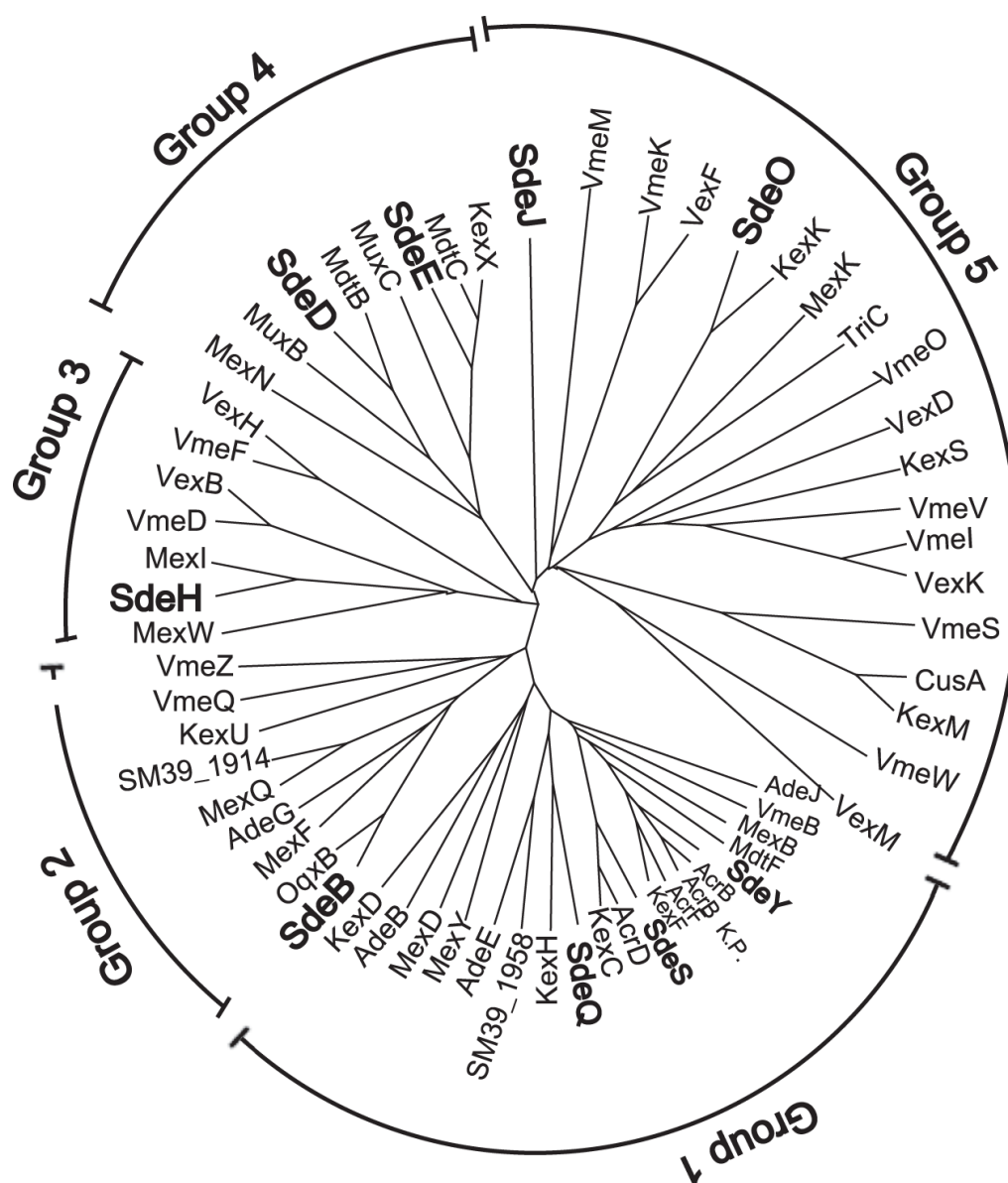


Figure 16. Unrooted phylogenetic tree of the IMP of RND efflux pumps.

The phylogenetic tree was obtained using CLUSTALW (<https://clustalw.ddbj.nig.ac.jp>). *E. coli*: AcrB, AcrD, AcrF, MdtB, MdtC, MdtF, and CusA. *V. parahaemolyticus*: VmeB, VmeD, VmeF, VmeI, VmeK, VmeM, VmeO, VmeQ, VmeS, VmeW, VmeV, and VmeZ. *V. cholerae*: VexB, VexD, VexF, VexH, VexK, and VexM. *S. marcescens*: SdeB, SdeD, SdeE, SdeY, SdeH, SdeJ, SdeO, SdeQ, SdeS, SM39_1914, and SM39_1958. *A. baumannii*: AdeB, AdeE, AdeG, and AdeJ. *P. aeruginosa*: MexD, MexF, MexI, MexK, MexN, MexQ, MexW, MexY, MuxB, MuxC, and TriC. *K. pneumoniae*: AcrB(K.P.), KexD, OqxB, KexC(KPN_RS15040), KexF(KPN_RS19875), KexH(KPN_RS21805), KexK(KPN_RS11560), KexM(KPN_RS25535), KexS(KPN_RS04245), KexU(KPN_RS03035), KexW(KPN_RS13595), and KexX(KPN_RS13600).

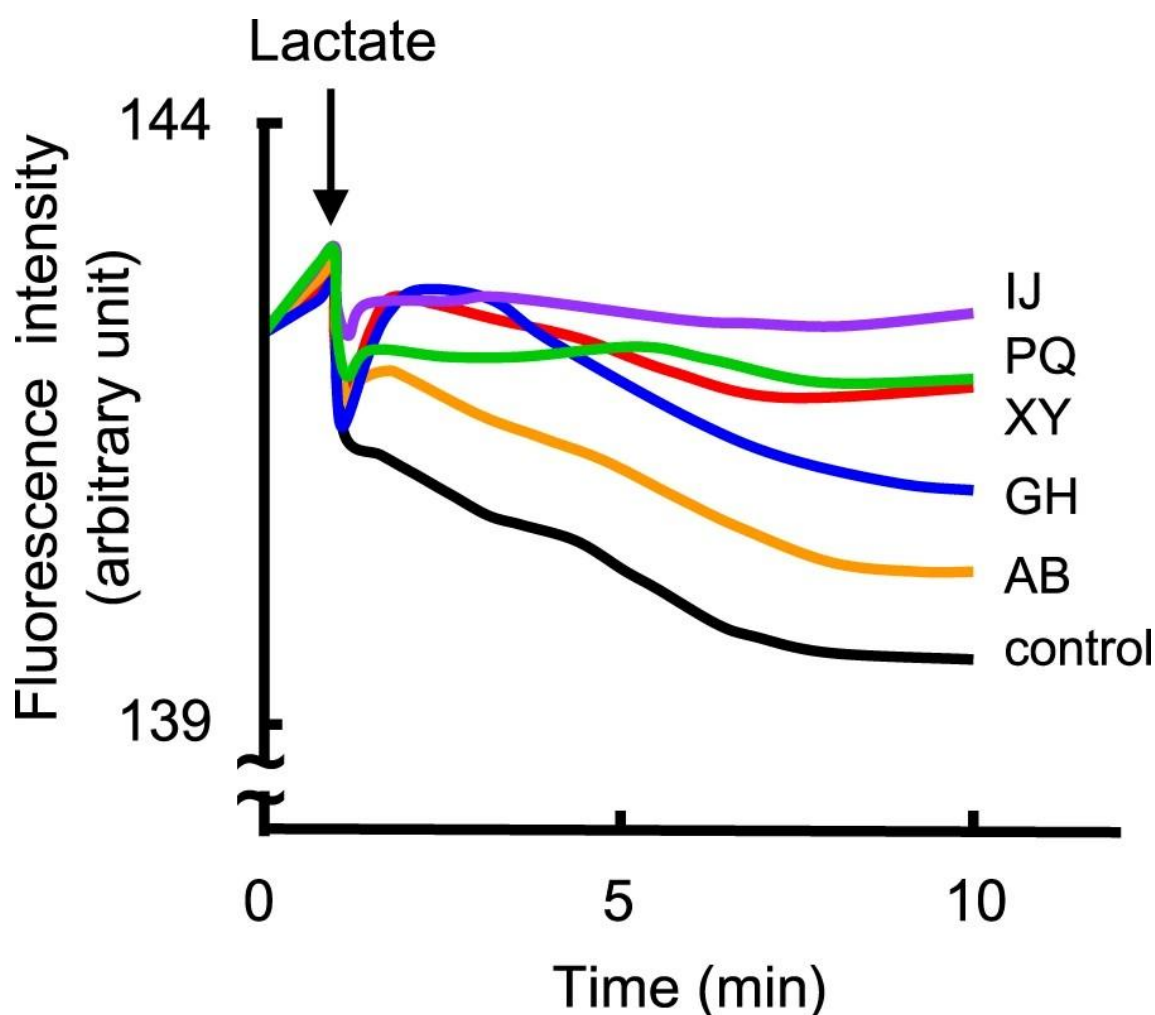


Figure 17. Rhodamine 6G efflux assay.

Energy-starved cells of *E. coli* KAM32 strains that express *S. marcescens* RND efflux systems were prepared as described in the Materials and Methods. Energy-starved cells were resuspended in PBS containing 5 mM MgSO_4 and 1 μM rhodamine 6G. At the time point indicated by the arrow, 20 mM potassium lactate (K-Lactate) was added to energize cells. Intracellular rhodamine 6G levels were monitored continuously by measuring the fluorescence of rhodamine 6G at excitation and emission wavelengths of 529 and 553 nm, respectively. IJ; *E. coli* KAM32/pURS6 (*sdeIJ*), PQ; *E. coli* KAM32/pURS8 (*sdePQ-omsA*), XY; *E. coli* KAM32/pURS2 (*sdeXY*), GH; *E. coli* KAM32/pURS5 (*sdeGH*), AB; *E. coli* KAM32/pURS3 (*sdeAB*), control; *E. coli* KAM32/pUC19.

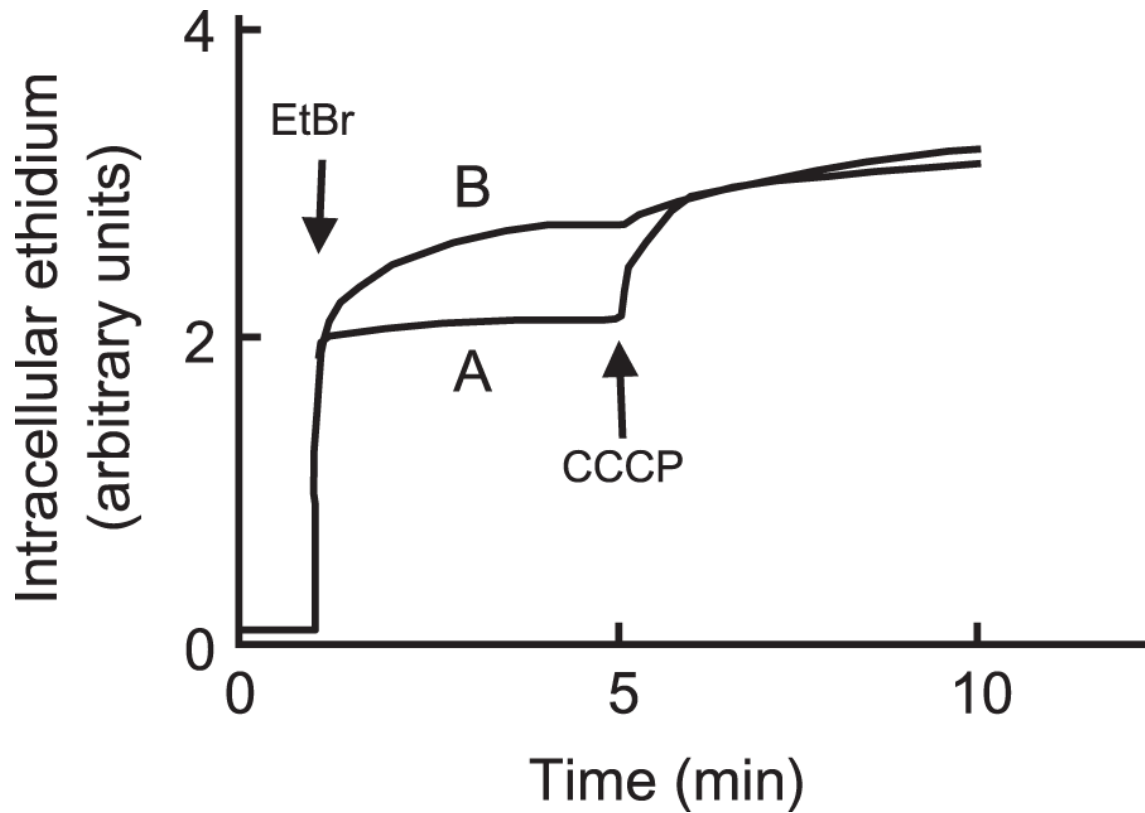


Figure 18. Ethidium efflux activity in *S. marcescens* cells.

The cells of the *S. marcescens* KS24 strain (A) and its KS24 Δ *sdeXY* (B) were prepared as described in the Materials and Methods. Ethidium bromide was added to cell suspensions at a final concentration of 10 μ M at the time point indicated by the first downward arrow. Intracellular ethidium levels were monitored continuously by measuring the fluorescence of ethidium at excitation and emission wavelengths of 500 and 580 nm, respectively. At the second downward arrow, CCCP was added to the suspensions at a final concentration of 100 μ M. Assays were performed at 37 °C.

Table 11. Bacterial strains and plasmids used in the present study.

Strain/plasmid	Description
<i>E. coli</i>	
KAM32	TG1, $\Delta acrB$, $\Delta ydhE$
KAM43	TG1, $\Delta acrAB$, $\Delta ydhE$, $\Delta tolC$
<i>S. marcescens</i>	
Db10	-
KS3	pSMC1-cured derivative of SM39
KS24	pSMC2-cured derivative of KS3
KS24 $\Delta sdeXY$	<i>sdeXY</i> deletion strain from KS24
Plasmid	
pUC18	Cloning vector, amp ^r
pUC19	Cloning vector, amp ^r
pSTV28	Cloning vector, Cm ^r
pBRFRTGM	pBR322 carrying the gentamicin resistance gene and FRT cassette
pKD46	Temperature-sensitive plasmid carrying lambda Red genes (<i>exo</i> , <i>bet</i> , <i>gam</i>)
pURS2	<i>sdeXY</i> ORF and promoter region cloned into pUC19
pURS3	<i>sdeAB</i> ORF and promoter region cloned into pUC19
pURS4	<i>sdeCDE</i> ORF and promoter region cloned into pUC19
pURS44	<i>sdeCD</i> ORF and promoter region cloned into pUC19
pURS45	<i>sdeCE</i> ORF and promoter region cloned into pUC19
pURS5	<i>sdeGH</i> ORF and promoter region cloned into pUC19
pURS6	<i>sdeIJ</i> ORF and promoter region cloned into pUC19
pURS7	<i>sdeNO</i> ORF and promoter region cloned into pUC19
pURS8	<i>sdePQ-omsA</i> ORF and promoter region cloned into pUC19
pURS82	<i>sdePQ</i> ORF and promoter region cloned into pUC19
pURS9	<i>sdeS</i> ORF and promoter region cloned into pUC19
pSOS2	<i>hasF</i> ORF cloned into pSTV28
pSMXY	<i>sdeXY</i> ORF and promoter region cloned into pSTV28

Table 12. Primers used in the present study.

The recognition site of each restriction enzyme was underlined.

SMA0370-0369 fw EcoRI	CGGAATTCGCACATATAATTAACGCAGCAATGG
SMA0370-0369 re XbaI	GTCTAGAGCATTAAAGCAACTGAAGCAAAAGAA
SMA0370-0369 re BglII	CAGAGATCTGCATTAAGCAACTGAAGCAAAAGAA
SMA1197-1196 fw XbaI	ATATCTAGAGGGAAAGCCCGGAAAACAGGG
SMA1197-1196 re EcoRI	CGAATTCGCCTGCTGGTATTTCAAGTCGG
SMA2945-2947F fw EcoRI	GGGAATTCACTTATTAAGACAATGCTGACGGTT
SMA2945-2947F re BamHI	TTGGATCCGTCCCTTGGCATATTGAGATTTTTTT
SMA2945-2947B fw EcoRI	GATCGAATTCATGGCAAGTGGCTGAAAAC
SMA2945-2947B re BamHI	ATCCAGGGATCCATAAAGAAGCCGAATGC
SMA1059-1060 fw EcoRI	CGGAATTCGCTTTGGCTGTAAAATCAGGAAGA
SMA1059-1060 re KpnI	AAGGTACCGGCTGCAAAAACGACGAAAAAAC
SMA1255-1254 fw KpnI	TGGGTACCCGACAAATCATTACCTTATGG
SMA1255-1254 re EcoRI	CGGAATTCCTTATCAGCGCTTAACCTTTTCG
SMA1698-1699 fw XbaI	TTTCTAGATAATTAAGGGAACCCATGCTCGGA
SMA1698-1699 re KpnI	CCGGTACCATTATTTTATCCTTCAGACGTG
SMA1743-1741 fw XbaI	CGTCTAGAACTTTTACCGAATCCTTACGCAAC
SMA1743-1741 re EcoRI	CCGAATTCGTTTTCTGTTTCTTACACTTTCCA
SMA2891 fw KpnI	CGGGGTACCTCCTTTTTTCTCCACGCTTT
SMA2891 re EcoRI	CGGAATTCAGTAAACACCTTGGGAACGAG

(Table 12 continued)

SMA3509 fw EcoRI	GGGAATTCGGCAACTATTTTCGTCTATCG
SMA3509 re BamHI	CCGGATCCAAACGAGAGTGAAGATGATA
sdeXY-FRTfw	TTCAGGCAGCTTAGTGCTAACAGGATGTAACGATAAAGAAGCTTTTCTGTGACTGGTGAG
sdeXY-FRTre	CACGTTGTCCTTTTCCTTGTTTCAGGAAGTAGTCGGAGACTCTATTCTCAGAATGACTTGG
sdeXY-Nfw	ATTGAACAAGGATTCATGGC
sdeXY-Nre	TTCTTTATCGTTACATCCTG
sdeXY-Cfw	AGTCTCCGACTACTTCCTGA
sdeXY-Cre	ACCTGGAAGTACACGTCGTT

Table 13. MICs of antimicrobial agents in *E. coli* KAM32 harboring each RND-type efflux pump.

N.D. indicates not determined.

Antimicrobial agent	MIC (µg/mL)											
	KAM32/ pUC19 (control)	Group 1				Group 2	Group 3	Group 4			Group 5	
		KAM32/ pURS2	KAM32/ pURS8	KAM32/ pURS82	KAM32/ pURS9	KAM32/ pURS3	KAM32/ pURS5	KAM32/ pURS4	KAM32/ pURS44	KAM32/ pURS45	KAM32/ pURS6	KAM32/ pURS7
		(<i>sdeXY</i>)	(<i>sdePQ-omsA</i>)	(<i>sdePQ</i>)	(<i>sdeS</i>)	(<i>sdeAB</i>)	(<i>sdeGH</i>)	(<i>sdeCDE</i>)	(<i>sdeCD</i>)	(<i>sdeCE</i>)	(<i>sdeIJ</i>)	(<i>sdeNO</i>)
Norfloxacin	0.016	0.06	0.13	0.06	0.016	0.06	0.13	0.03	0.03	0.03	0.016	0.016
Erythromycin	4	64	8	8	8	4	4	4	4	4	4	4
Chloramphenicol	1	8	1	2	1	8	1	1	1	1	1	1
Tetracycline	0.5	4	0.25	0.25	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.5
Benzalkonium Cl	4	32	8	4	4	16	32	4	4	4	32	4
Triclosan	0.5	2	0.25	0.13	0.5	4	8	0.5	N.D.	N.D.	0.5	0.5
Novobiocin	2	>128	16	16	8	4	4	8	2	2	2	2
SDS	128	>8200	>8200	256	>8200	256	1020	256	256	256	128	512
Deoxycholate	2050	32800	>32800	>32800	16400	2050	32800	2050	2050	2050	2050	2050
Ethidium bromide	4	>128	16	8	4	128	128	4	4	4	4	4
Rhodamine 6G	8	>128	>128	128	8	64	>128	8	8	8	>128	8

Table 14. Effects of HasF on antimicrobial sensitivity.

Antimicrobial agent	MIC (µg/mL)										
			Group 1							Group 2	
	KAM43/ pUC19	KAM43/ pUC19/ pSOS2	KAM43/ pURS2	KAM43/ pURS2/ pSOS2	KAM43/ pURS82	KAM43/ pURS82/ pSOS2	KAM43/ pURS8	KAM43/ pURS9	KAM43/ pURS9/ pSOS2	KAM43/ pURS3	KAM43/ pURS3/ pSOS2
	(control)		(<i>sdeXY</i>)	(<i>sdeXY</i>)	(<i>sdePQ</i>)	(<i>sdePQ</i>)	(<i>sdePQ</i>)	(<i>sdeS</i>)	(<i>sdeS</i>)	(<i>sdeAB</i>)	(<i>sdeAB</i>)
		(<i>hasF</i>)		(<i>hasF</i>)		(<i>hasF</i>)	(<i>omsA</i>)		(<i>hasF</i>)		(<i>hasF</i>)
Norfloxacin	0.016	0.032	0.016	0.064	0.016	0.016	0.032	0.016	0.016	0.016	0.032
Erythromycin	4	4	4	128	4	4	32	2	4	2	2
Benzalkonium	2	2	4	8	4	2	8	2	2	4	4
Novobiocin	0.25	4	0.5	32	0.5	4	32	0.5	2	0.5	2
SDS	32	64	32	>512	32	128	>512	32	128	32	128
Ethidium bromide	1	2	2	128	1	4	32	1	2	1	1
Rhodamine 6G	4	8	4	>128	4	32	256	4	4	4	64
Antimicrobial agent	MIC (µg/mL)										
			Group 3		Group 4		Group 5				
	KAM43/ pUC19	KAM43/ pUC19/ pSOS2	KAM43/ pURS5	KAM43/ pURS5/ pSOS2	KAM43/ pURS4	KAM43/ pURS4/ pSOS2	KAM43/ pURS6	KAM43/ pURS6/ pSOS2	KAM43/ pURS7	KAM43/ pURS7/ pSOS2	
	(control)		(<i>sdeGH</i>)	(<i>sdeGH</i>)	(<i>sdeCDE</i>)	(<i>sdeCDE</i>)	(<i>sdeIJ</i>)	(<i>sdeIJ</i>)	(<i>sdeNO</i>)	(<i>sdeNO</i>)	
		(<i>hasF</i>)		(<i>hasF</i>)		(<i>hasF</i>)		(<i>hasF</i>)		(<i>hasF</i>)	
Norfloxacin	0.016	0.032	0.016	0.128	0.016	0.016	0.016	0.032	0.016	0.032	
Erythromycin	4	4	4	8	2	4	4	8	4	4	
Benzalkonium	2	2	4	16	2	4	4	4	2	4	
Novobiocin	0.25	4	0.5	4	0.5	8	0.5	4	0.5	4	
SDS	32	64	16	>512	32	64	32	256	32	256	
Ethidium bromide	1	2	2	64	2	4	1	16	1	32	
Rhodamine 6G	4	8	4	>128	4	4	4	32	4	32	

Table 15. MICs of antimicrobial agents in *E. coli* KAM33 harboring *sdeS*.

KAM33, derived from *E. coli* TG1, lacks *acrAB* and *ydhE*.

Antimicrobial agent	MIC (µg/mL)	
	KAM33/pUC19	KAM33/pURS9
	(control)	(<i>sdeS</i>)
Erythromycin	4	4
Tetracycline	0.25	0.25
Benzalkonium Cl	4	4
Triclosan	0.5	0.5
Novobiocin	2	2
SDS	128	128
Deoxycholate	1024	1024
Ethidium bromide	4	4
Rhodamine 6G	8	8

Table 16. MICs of antimicrobial agents in *S. marcescens*.

N.D. indicates not determined.

Antimicrobial agent	MIC (µg/mL)			
	KS24	KS24 Δ sdeXY	KS24 Δ sdeXY/ pSTV28	KS24 Δ sdeXY/ pSMXY
Cloxacillin	512	16	0.5	>512
Oxacillin	512	16	0.5	512
Erythromycin	256	2	2	256
Tetracycline	8	1	1	8
Chloramphenicol	8	1	N.D.	N.D.
Novobiocin	64	1	1	64
Norfloxacin	16	1	1	16
Ofloxacin	4	0.25	0.25	8
Ciprofloxacin	4	0.25	0.25	4
Benzalkonium chloride	32	2	2	32
Chlorhexidine gluconate	16	2	2	16
Triclosan	>1024	256	256	>1024
Acriflavine	128	16	16	256
Ethidium bromide	512	1	2	512
Hoechst33342	16	0.13	0.063	16
Rhodamine6G	1024	4	4	1024
TPP	4096	4	4	4096
Sodium cholate	>51200	3200	800	>51200
Sodium deoxycholate	3200	800	400	3200
SDS	51200	50	<25	51200

Discussion

Previous studies have suggested that RND efflux systems play a major role in multidrug resistance in *S. marcescens* (66–70). Since Gram-negative bacteria have been reported to possess multiple active RND efflux systems (59–65), it was hypothesized that *S. marcescens* harbors additional active RND efflux systems. To investigate this hypothesis, all putative RND efflux systems from the *S. marcescens* Db10 strain were cloned, characterized in *E. coli*, and further evaluated to identify active systems with broad substrate specificities.

A previous study indicated that SdeAB functions as the primary RND efflux system in *S. marcescens*, as the *sdeB* mutant exhibited hypersensitivity to multiple antimicrobial agents, similar to the *hasF* mutant (69). However, the present findings demonstrated that SdeAB possesses narrower substrate specificity compared with SdeXY, SdePQ, and SdeGH. In addition, the *sdeXY* mutant was found to be hypersensitive to a broad spectrum of antimicrobial agents. Furthermore, an independent study reported that *sdeAB* is not expressed in the wild-type strain of *S. marcescens*, and its expression is induced by the biocide cetylpyridinium chloride (70). Thus, SdeAB may play a primary role in multidrug resistance only in specific strains of *S. marcescens* and/or under conditions involving exposure to particular biocides.

All *S. marcescens* RND efflux systems were found to utilize TolC and HasF when expressed in *E. coli*. However, certain RND pumps exhibited differences in substrate specificity depending on whether they were expressed with TolC of *E. coli* or HasF of *S. marcescens*. For example, SdeNO expressed with HasF in KAM43 conferred higher MICs than when expressed in KAM32 with TolC, whereas SdeIJ and SdeS conferred lower MICs under the same conditions. These findings suggest that compatibility between OMPs and other components of RND pumps is critical for efflux activity. As previously reported by our group, only VmeAB in *V. parahaemolyticus* conferred high MICs when expressed with TolC of *E. coli*, while other RND pumps in *V. parahaemolyticus* exhibited markedly higher MICs when expressed with VpoC, an ortholog of TolC (60). This observation may provide important insights into the interaction between outer membrane proteins and other components of RND efflux systems.

In summary, the present study demonstrated that *S. marcescens* possesses multiple RND efflux systems with the potential to confer multidrug resistance. Among these systems, SdeXY was identified as playing a major role in the intrinsic multidrug resistance in *S. marcescens*. SdePQ-OmsA and SdeGH exhibited broad substrate

specificities similar to SdeXY; however, these systems appear to be inducible and do not contribute significantly to the intrinsic multidrug resistance of *S. marcescens*.

Conclusion

This dissertation addressed two major challenges in infectious disease therapeutics: the lack of effective antiviral agents for emerging and re-emerging viral infections, and the growing threat of MDR bacterial pathogens. Through three complementary studies, diverse approaches were explored to overcome these global health concerns.

First, the potential of CENi as broad-spectrum antivirals was demonstrated. Screening of a CENi library identified structural analogs with 100- to 1,000-fold greater activity against bunyaviruses, including LASV, LCMV, and JUNV, compared with RBV. Mechanistic studies revealed that resistance-associated mutations localized to the CEN $\alpha 3$ -helix loop, analogous to influenza virus findings, suggesting a conserved inhibitory mechanism. Structural analysis highlighted the critical role of the carboxyl group side chain for antiviral potency. *In vivo* studies confirmed therapeutic efficacy, with significant reductions in viral load, mitigation of clinical symptoms, and improved survival in LCMV-infected mice. These findings underscore the potential of CENi compounds as broad-spectrum therapeutics for severe influenza and hemorrhagic fevers.

Second, host protease inhibitors were evaluated as antiviral agents against SARS-CoV-2. Comparative analyses of WT and furin-site deletion mutant (del2) revealed distinct entry pathways mediated by TMPRSS2 and cathepsins. The TMPRSS2 inhibitor camostat effectively blocked WT virus entry, whereas the cathepsin inhibitor K11777 exhibited potent activity against the del2 mutant. Escape mutants confirmed the role of S2' cleavage in viral entry. Importantly, combination therapy with camostat and K11777 synergistically inhibited SARS-CoV-2 infection *in vitro* and *in vivo*, highlighting the therapeutic potential of dual-target strategies against coronavirus infections.

Finally, the role of RND efflux systems in intrinsic multidrug resistance in *Serratia marcescens* was elucidated. Eight putative RND efflux genes were identified, six of which conferred multidrug resistance in *E. coli*. Functional assays demonstrated energy-dependent efflux activity and revealed that HasF, the TolC ortholog in *S. marcescens*, is essential for pump function. Moreover, deletion of *sdeXY* in a clinically derived MDR strain resulted in hypersensitivity to multiple antimicrobials, confirming its role as a major determinant of intrinsic resistance. These findings establish RND efflux systems as critical targets for novel antimicrobial strategies.

Collectively, these studies emphasize the importance of integrated approaches, combining conventional screening, drug repositioning, and mechanistic studies, to accelerate therapeutic development for viral and bacterial infections. The outcomes

presented herein not only advance our understanding of antiviral and antibacterial resistance mechanisms but also provide a framework for future research aimed at improving global preparedness against emerging pathogens and MDR organisms.

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Summary in Japanese (和文要旨)

人類を取り巻く感染症は、ウイルス、細菌などの様々な病原体によって引き起こされる。

ウイルス感染症においては、未だに有効な治療薬が存在しない感染症が存在している。近年、ヒトでの局所的な流行を繰り返しているラッサ熱は有効かつ安全な治療薬が確立されていない疾患の一つである。本感染症は重症化すると消化器出血、脳症、ショックなどの重篤な症状を示し、致死につながることから治療薬の開発が望まれている。また 2019 年以降に世界的な流行が問題となった新型コロナウイルス感染症 (COVID-19) 等の、新興感染症に対しては、流行が発生した時点で有効な治療薬は存在していない。パンデミックが発生した際には、有効な治療薬の迅速な開発が重要となる。そのため通常の創薬に加えて、既に開発・承認が進められており薬理作用や安全性などの情報が明らかである化合物を用いた、ドラッグリポジショニングの有用性が注目されている。

一方、細菌感染症においては多剤耐性菌の出現が問題となっており、これらは院内感染や日和見感染の原因となる。特に免疫力が低下した易感染状態の症例では日和見感染は大きなリスクとなる。そのため多剤耐性菌に対する治療薬開発は急務である。多剤耐性の獲得には複数の機構が関与している。その中でも多剤排出ポンプは、細菌が細胞外へ薬剤を能動的に排出することにより、複数の薬剤に対して同時に耐性を示す主要な因子として注目されている。多剤排出ポンプに関する研究は、従来の抗菌薬開発とは異なり薬剤感受性の回復といった新たな治療戦略の構築に繋がる可能性がある。また近年、多剤排出ポンプの立体構造解析や阻害剤のスクリーニング技術の進展によって、創薬標的としての有用性が高まっている。

上述の課題に対する研究として、第 1 章ではラッサウイルスを含むブニヤウイルスに対する阻害剤の同定を試みた。ブニヤウイルスの複製にはキャップ依存性エンドヌクレアーゼ (CEN) が必須であることから、本研究では CEN 阻害剤 (CENi) ライブラリーを用いて化合物スクリーニングを実施し、リバビリンに比べて最大 1,000 倍の活性を示す化合物を複数同定した。これらの化合物は、ラッサウイルス、リンパ球性脈絡髄膜炎ウイルス (LCMV)、フニンウイルスを含む複数のブニヤウイルスに対して活性を有していた。阻害メカニズムを明らかにするために薬剤耐性ウイルスの選択を実施し、薬剤耐性ウイルスの CEN 領域におけるアミノ酸置換を同定した。さらに LCMV 感染マウスモデルを用いた試験において、同定した化合物治療によって、血液中のウイルス RNA 量が大幅に減少し、感染による血小板減少症や肝機能障害が抑制され、さらにマウスの生存率が向上することが明らかとなった。これらの結果から、CEN 阻害剤

がブニヤウイルス感染症の広域治療薬としての可能性を有することが示され、有効かつ安全な治療薬が確立されていない疾患の一つであるラッサ熱の治療薬候補となり得る化合物を見出した。

COVID-19 の原因ウイルスである重症急性呼吸器症候群コロナウイルス 2 (SARS-CoV-2) は、2 型膜貫通型セリンプロテアーゼ (TMPRSS2) およびカテプシンプロテアーゼという 2 つの異なる経路で細胞に侵入することが知られている。また S 遺伝子のフーリン切断部位を欠失した SARS-CoV-2 変異株 (del2) では、野生株と細胞指向性が異なることが報告されていた。第 2 章では複数の異なる細胞株を用いて、SARS-CoV-2 野生株および del2 変異株に対するプロテアーゼ阻害剤の抗ウイルス活性を評価した。阻害剤にはセリンプロテアーゼ阻害剤として開発されたカモスタットや寄生虫感染症に対する治療薬候補として開発が進められていたカテプシン L 阻害剤である K11777 を含む 4 種類の化合物を用いた。その結果、TMPRSS2 阻害剤であるカモスタットは、野生株に対して強い抗ウイルス活性を示したが、del2 変異株に対しては活性を示さなかった。一方、カテプシン B/L 阻害剤である K11777 は、del2 ウイルスに対して強力な抗ウイルス活性を示した。次に SARS-CoV-2 および SARS-CoV の K11777 耐性変異ウイルスを単離し、これらのウイルスが有する変異がカテプシン L を介した S タンパク質における S2'部位での切断を促進することを明らかにした。最後に、SARS-CoV-2 感染ハムスターモデルにおいて K11777 とカモスタットの併用治療によって、SARS-CoV-2 WK-521 株の感染が強力に阻害されることを示した。本研究結果から SARS-CoV-2 の細胞侵入過程における S2'部位の重要性が明らかとなり、COVID-19 に対する治療戦略として、TMPRSS2 およびカテプシンプロテアーゼを標的とした併用療法の有用性が示唆された。

第 3 章では院内の日和見感染の原因菌として重要な *Serratia marcescens* (*S. marcescens*)に着目し、広範な化合物を基質とすることが知られ、多剤耐性発現において重要な因子である RND 型多剤排出ポンプの機能を評価した。*S. marcescens* Db10 株のゲノムから 8 つの RND 型多剤排出ポンプ遺伝子を同定した。これら 8 つの遺伝子をそれぞれ薬剤感性大腸菌である KAM32 株で発現させた場合、8 つの内 6 つにおいて複数の抗菌薬および消毒薬に対する最小発育阻止濃度 (MIC) が上昇した。それらのうち 5 つの遺伝子はベンザルコニウム耐性にも寄与し、これらの RND 型多剤排出ポンプが消毒薬耐性をもたらすことが示唆された。また、臨床分離多剤耐性 *S. marcescens* 株の派生株から RND 型多剤排出ポンプの構成遺伝子である *sdeXY* の欠損株を構築した。本株に対する抗菌薬の MIC 測定結果から、この *sdeXY* 欠損株が広範囲の抗菌薬に感受性を示すことが明らかとなった。本結果から、多剤耐性 *S. marcescens* においても多剤排出ポンプを阻害することで、耐性を示す抗菌薬の感受性を回復させうることが示唆された。

以上の様に本研究では、いずれもウイルスまたは細菌の複製・維持に必要な分子機構を標的とした研究を行った。第 1 章ではウイルスの複製に必須である酵素である CEN、第 2 章ではウイルスが細胞に侵入する際に必要な酵素である TMPRSS2 およびカテプシンプロテアーゼ、また第 3 章では細菌の多剤耐性に寄与している RND 型多剤排出ポンプを対象とした創薬開発の基盤となる研究を実施した。本研究の新規性は、第 1 章および第 2 章において阻害剤探索から耐性機構解析、さらに *in vivo* における薬効評価までを体系的に行い、それぞれの創薬標的としての有用性を実証した点にある。第 3 章では、*S. marcescens* における RND 型多剤排出ポンプの全体像を明らかにし、主要な RND 型多剤排出ポンプが薬剤耐性に与える影響を解明した点が特徴である。これらの研究によって、対象としたウイルスおよび細菌において未検証であった標的分子について、創薬標的としての有用性を明らかにした。これにより、ウイルスおよび細菌感染症に対する新たな治療戦略の構築に貢献するものであり、今後の感染症対策において基盤となる知見を提供するものである。