ゾコーバ錠 125mg

第 2 部 (モジュール 2) : CTD の概要 (サマリー)

2.6 非臨床試験の概要文及び概要表

2.6.3 薬理試験概要表

塩野義製薬株式会社

目次

略号及び用語定義一覧表

略号	英語
3CL	3C-like
A234S	alanine-to-serine at amino acid position 234
ACE2	angiotensin-converting enzyme 2
ATP	adenosine triphosphate
$AUC_{0\text{-}24\ (48)\ hr}$	area under the plasma concentration-time curve from time 0 to 24 (48) hours post-dose
bid	bis in die, twice daily
$C_{24\ (48)\ hr}$	plasma concentration at 24 (48) hours after first administration
CCK	cholecystokinin
CC_{50}	50% cytotoxicity concentration
CHO	chinese hamster ovary
CI	combination index
C_{max}	maximum concentration after first administration/maximum plasma concentration
CPE	cytopathic effect
D48G	asparatic acid-to-glycine at amino acid position 48
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
$EC_{50\ (90,\ 99,\ 99.9)}$	50% (90%, 99%, 99.9%) effective concentration
Exp	experiment
F	phenylalanine
F/L	mixture of phenylalanine and leucine
FOB	functional observation battery
G15S	glycine-to-serine at amino acid position 15
GISAID	Global Initiative on Sharing All Influenza Data
GLP	Good Laboratory Practice
hERG	human ether-à-go-go related gene
H246Y	histidine-to-tyrosine at amino acid position 246
HIV	human immunodeficiency virus
HS	human serum
HR	heart rate
IC_{50}	50% inhibitory concentration
IP	inositol phosphate
K90R	lysine-to-arginine at amino acid position 90
L75	leucin at amino acid position 75
L87F	leucine-to-phenylalanine at amino acid position 87
L89F	leucine-to-phenylalanine at amino acid position 89
LC/MS/MS	liquid chromatography with tandem mass spectrometry

略号	英語
M	male
M49L	methionine-to-leucine at amino acid position 49
M49(M/L)	mixture of no substation and methionine-to-leucine at amino acid position 49
MC	methylcellulose aqueous solution
MS	mouse serum
N/A	not applicable
NC	not calculated
NR	not required
nsp5	non-structual protein 5
NT	not tested
P52S	proline-to-serine at amino acid position 52
P108S	proline-to-serine at amino acid position 108
P132H	proline-to-histidine at amino acid position 132
PA-EC_{50}	protein-adjusted EC_{50}
PBS	phosphate buffered saline
PD	pharmacodynamics
PDE	phosphodiesterase
PK	pharmacokinetics
PS	potency shift
qd	quaque die, once daily
qid	quarter in die, 4 times daily
QTc	corrected QT interval
r^2	coefficients of determination
RT-PCR	reverse transcription polymerase chain reaction
S144A	serine-to-alanine at amino acid position 144
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SD	standard deviation
T198I	threonine-to-isoleucine at amino acid position 198
T21I	threonine-to-isoleucine at amino acid position 21
$TCID_{50}$	50% tissue culture infectious dose
tid	three times daily
$Time_{High}$	total time above the target plasma concentration
T_{max}	time to maximum plasma concentration
TMPRSS2	transmembrane protease, serine 2

2.6.3 薬理試験概要表

2.6.3.1 薬理試験：一覧表

Test Article: S-217622 fumaric acid

Type of Study	Test System	Method of Administration	Testing Facility	Study Number	Location in CTD
Primary Pharmacodynamics					
Inhibitory Effect of S-217622 on SARS-CoV-2 3CL Protease	Enzyme: Recombinant SARS-CoV-2 3CL protease Substrate: Dabcyl-KTSAVLQSGFRKME (Edans) -NH_2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-036-N	4.2.1.1-01
Inhibitory Effect of S-217622 on Cytopathic Effect in SARS-CoV-2 Infected Cells	Virus: hCoV-19/Japan/TY/WK-521/2020, hCoV-19/Japan/QK002/2020, hCoV-19/Japan/QHN001/2020, hCoV-19/Japan/QHN002/2020, hCoV-19/Japan/TY7-501/2021, hCoV-19/Japan/TY7-503/2021, hCoV-19/Japan/TY8-612/2021 Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-031-N	4.2.1.1-02
Inhibitory Effect of S-217622 on Cytopathic Effect in SARS-CoV-2 Infected Cells (2)	Virus: hCoV-19/Japan/TY11-927-P1/2021 Cell: VeroE6/TMPRSS2, HEK293T/ACE2-TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-082-N	4.2.1.1-03
Inhibitory Effect of S-217622 on Cytopathic Effect in SARS-CoV-2 Infected Cells (3)	Virus: SARS-CoV-2 MA-P10 Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-104-N	4.2.1.1-04
Inhibitory Effect of S-217622 on Cytopathic Effect in SARS-CoV-2 Infected Cells (4)	Virus: hCoV-19/Japan/TY38-873/2021 Cell: VeroE6/TMPRSS2, HEK293T/ACE2-TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-132-N	4.2.1.1-05
Inhibitory Effect of S-217622 on Cytopathic Effect in SARS-CoV-2 Infected HEK293T/ACE2-TMPRSS2 Cells	Virus: hCoV-19/Japan/TY/WK-521/2020, hCoV-19/Japan/QK002/2020, hCoV-19/Japan/TY7-501/2021, hCoV-19/Japan/TY8-612/2021 Cell: HEK293T/ACE2-TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-068-N	4.2.1.1-06
Inhibitory Effect of S-217622 on SARS-CoV-2 Replication in Primary Human Nasal Epithelial Cells	Virus: hCoV 19/Japan/TY11-927-P1/2021 Cell: MucilAir™ Nasal cavity, Human	In vitro	Shionogi & Co., Ltd.	S-217622-EB-122-N	4.2.1.1-07
Inhibitory Effect of S-217622 on Replication of SARS-CoV-2 in Cultured Cells in the Presence of Serum	Virus: hCoV-19/Japan/TY7-501/2021 Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-032-N	4.2.1.1-08

Type of Study	Test System	Method of Administration	Testing Facility	Study Number	Location in CTD
Isolation of SARS-CoV-2 Resistant to S-217622 in Cultured Cells	Virus: hCoV-19/Japan/TY7-501/2021 Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-074-N	4.2.1.1-09
Isolation of SARS-CoV-2 Resistant to S-217622 in Cultured Cells (2)	Virus: hCoV-19/Japan/TY/WK-521/2020, hCoV-19/Japan/QHN001/2020, hCoV-19/Japan/TY8-612/2021, hCoV-19/Japan/TY11-927-P1/2021 Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-079-N	4.2.1.1-10
Drug Susceptibility Testing of SARS-CoV-2 Mutants to S-217622 in Cultured Cells	Virus: hCoV-19/Japan/TY/WK-521/2020, hCoV-19/Japan/QHN001/2020, hCoV-19/Japan/TY8-612/2021, hCoV-19/Japan/TY7-501/2021 hCoV-19/Japan/TY11-927-P1/2021 Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-090-N	4.2.1.1-11
Replicative Capacity and Drug Susceptibility Testing of Reverse Genetics-derived SARS-CoV-2 Mutants to S-217622 in Cultured Cell	Virus: rgSARS-CoV-2/Hu/DP/Kng/19-020 (wild-type), rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/M49L, rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/S144A, rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/M49L/S144A Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-103-N	4.2.1.1-12
Replicative Capacity and Drug Susceptibility Testing of Reverse Genetics-derived SARS-CoV-2 Mutants to S-217622 in Cultured Cell (2)	Virus: rgSARS-CoV-2/Hu/DP/Kng/19-020 (wild-type), rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/D48G, rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/P52S Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-116-N	4.2.1.1-13
Inhibitory Effect of S-217622 in Combination with Anti-SARS-CoV-2 Drugs on Replication of SARS-CoV-2 in Cultured Cells	Virus: hCoV-19/Japan/TY11-927-P1/2021 Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-094-N	4.2.1.1-14
Inhibitory Effect of S-217622 in Combination with Anti-SARS-CoV-2 Drugs on Replication of SARS-CoV-2 in Cultured Cells (2)	Virus: rgSARS-CoV-2/Hu/DP/Kng/19-020 (wild-type) Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-136-N	4.2.1.1-15
Pharmacokinetic and Pharmacodynamic Analysis of S-217622 on SARS-CoV-2 Replication in Mice	Animal: Mouse Virus: hCoV-19/Japan/TY7-501/2021	Oral	Shionogi & Co., Ltd.	S-217622-EB-018-N	4.2.1.1-16

Type of Study	Test System	Method of Administration	Testing Facility	Study Number	Location in CTD
Pharmacokinetic and Pharmacodynamic Analysis of Delayed Treatment with S-217622 on SARS-CoV-2 Replication in Mice	Animal: Mouse Virus: hCoV-19/Japan/TY7-501/2021	Oral	Shionogi & Co., Ltd.	S-217622-EB-081-N	4.2.1.1-17
Therapeutic Effect of Delayed Treatment with S-217622 in Mice Infected with SARS-CoV-2	Animal: Mouse Virus: SARS-CoV-2 MA-P10	Oral	Shionogi & Co., Ltd.	S-217622-EB-099-N	4.2.1.1-18
Polymorphism Analysis of Amino Acid Positions Associated with S-217622 Low Susceptibility in SARS-CoV-2 3CL Protease (2)	Database: GISAID	-	Shionogi & Co., Ltd.	S-217622-EB-172-N	4.2.1.1-19
Inhibitory Effect of S-217622 on Mutants of SARS-CoV-2 3CL Protease	Enzyme: Recombinant SARS-CoV-2 3CL protease (G15S, T21I, L89F, K90R, P108S, and P132H) Substrate: Dabcyl-KTSAVLQSGFRKME (Edans) -NH_2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-159-N	4.2.1.1-20
Inhibitory Effect of S-217622 on Cytopathic Effect in SARS-CoV-2 Infected Cells (5)	Virus: hCoV-19/Japan/TY38-871/2021, hCoV-19/Japan/TY40-385/2022 Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-168-N	4.2.1.1-21
Inhibitory Effect of S-217622 on Cytopathic Effect in SARS-CoV-2 Infected Cells (6)	Virus: hCoV-19/Japan/TY28-444/2021, hCoV-19/Japan/TY33-456/2021, hCoV-19/Japan/TY26-717/2021, hCoV-19/Japan/TY41-686/2022 Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-174-N	4.2.1.1-22
Inhibitory Effect of S-217622 on Cytopathic Effect in SARS-CoV-2 Infected Cells (7)	Virus: hCoV-19/Japan/TY41-703/2022, hCoV-19/Japan/TY41-702/2022 Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-177-N	4.2.1.1-23
Inhibitory Effect of S-217622 on Cytopathic Effect in SARS-CoV-2 Infected Cells (8)	Virus: hCoV-19/Japan/TY41-716/2022 Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-184-N	4.2.1.1-24

Type of Study	Test System	Method of Administration	Testing Facility	Study Number	Location in CTD
Drug Susceptibility Testing of Reverse Genetics-derived SARS-CoV-2 Mutants to S-217622 in Cultured Cell	Virus: rgSARS-CoV-2/Hu/DP/Kng/19-020 (wild-type), rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/L87F, rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/T198I, rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/A234S, rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/H246Y Cell: VeroE6/TMPRSS2	In vitro	Shionogi & Co., Ltd.	S-217622-EB-178-N	4.2.1.1-25
Secondary Pharmacodynamics					
Study for specific Interaction of S-217622 on various Receptors and Enzymes in vitro	Various receptors, channels, transporters and enzymes	In vitro	[redacted]	S-217622-EF-046-N	4.2.1.2-01
Cytotoxicity Study of S-217622 fumaric acid in Human Primary Cells	Human primary cells: Human hepatocytes, Normal human mixed renal epithelial cells, Human peripheral blood $CD4^+$ T cells	In vitro	[redacted]	S-217622-EF-084-N	4.2.1.2-02
Cytotoxicity Study of S-217622 fumaric acid in Cells from Multiple Human Tissues	Human cell lines: Hepatocellular carcinoma (HepG2), Renal glomeruli (HK-2), Embryonic kidney (HEK293), Neuroblastoma (SH-SY-5Y), Neuroblastoma (SK-N-SH), T cell leukemia (Jurkat), Prostate carcinoma (E006AA-hT), Vascular endothelial (HUVEC) Lung fibroblast (MRC-5)	In vitro	[redacted]	S-217622-EF-085-N	4.2.1.2-03
Mitochondrial Toxicity Test of S-217622 fumaric acid in Human Cell Line	HepG2 cells	In vitro	[redacted]	S-217622-EF-077-N	4.2.1.2-04
Safety Pharmacology					
Central nervous system					
Effects of S-217622 fumaric acid on Central Nervous System in Rats	Rat/Crl:CD(Sprague Dawley)	Oral gavage	[redacted]	S-217622-SF-035-L	4.2.1.3-01
Cardiovascular system					

Type of Study	Test System	Method of Administration	Testing Facility	Study Number	Location in CTD
Effects of S-217622 fumaric acid on Potassium Current in hERG Transfected Cells	CHO cells expressing hERG channels	In vitro	[redacted]	S-217622-SF-034-L	4.2.1.3-02
Cardiovascular and respiratory system					
Effects of S-217622 fumaric acid on Cardiovascular and Respiratory Systems in Monkeys	Cynomolgus monkey	Oral gavage	[redacted]	S-217622-SF-033-L	4.2.1.3-03
3CL = 3C-like; hERG = human ether-à-go-go-related gene; nsp5 = non-structual protein 5; CHO = chinese hamster ovary; GISAID = Global Initiative on Sharing All Influenza Data (https://www.gisaid.org/), SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2; TMPRSS2 = transmembrane protease, serine 2					

2.6.3.2 効力を裏付ける試験

2.6.3.2.1 SARS-CoV-2 3CL プロテアーゼ活性に対する阻害効果

Test Article: S-217622 fumaric acid

Enzyme	Recombinant SARS-CoV-2 3CL protease (expressed in *Escherichia coli*; R&D Systems, Inc., Lot 03581921A)
Concentration as free form (nmol/L)	0.127, 0.381, 1.14, 3.43, 10.3, 30.9, 92.6, 278, 833, 2500
Reference substance (Final concentration)	N/A
Vehicle	DMSO
Evaluation Items	Inhibitory effect of S-217622 fumaric acid on SARS-CoV-2 3CL protease activity.
Evaluation Method	Recombinant SARS-CoV-2 3CL protease was incubated with substrate and S-217622 fumaric acid. Dabcyl-KTSAVLQ as a reaction product, was quantified by mass spectroscopy (6550 iFunnel Q TOF, Agilent technologies) interfaced with liquid chromatography (RapidFire^{TM} system, Agilent technologies). Inhibitory activity was expressed as IC_{50} calculated using Excel XLfit software.
Results	IC_{50} (nmol/L) = 13.2 ± 1.1
DMSO = Dimethyl sulfoxide; IC_{50} = 50% inhibitory concentration; N/A = not applicable Data are expressed as the mean ±standard deviation (SD) in 3 independent experiments.	
Study Number [Location in CTD]	S-217622-EB-036-N [4.2.1.1-01]

2.6.3.2.2 SARS-CoV-2 感染細胞に対する細胞変性抑制効果

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with SARS-CoV-2 clinical isolates (hCoV-19/Japan/TY/WK-521/2020 (Pango Lineage: A), hCoV-19/Japan/QK002/2020 (Pango Lineage: B.1.1.7), hCoV-19/Japan/QHN001/2020 (Pango Lineage: B.1.1.7), hCoV-19/Japan/QHN002/2020 (Pango Lineage: B.1.1.7), hCoV-19/Japan/TY7-501/2021 (Pango Lineage: P.1), hCoV-19/Japan/TY7-503/2021 (Pango Lineage: P.1), and hCoV-19/Japan/TY8-612/2021 (Pango Lineage: B.1.351)
Final concentration as free form (μmol/L)	Viral replication inhibition assay: 0.0046, 0.014, 0.041, 0.12, 0.37, 1.1, 3.3, 10 Cytotoxicity assay: 0.046, 0.14, 0.41, 1.2, 3.7, 11, 33, 100
Reference substance (Final concentration [μmol/L])	Remdesivir Viral replication inhibition assay: 0.0091, 0.027, 0.082, 0.25, 0.74, 2.2, 6.7, 20 Cytotoxicity assay: 0.046, 0.14, 0.41, 1.2, 3.7, 11, 33, 100
Vehicle	0.5% DMSO
Evaluation item	EC_{50}: The concentration achieving 50% of inhibition on CPE induced by SARS-CoV-2, CC_{50}: The concentration achieving 50% of cytotoxicity
Evaluation Method	Viral replication inhibition assay: One hundred microliters of serially diluted substance solutions were added to a 96-well plate. SARS-CoV-2 virus stock solutions were diluted to appropriate concentrations in advance, and each 50 μL/well was dispensed on the 96-well plate containing a test or reference substance. The infectious dose of each virus was as follows: 30 $TCID_{50}$/well for hCoV-19/Japan/TY8-612/2021, 1000 $TCID_{50}$/well for other viruses. Then, each 50 μL/well of VeroE6/TMPRSS2 cells which were adjusted to 3.0×10^5 cells/mL was dispensed on the plate. The plates were mixed with a plate mixer and were incubated at 37°C in a CO_2 incubator for 3 days. The inhibitory effects of the test substance on CPE induced by SARS-CoV-2 virus were measured by cell viability. Cytotoxicity assay: One hundred microliters of serially diluted substance solutions and 50 μL of the viral assay medium were added to a 96-well plate. Each 50 μL/well of VeroE6/TMPRSS2 cells which were adjusted to 3.0×10^5 cells/mL was dispensed on the 96-well plate containing a test or reference substance. The plate was mixed with a plate mixer and was incubated at 37°C in a CO_2 incubator for 3 days. The cytotoxicity of the test substance in VeroE6/TMPRSS2 cells was measured by cell viability. Measurement of cell viability: After 3 days incubation of the plates prepared in the viral replication inhibition assay or cytotoxicity assay, 60 μL of CellTiter-Glo® 2.0 Reagent was added into each well. After mixing with a plate mixer, plates were incubated at room temperature for approximately 30 minutes, and each 200 μL was dispensed on a new white 96-well plate. Luminescent signal was measured by a multiplate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).

Results			
Strains	Pango Lineage	EC_{50} (μmol/L)	
		S-217622 fumaric acid	Remdesivir
hCoV-19/Japan/TY/WK-521/2020	A	0.37 ± 0.060	1.9 ± 0.14
hCoV-19/Japan/QK002/2020	B.1.1.7	0.33 ± 0.050	0.87 ± 0.027
hCoV-19/Japan/QHN001/2020	B.1.1.7	0.31 ± 0.070	0.97 ± 0.14
hCoV-19/Japan/QHN002/2020	B.1.1.7	0.46 ± 0.044	0.99 ± 0.18
hCoV-19/Japan/TY7-501/2021	P.1	0.50 ± 0.048	2.1 ± 0.39
hCoV-19/Japan/TY7-503/2021	P.1	0.43 ± 0.00085	1.0 ± 0.16
hCoV-19/Japan/TY8-612/2021	B.1.351	0.40 ± 0.048	1.2 ± 0.30
Cells		CC_{50} (μmol/L)	
		S-217622 fumaric acid	Remdesivir
VeroE6/TMPRSS2		>100	>100
CC_{50} = 50% cytotoxicity concentration; CPE = cytopathic effect; EC_{50} = 50% effective concentration; $TCID_{50}$ = 50% tissue culture infectious dose; TMPRSS2 = transmembrane protease, serine 2 Data are expressed as the mean ± SD in 3 independent experiments.			
Study Number [Location in CTD]		S-217622-EB-031-N [4.2.1.1-02]	

2.6.3.2.3 SARS-CoV-2 感染細胞に対する細胞変性抑制効果 (2)

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells or HEK293T/ACE2-TMPRSS2 cells infected with SARS-CoV-2 clinical isolates [hCoV-19/Japan/TY11-927-P1/2021 (Pango Lineage: B.1.617.2)]
Final concentration (μmol/L)	In VeroE6/TMPRSS2 cells: 0.0046, 0.014, 0.041, 0.12, 0.37, 1.1, 3.3, 10 In HEK293T/ACE2-TMPRSS2 cells: 0.00046, 0.0014, 0.0041, 0.012, 0.037, 0.11, 0.33, 1.0
Reference substance (Final concentration [μmol/L])	Remdesivir In VeroE6/TMPRSS2 cells: 0.0091, 0.027, 0.082, 0.25, 0.74, 2.2, 6.7, 20 In HEK293T/ACE2-TMPRSS2 cells: 0.00023, 0.00069, 0.0021, 0.0062, 0.019, 0.056, 0.17, 0.50
Vehicle	0.5% DMSO
Evaluation item	EC_{50}: The concentration achieving 50% of inhibition on CPE induced by SARS-CoV-2
Evaluation Method	Viral replication inhibition assay: One hundred microliters of serially diluted substance solutions were added to a 96-well plate. SARS-CoV-2 virus stock solutions were diluted to appropriate concentrations in advance, and each 50 μL/well was dispensed on the 96-well plate containing a test or reference substance. The infectious dose of virus was as follows: 1000 $TCID_{50}$/well for VeroE6/TMPRSS2 cells, 3000 $TCID_{50}$/well for HEK293T/ACE-TMPRSS2 cells. Then, each 50 μL/well of VeroE6/TMPRSS2 cells or HEK293T/ACE2-TMPRSS2 cells which were adjusted to 3.0×10^5 cells/mL was dispensed on the plate. The plates were mixed with a plate mixer and were incubated at 37°C in a CO_2 incubator for 3 days. The inhibitory effects of the test substance on CPE induced by SARS-CoV-2 virus were measured by cell viability. Measurement of cell viability: After 3 days incubation of the plates prepared in the viral replication inhibition assay or cytotoxicity assay, 60 μL of CellTiter-Glo® 2.0 Reagent was added into each well. After mixing with a plate mixer, plates were incubated at room temperature for approximately 30 minutes, and each 200 μL was dispensed on a new white 96-well plate. Luminescent signal was measured by a multiplate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).

Results			
Strains	Pango Lineage	EC_{50} (μmol/L) infected VeroE6/TMPRSS2 cell	
		S-217622 fumaric acid	Remdesivir
hCoV-19/Japan/TY11-927-P1/2021	B.1.617.2	0.41 ± 0.014	1.6 ± 0.22
Strains	Pango Lineage	EC_{50} (μmol/L) infected HEK293T/ACE2-TMPRSS2 cell	
		S-217622 fumaric acid	Remdesivir
hCoV-19/Japan/TY11-927-P1/2021	B.1.617.2	0.058 ± 0.0073	0.0094 ± 0.0022
ACE2 = angiotensin-converting enzyme 2 Data are expressed as the mean ± SD in 3 independent experiments.			
Study Number [Location in CTD]	S-217622-EB-082-N [4.2.1.1-03]		

2.6.3.2.4 SARS-CoV-2 感染細胞に対する細胞変性抑制効果 (3)

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with mouse-adapted SARS-CoV-2 strain (SARS-CoV-2 MA-P10)
Final concentration as free form (μmol/L)	0.0046, 0.014, 0.041, 0.12, 0.37, 1.1, 3.3, 10
Reference substance (Final concentration [μmol/L])	Remdesivir Viral replication inhibition assay: 0.0091, 0.027, 0.082, 0.25, 0.74, 2.2, 6.7, 20
Vehicle	0.5% DMSO
Evaluation item	EC_{50}: The concentration achieving 50% of inhibition on CPE induced by SARS-CoV-2
Evaluation Method	Viral replication inhibition assay: One hundred microliters of serially diluted substance solutions were added to a 96-well plate. SARS-CoV-2 virus stock solutions were diluted to appropriate concentrations in advance, and each 50 μL/well was dispensed on the 96-well plate containing a test or reference substance. The infectious dose of virus was 1000 $TCID_{50}$/well. Then, each 50 μL/well of VeroE6/TMPRSS2 cells which were adjusted to 3.0×10^5 cells/mL was dispensed on the plate. The plates were mixed with a plate mixer and were incubated at 37°C in a CO_2 incubator for 3 days. The inhibitory effects of the test substance on CPE induced by SARS-CoV-2 virus were measured by cell viability. Measurement of cell viability: After 3 days incubation of the plates prepared in the viral replication inhibition assay, 60 μL of CellTiter-Glo® 2.0 Reagent was added into each well. After mixing with a plate mixer, plates were incubated at room temperature for approximately 30 minutes, and each 200 μL was dispensed on a new white 96-well plate. Luminescent signal was measured by a multiplate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).

Results		
Strains	EC_{50} (μmol/L)	
	S-217622 fumaric acid	Remdesivir
SARS-CoV-2 MA-P10	0.12 ± 0.040	1.6 ± 0.11
Data are expressed as the mean ± SD in 3 independent experiments.		
Study Number [Location in CTD]	S-217622-EB-104-N [4.2.1.1-04]	

2.6.3.2.5 SARS-CoV-2 感染細胞に対する細胞変性抑制効果 (4)

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells or HEK293T/ACE2-TMPRSS2 cells infected with SARS-CoV-2 clinical isolates [hCoV-19/Japan/TY38-873/2021 (Pango Lineage: B.1.1.529)]
Final concentration (μmol/L)	In VeroE6/TMPRSS2 cells: 0.0046, 0.014, 0.041, 0.12, 0.37, 1.1, 3.3, 10 In HEK293T/ACE2-TMPRSS2 cells: 0.00046, 0.0014, 0.0041, 0.012, 0.037, 0.11, 0.33, 1.0
Reference substance (Final concentration [μmol/L])	Remdesivir In VeroE6/TMPRSS2 cells: 0.0091, 0.027, 0.082, 0.25, 0.74, 2.2, 6.7, 20 In HEK293T/ACE2-TMPRSS2 cells: 0.00023, 0.00069, 0.0021, 0.0062, 0.019, 0.056, 0.17, 0.50
Vehicle	0.5% DMSO
Evaluation item	EC_{50}: The concentration achieving 50% of inhibition on CPE induced by SARS-CoV-2
Evaluation Method	Viral replication inhibition assay: One hundred microliters of serially diluted substance solutions were added to a 96-well plate. SARS-CoV-2 virus stock solutions were diluted to appropriate concentrations in advance, and each 50 μL/well was dispensed on the 96-well plate containing a test or reference substance. The infectious dose of virus was as follows: 3000 $TCID_{50}$/well for VeroE6/TMPRSS2 cells, 9000 $TCID_{50}$/well for HEK293T/ACE-TMPRSS2 cells. Then, each 50 μL/well of VeroE6/TMPRSS2 cells or HEK293T/ACE2-TMPRSS2 cells which were adjusted to 3.0×10^5 cells/mL was dispensed on the plate. The plates were mixed with a plate mixer and were incubated at 37°C in a CO_2 incubator for 4 days for VeroE6/TMPRSS2 cells and for 3 days for HEK293T/ACE2-TMPRSS2 cells. The inhibitory effects of the test substance on CPE induced by SARS-CoV-2 virus were measured by cell viability. Cytotoxicity assay: One hundred microliters of serially diluted substance solutions and 50 μL of the viral assay medium were added to a 96-well plate. Each 50 μL/well of VeroE6/TMPRSS2 cells which were adjusted to 3.0×10^5 cells/mL was dispensed on the 96-well plate containing a test or reference substance. The plate was mixed with a plate mixer and was incubated at 37°C in a CO_2 incubator for 4 days. The cytotoxicity of the test substance in VeroE6/TMPRSS2 cells was measured by cell viability. Measurement of cell viability: After 3 or 4 days incubation of the plates prepared in the viral replication inhibition assay or cytotoxicity assay, 60 μL of CellTiter-Glo® 2.0 Reagent was added into each well. After mixing with a plate mixer, plates were incubated at room temperature for approximately 30 minutes, and each 200 μL was dispensed on a new white 96-well plate. Luminescent signal was measured by a multiplate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).

Results			
Strains	Pango Lineage	EC_{50} (μmol/L) infected VeroE6/TMPRSS2 cell	
		S-217622 fumaric acid	Remdesivir
hCoV-19/Japan/TY38-873/2021	B.1.1.529	0.29 ± 0.054	1.1 ± 0.28
Strains	Pango Lineage	EC_{50} (μmol/L) infected HEK293T/ACE2-TMPRSS2 cell	
		S-217622 fumaric acid	Remdesivir
hCoV-19/Japan/TY38-873/2021	B.1.1.529	0.064 ± 0.033	0.015 ± 0.0051
Cells		CC_{50} (μmol/L)	
		S-217622 fumaric acid	Remdesivir
VeroE6/TMPRSS2		>100	>100
Data are expressed as the mean ± SD in 3 independent experiments.			
Study Number [Location in CTD]		S-217622-EB-0132-N [4.2.1.1-05]	

2.6.3.2.6 SARS-CoV-2 感染 HEK293T/ACE2-TMPRSS2 細胞における細胞変性効果に対する S-217622 フマル酸共結晶の阻害効果

Test Article: S-217622 fumaric acid

Species/Biological material	HEK293T/ACE2-TMPRSS2 cells infected with SARS-CoV-2 clinical isolates (hCoV-19/Japan/TY/WK-521/2020 (Pango Lineage: A), hCoV-19/Japan/QK002/2020 (Pango Lineage: B.1.1.7), hCoV-19/Japan/TY7-501/2021 (Pango Lineage: P.1), and hCoV-19/Japan/TY8-612/2021 (Pango Lineage: B.1.351)
Final concentration as free form (μmol/L)	Viral replication inhibition assay: 0.00046, 0.0014, 0.0041, 0.012, 0.037, 0.11, 0.33, 1.0 Cytotoxicity assay: 0.046, 0.14, 0.41, 1.2, 3.7, 11, 33, 100
Reference substance (Final concentration [μmol/L])	Remdesivir Viral replication inhibition assay: 0.00023, 0.00069, 0.0021, 0.0062, 0.019, 0.056, 0.17, 0.5 Cytotoxicity assay: 0.046, 0.14, 0.41, 1.2, 3.7, 11, 33, 100
Vehicle	0.5% DMSO
Evaluation item	EC_{50}: The concentration achieving 50% of inhibition on CPE induced by SARS-CoV-2, CC_{50}: The concentration achieving 50% of cytotoxicity
Evaluation Method	Viral replication inhibition assay: One hundred microliters of serially diluted substance solutions were added to a 96-well plate. SARS-CoV-2 virus stock solutions were diluted to appropriate concentrations in advance, and each 50 μL/well was dispensed on the 96-well plate containing a test or reference substance. The infectious dose of each virus was as follows: 9000 $TCID_{50}$/well for hCoV-19/Japan/QK002/2020, 3000 $TCID_{50}$/well for other viruses. Then, each 50 μL/well of HEK293T/ACE2-TMPRSS2 cells which were adjusted to 3.0×10^5 cells/mL was dispensed on the plate. The plates were mixed with a plate mixer and were incubated at 37°C in a CO_2 incubator for 3 days. The inhibitory effects of the test substance on CPE induced by SARS-CoV-2 virus were measured by cell viability. Cytotoxicity assay: One hundred microliters of serially diluted substance solutions and 50 μL of the viral assay medium were added to a 96-well plate. Each 50 μL/well of HEK293T/ACE2-TMPRSS2 cells which were adjusted to 3.0×10^5 cells/mL was dispensed on the 96-well plate containing a test or reference substance. The plate was mixed with a plate mixer and was incubated at 37°C in a CO_2 incubator for 3 days. The cytotoxicity of the test substance in VeroE6/TMPRSS2 cells was measured by cell viability. Measurement of cell viability: After 3 days incubation of the plates prepared in the viral replication inhibition assay or cytotoxicity assay, 60 μL of CellTiter-Glo® 2.0 Reagent was added into each well. After mixing with a plate mixer, plates were incubated at room temperature for approximately 30 minutes, and each 200 μL was dispensed on a new white 96-well plate. Luminescent signal was measured by a multiplate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).

Results

Strains	Pango Lineage	EC_{50} (μmol/L)	
		S-217622 fumaric acid	Remdesivir
hCoV-19/Japan/TY/WK-521/2020	A	0.027 ± 0.0018	0.012 ± 0.0020
hCoV-19/Japan/QK002/2020	B.1.1.7	0.044 ± 0.0068	0.011 ± 0.0024
hCoV-19/Japan/TY7-501/2021	P.1	0.026 ± 0.0051	0.0061 ± 0.0011
hCoV-19/Japan/TY8-612/2021	B.1.351	0.038 ± 0.0059	0.010 ± 0.00045

Cells	CC_{50} (μmol/L)	
	S-217622 fumaric acid	Remdesivir
HEK293T/ACE2-TMPRSS2	55 ± 11	6.1 ± 0.36
Data are expressed as the mean ± SD in 3 independent experiments.		
Study Number [Location in CTD]	S-217622-EB-068-N [4.2.1.1-06]	

2.6.3.2.7 初代ヒト鼻腔上皮細胞における SARS-CoV-2 の増殖性に対する S-217622 フマル酸共結晶の阻害効果

Test Article: S-217622 fumaric acid

Species/Biological material	MucilAir™ (Nasal cavity, Human) infected with SARS-CoV-2 clinical isolates [hCoV-19/Japan/TY11-927-P1/2021 (Pango Lineage: B.1.617.2)].
Final concentration as free form (μmol/L)	0.00741, 0.0222, 0.0667, 0.200, 0.600
Reference substance (Final concentration [μmol/L])	-
Vehicle	0.5% DMSO
Evaluation item	$EC_{50, 90, 99. or 99.9}$: The concentration achieving 50, 90, 99, or 99.9% of inhibition against SARS-CoV-2
Evaluation Method	Viral replication inhibition assay: MucilAir™ (about 5.0×10^5 cells/well) seeded in transwell on a 24-well plate was infected with hCoV-19/Japan/TY11-927-P1/2021 strain at 5000 $TCID_{50}$/well (multiplicity of infection of about 0.01). The cells were incubated at 37°C in a 5% CO_2 incubator for 2 hours. After incubation, the cells were washed with the viral assay medium to remove unabsorbed viruses, followed by transferring the transwell to a 24-well plate containing 700 μL of serially diluted substance solutions. Afterwards, the infected cells were incubated at 37°C in a 5% CO_2 incubator. The cell culture fluids were collected at 2 and 3 days after inoculation by adding the viral assay medium into transwell, and collected samples were stored in a deep freezer until use. The collected samples were subjected to the viral titration. The assay was performed duplicate for each substance concentration. Measurement of virus titers: The culture fluids were diluted with the virus assay medium using a 10-fold serial dilution scheme. Then, 25 μL of serially diluted culture fluids were added to the 96-well plates, and 175 μL of VeroE6/TMPRSS2 cell suspension (0.86×10^5 cells/mL) were dispensed on the plates. The plates were incubated at 37°C in a 5% CO_2 incubator for 4 days. After incubation, the presence of virus-induced CPE was observed under a microscope. The virus titers were expressed as log $TCID_{50}$/mL.

Results

Strains	Pango Lineage	Day		S-217622 fumaric acid	
				μmol/L	μg/mL
hCoV-19/Japan/TY11-927-P1/2021	B.1.617.2	2	EC_{50}	0.0177 ± 0.00984	0.00940 ± 0.00523
			EC_{90}	0.0514 ± 0.0182	0.0274 ± 0.00968
			EC_{99}	0.121 ± 0.00460	0.0645 ± 0.00245
			$EC_{99.9}$	0.266 ± 0.0833	0.142 ± 0.0443
		3	EC_{50}	0.0570 ± 0.0307	0.0303 ± 0.0163
			EC_{90}	0.117 ± 0.0255	0.0623 ± 0.0136
			EC_{99}	0.207 ± 0.0707	0.110 ± 0.0376
			$EC_{99.9}$	0.329 ± 0.105	0.175 ± 0.0558
Data are expressed as the mean ± SD in 3 independent experiments.					
Study Number [Location in CTD]	S-217622-EB-122-N [4.2.1.1-07]				

2.6.3.2.8 抗 SARS-CoV-2 活性に対するヒト血清及びマウス血清の影響

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with SARS-CoV-2 clinical isolates hCoV-19/Japan/TY7-501/2021 (Pango Lineage: P.1)
Final concentration as free form (μmol/L)	0.02, 0.07, 0.21, 0.62, 1.85, 5.56, 16.67, 50
Reference substance (Final concentration [μmol/L])	Remdesivir: 0.02, 0.07, 0.21, 0.62, 1.85, 5.56, 16.67, 50
Vehicle	0.5% DMSO
Evaluation item	EC_{50}: The concentration achieving 50% of inhibition on CPE induced by SARS-CoV-2, PA-EC_{50} extrapolated to 100% serum, PS extrapolated to 100% serum
Evaluation Method	Viral replication inhibition assay: The test and reference substances were diluted with DMSO and various concentrations of HS or MS assay medium. One hundred microliters of serially diluted substance solutions were added to a 96-well plate, and these solutions were incubated at room temperature for about 1 hour. Each 50 μL/well of VeroE6/TMPRSS2 cells which were adjusted to 3.0×10^5 cells/mL with the viral assay medium was dispensed on the plate. Then, SARS-CoV-2 virus stock solution was diluted the viral assay medium to 1000 (HS) or 10000 (MS) $TCID_{50}$, and each 50 μL/well was dispensed on the 96-well plate containing a test or reference substance. Then, the plates were mixed with a plate mixer, and were incubated at 37°C in a CO_2 incubator for 3 days. The inhibitory effects of the test and reference substances on CPE induced by SARS-CoV-2 virus were measured by cell viability. Measurement of cell viability: After 3 days incubation of the plates prepared in the viral replication inhibition assay, 60 μL of CellTiter-Glo® 2.0 Reagent was added into each well. After mixing with a plate mixer, plates were incubated at room temperature for approximately 30 minutes, and each 200 μL was dispensed on a new white 96-well plate. Luminescent signal was measured by a multiplate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).

Results

Strains	Pango Lineage	Substance	Exp	EC_{50} (μmol/L)				
				HS 25%	HS 12.5%	HS 6.25%	HS 3.125%	HS 0%
hCoV-19/Japan/TY7-501/2021	P.1	S-217622 fumaric acid	1	1.41	1.08	0.77	0.69	0.62
			2	1.01	0.72	0.63	0.64	0.44
			3	0.99	1.37	0.98	0.70	0.52
		Remdesivir	1	2.08	2.07	2.22	2.74	2.06
			2	1.99	2.13	2.11	2.06	1.80
			3	1.96	2.15	2.03	1.98	2.25
Strains	Pango Lineage	Substance	Exp	EC_{50} (μmol/L)				
				MS 12.5%	MS 6.25%	MS 3.125%	MS 1.5625%	MS 0%
hCoV-19/Japan/TY7-501/2021	P.1	S-217622 fumaric acid	1	1.31	0.73	0.67	0.76	0.71
			2	0.75	0.66	0.55	0.66	0.59
			3	1.17	0.75	0.67	0.68	0.67
		Remdesivir	1	23.89	18.67	12.27	11.94	5.60
			2	32.87	22.90	9.86	6.78	3.23
			3	34.47	23.16	15.66	6.76	5.09

Strains	Pango Lineage	S-217622 fumaric acid				Remdesivir			
		Extrapolated values to 100% human serum		Extrapolated values to 100% mouse serum		Extrapolated values to 100% human serum		Extrapolated values to 100% mouse serum	
		PA-EC_{50} (μmol/L)	Potency shift	PA-EC_{50} (μmol/L)	Potency shift	PA-EC_{50} (μmol/L)	Potency shift	PA-EC_{50} (μmol/L)	Potency shift
hCoV-19/Japan/TY7-501/2021	P.1	3.02 ± 0.76	6 ± 1	3.93 ± 1.88	6 ± 2	1.67 ± 0.60	1 ± 0	213.98 ± 60.31	51 ± 26
Exp = experiment; PA-EC_{50} = protein-adjusted EC_{50}; HS = human serum; MS = mouse serum; PS = potency shift Data are expressed as the mean ± SD in 3 independent experiments. PA-EC_{50} extrapolated to 100% serum was calculated by linear regression using the EC_{50} value of each serum concentration. PS extrapolated to 100% serum were calculated by dividing PA-EC_{50} (extrapolated value of 100% HS or MS) by EC_{50} (in the presence of 0% HS or MS).									
Study Number [Location in CTD]		S-217622-EB-032-N [4.2.1.1-08]							

2.6.3.2.9 S-217622 フマル酸共結晶に対する SARS-CoV-2 耐性分離試験

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with SARS-CoV-2 clinical isolates hCoV-19/Japan/TY7-501/2021 (Pango Lineage: P.1)
Initial concentration as free form (μmol/L)	0.041, 0.12, 0.37, 1.1, 3.3, 10 When CPE was observed, 1/10 of the cell suspensions was passaged by adding fresh cells containing or not containing same concentration range of test substance.
Reference article (Final concentration)	N/A
Vehicle	DMSO
Evaluation items	Amino acid substitution of SARS-CoV-2 (hCoV-19/Japan/TY7-501/2021) nsp5 region
Evaluation method	The day after seeding and culturing 3.0×10^6 cells of VeroE6/TMPRSS2 cells in 75 cm^2 flask, VeroE6/TMPRSS2 cells were infected with SARS-CoV-2 (75,000 $TCID_{50}$) and cultured at 37°C, 5% CO_2. Since cytopathic effect was observed after culturing for 2 days, suspension of SARS-CoV-2 infected VeroE6/TMPRSS2 cells was used for isolation of drug resistant viruses. For the initial passage, assay medium containing or not containing test substance dilutions (250 μL/well) was added to a 12-well plate. Five hundred microliters of VeroE6/TMPRSS2 cells suspension (4×10^5 cells/mL) was added to each well, and then suspension of SARS-CoV-2 infected VeroE6/TMPRSS2 cells was added to each well (250 μL/well). The plate was incubated at 37°C, 5% CO_2 and SARS-CoV-2 infected VeroE6/TMPRSS2 cells were observed under microscopy. When CPE was observed, 100 μL of the cell suspensions was passaged by adding 2×10^5 cells of fresh VeroE6/TMPRSS2 cells in assay medium containing or not containing test substance (900 μL/well). In this study, carrying over the test substance during passage was not considered.To isolate the drug resistant viruses, the CPE positive wells up to three points from the higher concentration of test substance was passaged at every 2 or 3 days. This passage was repeated for 4 times. At the end of culture, culture fluid of cells indicating CPE was collected. DNA of nsp5 region was amplified by RT-PCR using viral RNA extracted from the collected culture fluid and sanger sequencing of the RT-PCR products were conducted. When the second peak was identified at more than 10% of the first peak in the sequencing data, the nucleotide was judged as mixed base. Alignment of the obtained RT-PCR product sequences and SARS-CoV-2 nsp5 region sequence of virus used in this study was conducted by using software GENETYX ver.14.1.0 and amino acid substitutions of each sample were identified.

Results

Amino Acid Substitution in the nsp5 Region of SARS-CoV-2 Variants Isolated in the Resistant SARS-CoV-2 Isolation Study of S-217622 Fumaric Acid in VeroE6/TMPRSS2 Cells

ID	Concentration of S-217622 fumaric acid at culture (μmol/L)				CPE of Passage 4 (%)	amino acid substitution	nucleotide change of codon	synonymous substitution (L75)	294th amino acid
	Passage 1	Passage 2	Passage 3	Passage 4					
hCoV-19/Japan/TY7-501/2021/P4-1	10	10	10	10	0~50	S144A	TCA → GCA	+	F
hCoV-19/Japan/TY7-501/2021/P4-2	10	3.3	10	10	50~90	S144A	TCA → GCA	+	F
hCoV-19/Japan/TY7-501/2021/P4-3	10	1.1	10	10	0~50	M49L	ATG → CTT	−	L
hCoV-19/Japan/TY7-501/2021/P4-4	3.3	10	10	10	90~100	M49L	ATG → YTG	−	F/L
hCoV-19/Japan/TY7-501/2021/P4-5	3.3	3.3	10	10	90~100	S144A	TCA → GCA	+ / −	F/L
hCoV-19/Japan/TY7-501/2021/P4-6	3.3	1.1	10	10	50~90	M49(M/L)/ S144A	M49(M/L): ATG → MTG, S144A: TCA → GCA	+	F
hCoV-19/Japan/TY7-501/2021/P4-7	1.1	10	10	10	0~50	S144A	TCA → GCA	+	F
hCoV-19/Japan/TY7-501/2021/P4-8	1.1	3.3	10	10	90~100	S144A	TCA → GCA	−	F
hCoV-19/Japan/TY7-501/2021/P4-9	1.1	1.1	10	10	90~100	M49L	ATG → TTG	−	F/L
hCoV-19/Japan/TY7-501/2021/P4-10	0	0	0	0	90~100	No mutation	−	−	F/L
hCoV-19/Japan/TY7-501/2021/P4-11	0	0	0	0	90~100	No mutation	−	−	F/L

F = phenylalanine; F/L = mixture of phenylalanine and leucine; L75 = leucin at amino acid position 75; M49L = methionine-to-leucine at amino acid position 49;
M49(M/L) = mixture of no substation and methionine-toleucine at amino acid position 49; RT-PCR = reverse transcription polymerase chain reaction;
S144A = serine-to-alanine at amino acid position 144

CPE of Passage 4 indicated that the range of percentage of CPE in whole well in observation under microscopy at passage 4.

Amino acids indicated by slash means multiple amino acid substitutions were identified. Two amino acids indicated by slash in parentheses means mixture of 2 amino acid sequence which was identified in same position.

In some samples, the nucleotide substitution at amino acid position L75 was identified as a synonymous substitution, and the results were shown as follows: '+'; synonymous substitution, '−'; no substitution, '+ / −'; mixture with parent base and synonymous substitution.

Mixed base: Y → C and T, M→ A and C

The 294th amino acid of parent SARS-CoV-2 (hCoV-19/Japan/TY7-501/2021) nsp5 region was a mixture of F and L. The amino acid after the passage study was shown.

Study number [Location in CTD]	S-217622-EB-074-N [4.2.1.1-09]

2.6.3.2.10 S-217622 フマル酸共結晶に対する SARS-CoV-2 耐性分離試験 (2)

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with SARS-CoV-2 clinical isolates hCoV-19/Japan/TY/WK-521/2020 (Pango Lineage: A), hCoV-19/Japan/QHN001/2020 (Pango Lineage: B.1.1.7), hCoV-19/Japan/TY8-612/2021 (Pango Lineage: B.1.351), and hCoV-19/Japan/TY11-927-P1/2021 (Pango Lineage: B.1.617.2)
Initial concentration as free form (μmol/L)	0.041, 0.12, 0.37, 1.1, 3.3, 10 When CPE was observed, 1/10 of the cell suspensions was passaged by adding fresh cells containing or not containing same concentration range of test substance.
Reference article (Final concentration)	N/A
Vehicle	DMSO
Evaluation items	Amino acid substitution of SARS-CoV-2 nsp5 region
Evaluation method	The day after seeding and culturing 2.5×10^6 cells of VeroE6/TMPRSS2 cells in 75 cm^2 flask, VeroE6/TMPRSS2 cells were infected with SARS-CoV-2 (75,000 $TCID_{50}$) and cultured at 37°C, 5% CO_2. Since CPE was observed after culturing for 2 days, suspension of SARS-CoV-2 infected VeroE6/TMPRSS2 cells was used for isolation of drug resistant viruses. For the initial passage, assay medium containing or not containing test substance dilutions (250 μL/well) was added to a 12-well plate. Five hundred microliters of VeroE6/TMPRSS2 cells suspension (4×10^5 cells/mL) was added to each well, and then suspension of SARS-CoV-2 infected VeroE6/TMPRSS2 cells was added to each well (250 μL/well). The plate was incubated at 37°C, 5% CO_2 and SARS-CoV-2 infected VeroE6/TMPRSS2 cells were observed under microscopy. When CPE was observed, 100 μL of the cell suspensions was passaged by adding 2×10^5 cells of fresh VeroE6/TMPRSS2 cells in assay medium containing or not containing test substance (900 μL/well). In this study, carrying over the test substance during passage was not considered.To isolate the drug resistant viruses, the CPE positive wells up to 4 points from the higher concentration of test substance was passaged at every 2 or 3 days. This passage was repeated for 4 times under the condition with test substance. At the end of culture, culture fluid with the highest concentration of test substance among indicating more than 50% CPE was collected. DNA of nsp5 region was amplified by RT-PCR using viral RNA extracted from the collected culture fluid and sanger sequencing of the RT-PCR products were conducted. When the second peak was identified at more than 10% of the first peak in the sequencing data, the nucleotide was judged as mixed base. Alignment of the obtained RT-PCR product sequences and SARS-CoV-2 nsp5 region sequence of virus used in this study was conducted by using software GENETYX ver.14.1.0 and amino acid substitutions of each sample were identified.

Results									
Amino Acid Substitution in the nsp5 Region of SARS-CoV-2 in the Resistant Virus Isolation Study of S-217622 Fumaric Acid in VeroE6/TMPRSS2 Cells									
Strain	ID[a]	Concentration of S-217622 fumaric acid at each point (μmol/L)					CPE (%) at the end of culture	Amino acid substitutions	Nucleotide changes in identified amino acid substitutions
		Passage 1	Passage 2	Passage 3	Passage 4	Passage 5[b]			
hCoV-19/Japan/TY/WK-521/2020	−	10	10	10	0.041 - 10	−	1 - 50	NT	NT
	−	10	3.3	10	0.041 - 10	−	1 - 50	NT	NT
	−	3.3	10	10	0.041 - 10	−	1 - 50	NT	NT
	−	3.3	3.3	10	0.041 - 10	−	1 - 50	NT	NT
	P4-1	1.1	10	10	10	−	91 - 100	M49L	ATG → CTG
	P4-2	1.1	3.3	10	10	−	91 - 100	M49L	ATG → TTG
	P4-3	0.37	10	10	10	−	91 - 100	M49L	ATG → CTG
	P4-4	0.37	3.3	10	10	−	51 - 90	M49L	ATG → CTG
	P5-1	0	0	0	0	0	91 - 100	No mutation	No mutation
hCoV-19/Japan/QHN001/2020	−	10	10	10	0.041 - 10	−	1 - 50	NT	NT
	−	10	3.3	10	0.041 - 10	−	1 - 50	NT	NT
	−	3.3	10	10	0.041 - 10	−	1 - 50	NT	NT
	P4-1	3.3	3.3	10	10	−	91 - 100	M49L	ATG → CTG
	P4-2	3.3	3.3	3.3	10	−	91 - 100	M49L	ATG → CTG
	−	1.1	10	10	0.041 - 10	−	1 - 50	NT	NT
	P4-3	1.1	3.3	10	10	−	91 - 100	M49(M/L)/S144A	M49(M/L): ATG → WTG[c], S144A: TCA → GCA
	P4-4	1.1	3.3	3.3	10	−	91 - 100	S144A	TCA → GCA
	−	0.37	10	10	0.041 - 10	−	1 - 50	NT	NT
	−	0.37	3.3	10	0.041 - 10	−	1 - 50	NT	NT
	P4-5	0.37	3.3	3.3	10	−	51 - 90	D48G	GAC → GGC
	P4-6	0.37	3.3	1.1	10	−	51 - 90	D48G	GAC → GGC
	P5-1	0	0	0	0	0	91 - 100	No mutation	No mutation

Strain	ID[a]	Concentration of S-217622 fumaric acid at each point (μmol/L)					CPE (%) at the end of culture	Amino acid substitutions	Nucleotide changes in identified amino acid substitutions
		Passage 1	Passage 2	Passage 3	Passage 4	Passage 5[b]			
hCoV-19/Japan/TY8-612/2021	−	10	10	10	0.041 - 10	−	1 - 50	NT	NT
	P4-4	10	3.3	10	10	−	91 - 100	M49L	ATG → TTG
	−	3.3	10	10	0.041 - 10	−	1 - 50	NT	NT
	P4-5	3.3	3.3	10	10	−	91 - 100	P52S	CCT → TCT
	P4-1	1.1	10	10	10	−	51 - 90	M49L	ATG → TTG
	P4-2	1.1	3.3	10	10	−	51 - 90	M49L	ATG → TTG
	−	0.37	10	10	0.041 - 10	−	1 - 50	NT	NT
	P4-3	0.37	3.3	10	10	−	91 - 100	M49L	ATG → CTG
	P5-1	0	0	0	0	0	91 - 100	No mutation	No mutation
Strain	ID [a]	Concentration of S-217622 fumaric acid at each point (μmol/L)					CPE (%) at the end of culture	Amino acid substitutions	Nucleotide changes in identified amino acid substitutions
		Passage 1	Passage 2	Passage 3	Passage 4	Passage 5 [b]			
hCoV-19/Japan/TY11-927-P1/2021	−	10	10	10	0.041 - 10	−	1 - 50	NT	NT
	−	10	3.3	10	0.041 - 10	−	1 - 50	NT	NT
	−	3.3	10	10	0.041 - 10	−	1 - 50	NT	NT
	−	3.3	3.3	10	0.041 - 10	−	1 - 50	NT	NT
	P4-1	3.3	1.1	10	10	−	91 - 100	M49L	ATG → TTG
	P4-2	3.3	1.1	3.3	10	−	91 - 100	M49L	ATG → TTG
	−	1.1	10	10	0.041 - 10	−	1 - 50	NT	NT
	−	1.1	3.3	10	0.041 - 10	−	1 - 50	NT	NT
	P4-3	1.1	1.1	10	1.1	−	51 - 90	D48G	GAC → GGC
	P4-4	1.1	1.1	3.3	1.1	−	91 - 100	D48G	GAC → GGC
	P5-1	0	0	0	0	0	91 - 100	No mutation	No mutation

D48G = asparatic acid-to-glycine at amino acid position 48, NT = not tested, P52S = proline-to-serine at amino acid position 52

CPE (%) at the end of culture: the degree of CPE in whole well in observation under microscopy at the end of culture.

Amino acids indicated by slash means multiple amino acid substitutions were identified. Two amino acids indicated by slash in parentheses means mixture of 2 amino acid sequence which was identified in same position.

a Minus means that ID was not given because it was not subject to sequence analysis.

b Minus means that the passage was not conducted because it was completed in 4 times.

c Mixed base: W → A and T

Study number [Location in CTD]	S-217622-EB-079-N [4.2.1.1-10]

2.6.3.2.11 S-217622 フマル酸共結晶に対する SARS-CoV-2 変異体の薬剤感受性試験

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with SARS-CoV-2 mutants isolated in the presence of S-217622 fumaric acid
Final concentration as free form (μmol/L)	0.046, 0.14, 0.41, 1.2, 3.7, 11, 33, 100
Reference substance (Final concentration [μmol/L])	Remdesivir: 0.046, 0.14, 0.41, 1.2, 3.7, 11, 33, 100
Vehicle	0.5% DMSO
Evaluation item	EC_{50}: The concentration achieving 50% inhibition on cell death caused by SARS-CoV-2 infection Fold Change: The fold change against each SARS-CoV-2 strain was calculated as relative EC_{50} to reference strain
Evaluation Method	Antiviral assay: Fifty microliters of serially diluted substance solutions were added to 96-well plates. Then, 100 μL of VeroE6/TMPRSS2 cell suspension (1.5×10^5 cells/mL) was added to the 96-well plates. Finally, 50 μL of SARS-CoV-2 solution was added to the wells containing test or reference substance and virus control wells of the 96-well plates (30 $TCID_{50}$: hCoV-19/Japan/TY8-612/2021 or 1000 $TCID_{50}$: other strains). Only assay medium was added to cell control wells of the 96-well plates. These plates were incubated at 37°C, 5% CO_2 for 3 days. The inhibitory effects of the test or reference substance on cytopathic effect induced by SARS-CoV-2 infection was measured by cell viability. Measurement of cell viability: After 3 days incubation of the plates prepared in the antiviral assay, 60 μL of CellTiter-Glo® 2.0 Reagent was added into each well and incubated at room temperature for approximately 30 minutes. After incubation, 200 μL of solution in each well was transferred to new white 96-well plate. Luminescent signal was measured by a multimode plate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).

Results						
Strain	ID[a]	Amino acid substitution of nsp5 region[b]	EC_{50} (μmol/L)		Fold change	
			S-217622 fumaric acid	Remdesivir	S-217622 fumaric acid	Remdesivir
hCoV-19/Japan/TY/WK-521/2020	P4-1	M49L	5.9 ± 2.6	1.1 ± 0.2	12 ± 5	0.40 ± 0.03
	P5-1	No mutation	0.47 ± 0.02	2.8 ± 0.2	1.0	1.0
hCoV-19/Japan/QHN001/2020	P4-1	M49L	12 ± 1	1.6 ± 0.2	26 ± 1	0.58 ± 0.09
	P4-4	S144A	4.0 ± 0.3	1.4 ± 0.2	8.5 ± 0.4	0.49 ± 0.07
	P4-5	D48G	1.8 ± 0.4	2.0 ± 0.3	3.7 ± 0.7	0.71 ± 0.09
	P5-1	No mutation	0.47 ± 0.01	2.8 ± 0.7	1.0	1.0
hCoV-19/Japan/TY8-612/2021	P4-1	M49L	10 ± 1	1.1 ± 0.1	21 ± 1	0.66 ± 0.12
	P4-5	P52S	2.6 ± 0.8	1.4 ± 0.0	5.5 ± 1.5	0.86 ± 0.19
	P5-1	No mutation	0.48 ± 0.00	1.7 ± 0.4	1.0	1.0
hCoV-19/Japan/TY7-501/2021	P4-3[c]	M49L	13 ± 0	3.1 ± 0.8	27 ± 1	1.8 ± 0.2
	P4-6[d]	M49(M/L)/S144A	49 ± 5	1.6 ± 0.3	100 ± 10	0.97 ± 0.08
	P4-7[d]	S144A	5.4 ± 1.1	3.0 ± 1.1	11 ± 2	1.8 ± 0.2
	P4-10[c]	No mutation	0.48 ± 0.02	1.7 ± 0.4	1.0	1.0
hCoV-19/Japan/TY11-927-P1/2021	P4-2	M49L	13 ± 1	1.7 ± 0.3	41 ± 2	1.1 ± 0.1
	P4-4	D48G	2.0 ± 0.4	2.7 ± 0.2	6.5 ± 0.9	1.7 ± 0.2
	P5-1	No mutation	0.31 ± 0.03	1.6 ± 0.3	1.0	1.0

Fold change: EC_{50} value against each SARS-CoV-2 strain/EC_{50} value against reference strain
Data are expressed as the mean ± SD of 3 independent experiments.
a Corresponds to the samples obtained in the previous study (S-217622-EB-074-N, S-217622-EB-079-N).
b S-217622 fumaric acid treatment emerged amino acid substitutions are shown.
c ID P4-3 has amino acid substitution at position 294 (F294L). ID P4-10 is the mixture of F294F and F294L. Since parent strain used in the previous study is the mixture of F294F and F294L, it was not considered to be S-217622 fumaric acid treatment-induced amino acid substitution.
d Amino acid at position 75 has synonymous substitutions.
e M49(M/L)/S144A is the mixture of S144A and M49L/S144A.

Study Number [Location in CTD]	S-217622-EB-090-N [4.2.1.1-11]

2.6.3.2.12 S-217622 フマル酸共結晶に対するリバースジェネティクス由来 SARS-CoV-2 変異体の複製能力及び薬剤感受性試験

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with reverse genetics-derived SARS-CoV-2 mutants to S 217622 fumaric acid in cultured cell
Final concentration as free form (μmol/L)	0.011, 0.034, 0.10, 0.31, 0.93, 2.8, 8.3, 25 or 0.046, 0.14, 0.41, 1.2, 3.7, 11, 33, 100
Reference substance (Final concentration)	Remdesivir: 0.023, 0.069, 0.21, 0.62, 1.9, 5.6, 17, 50 μmol/L, PF-07321332: 0.0046, 0.014, 0.041, 0.12, 0.37, 1.1, 3.3, 10 μmol/L, REGN-COV2: REGN antibody cocktail (REGN10933 and REGN10987): 0.00091, 0.0027, 0.0082, 0.025, 0.074, 0.22, 0.67, 2.0 μg/mL
Vehicle	0.5% DMSO and 0.5% PBS
Evaluation item	The mean EC_{50} and the mean fold change in EC_{50} The virus titers
Evaluation Method	Antiviral assay: Fifty microliters of serially diluted substance solutions were added to 96-well plates. Then, 50 μL of SARS-CoV-2 solution was added to the wells containing test or reference substance and virus control wells of the 96-well plates (1000 $TCID_{50}$/well). Only viral assay medium was added to cell control wells of the 96-well plates. Finally, 100 μL of VeroE6/TMPRSS2 cell suspension (1.5×10^5 cells/mL) was added to the 96-well plates. These plates were incubated at 37°C, 5% CO_2 for 3 days. [redacted] Reporter Assay: After 3 days incubation of the plates prepared in the antiviral assay, 20 μL of [redacted] was added into each well and incubated at room temperature for approximately 15 minutes. After incubation, luminescent signal was measured by a multimode plate reader (EnSpire™, PerkinElmer). Viral replication Assay: VeroE6/TMPRSS2 cells were seeded on 6-well plates (4.5×10^5 cells/well). The next day, VeroE6/TMPRSS2 cells were infected with SARS-CoV-2 (1000 $TCID_{50}$/well) and incubated at 37°C, 5% CO_2 for 1 hour. After incubation, the cells were washed with viral assay medium. The viral assay medium was added to cells and the plates were incubated at 37°C, 5% CO_2. The cultures were performed in triplicate. The culture fluid of VeroE6/TMPRSS2 cells were collected at 0, 16, 24, 48, 72 hours after medium change. The collected samples were stored at −80°C until the viral titration was performed. Measurement of the Viral Titers: One hundred microliters of VeroE6/TMPRSS2 cell suspension (1.5×10^5 cells/mL) were added to the 96-well plates. The stored culture fluids were diluted by 10-fold serial dilution scheme with the viral assay medium. Then, 100 μL of the diluted samples were added into the plates and the plates were incubated at 37°C, 5% CO_2 for 4 days. Finally, virus-induced CPE was observed under a microscopy. Viral titers were expressed as log $TCID_{50}$/mL, and lower limit of detection of viral titer was defined as 1.5 log $TCID_{50}$/mL when no CPE was observed in the lowest dilution (10-fold).

Strain	EC_{50}[a]				Fold change in EC_{50} [b]			
	S-217622 fumaric acid	Remdesivir	PF-07321332[c]	REGN-COV2[d]	S-217622 fumaric acid	Remdesivir	PF-07321332[c]	REGN-COV2[d]
rgSARS-CoV-2/Hu/DP/Kng/19-020 (wild-type)	0.11 ± 0.04	1.6 ± 0.0	0.042 ± 0.004	0.22 ± 0.02	1.0	1.0	1.0	1.0
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/M49L	1.7 ± 0.3	0.85 ± 0.04	0.029 ± 0.007	0.21 ± 0.01	17 ± 6	0.52 ± 0.03	0.68 ± 0.15	0.93 ± 0.02
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/S144A	0.92 ± 0.35	1.5 ± 0.3	0.057 ± 0.003	0.22 ± 0.02	9.2 ± 5.8	0.90 ± 0.19	1.4 ± 0.2	1.0 ± 0.0
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/M49L/S144A	11 ± 1	0.86 ± 0.05	0.055 ± 0.004	0.21 ± 0.01	100 ± 30	0.53 ± 0.03	1.3 ± 0.0	0.95 ± 0.03

PBS = phosphate buffered saline
Fold change: EC_{50} value against each SARS-CoV-2 strain/EC_{50} value against wild-type virus
Data are expressed as the mean ± SD of 3 independent experiments.
a S-217622 fumaric acid, Remdesivir and PF-07321332: μmol/L, REGN-COV2: μg/mL
b EC_{50} value against each SARS-CoV-2 strain/EC_{50} value against wild-type virus.
c In the evaluation of PF-07321332, CP-100356 monohydrochloride (P-glycoprotein inhibitor) was added at the final concentration of 1 μmol/L.
d REGN-COV2: REGN antibody cocktail (REGN10933 and REGN10987)

strain	Time post-infection (hours)	Virus titers (log_{10} $TCID_{50}$/mL)	
		Mean	SD
rgSARS-CoV-2/Hu/DP/Kng/19-020 (wild-type)	0	1.50	0.00
	16	3.22	0.51
	24	5.44	0.19
	48	6.44	0.19
	72	5.44	0.10
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/M49L	0	1.50	0.00
	16	2.94	0.42
	24	4.83	0.29
	48	6.81	0.17
	72	5.61	0.10
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/S144A	0	1.50	0.00
	16	3.22	0.48
	24	5.31	0.57
	48	6.08	0.13
	72	5.44	0.10
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/M49L/S144A	0	1.50	0.00
	16	3.00	0.00
	24	4.67	0.29
	48	6.44	0.10
	72	5.67	0.17
Data represent the mean and SD of triplicate samples			
Study Number [Location in CTD]		S-217622-EB-103-N [4.2.1.1-12]	

2.6.3.2.13 S-217622 フマル酸共結晶に対するリバースジェネティクス由来 SARS-CoV-2 変異体の複製能力及び薬剤感受性試験 (2)

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with reverse genetics-derived SARS-CoV-2 mutants to S 217622 fumaric acid in cultured cell
Final concentration as free form (μmol/L)	0.011, 0.034, 0.10, 0.31, 0.93, 2.8, 8.3, 25
Reference substance (Final concentration)	Remdesivir: 0.023, 0.069, 0.21, 0.62, 1.9, 5.6, 17, 50 μmol/L, PF-07321332: 0.0046, 0.014, 0.041, 0.12, 0.37, 1.1, 3.3, 10 μmol/L, REGN-COV2: REGN antibody cocktail (REGN10933 and REGN10987): 0.00091, 0.0027, 0.0082, 0.025, 0.074, 0.22, 0.67, 2.0 μg/mL
Vehicle	0.5% DMSO and 0.5% PBS
Evaluation item	The mean EC_{50} and the mean fold change in EC_{50} The virus titers
Evaluation Method	Antiviral assay: Fifty microliters of serially diluted substance solutions were added to 96-well plates. Then, 50 μL of SARS-CoV-2 solution was added to the wells containing test or reference substance and virus control wells of the 96-well plates (1000 $TCID_{50}$/well). Only viral assay medium was added to cell control wells of the 96-well plates. Finally, 100 μL of VeroE6/TMPRSS2 cell suspension (1.5×10^5 cells/mL) was added to the 96-well plates. These plates were incubated at 37°C, 5% CO_2 for 3 days. [redacted] Reporter Assay: After 3 days incubation of the plates prepared in the antiviral assay, 20 μL of [redacted] was added into each well and incubated at room temperature for approximately 15 minutes. After incubation, luminescent signal was measured by a multimode plate reader (EnSpire™, PerkinElmer). Viral replication Assay: VeroE6/TMPRSS2 cells were seeded on 6-well plates (4.5×10^5 cells/well). The next day, VeroE6/TMPRSS2 cells were infected with SARS-CoV-2 (1000 $TCID_{50}$/well) and incubated at 37°C, 5% CO_2 for 1 hour. After incubation, the cells were washed with viral assay medium. The viral assay medium was added to cells and the plates were incubated at 37°C, 5% CO_2. The cultures were performed in triplicate. The culture fluid of VeroE6/TMPRSS2 cells were collected at 0, 16, 24, 48, 72 hours after medium change. The collected samples were stored at −80°C until the viral titration was performed. Measurement of the Viral Titers: One hundred microliters of VeroE6/TMPRSS2 cell suspension (1.5×10^5 cells/mL) were added to the 96-well plates. The stored culture fluids were diluted by 10-fold serial dilution scheme with the viral assay medium. Then, 100 μL of the diluted samples were added into the plates and the plates were incubated at 37°C, 5% CO_2 for 4 days. Finally, virus-induced CPE was observed under a microscopy. Viral titers were expressed as log $TCID_{50}$/mL, and lower limit of detection of viral titer was defined as 1.5 log $TCID_{50}$/mL when no CPE was observed in the lowest dilution (10-fold).

Strain	EC_{50}[a]				Fold change			
	S-217622 fumaric acid	Remdesivir	PF-07321332 [b]	REGN-COV2[c]	S-217622 fumaric acid	Remdesivir	PF-07321332[b]	REGN-COV2[c]
rgSARS-CoV-2/Hu/DP/Kng/19-020 (wild-type)	0.11 ± 0.03	1.7 ± 0.1	0.037 ± 0.008	0.21 ± 0.01	1.0	1.0	1.0	1.0
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/D48G	0.49 ± 0.25	1.7 ± 0.1	0.038 ± 0.014	0.19 ± 0.08	4.3 ± 1.1	1.0 ± 0.1	1.0 ± 0.3	0.91 ± 0.31
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/P52S	0.44 ± 0.33	1.8 ± 0.2	0.021 ± 0.013	0.19 ± 0.01	3.7 ± 1.9	1.0 ± 0.2	0.54 ± 0.20	0.91 ± 0.01

Fold change: EC_{50} value against each SARS-CoV-2 strain/EC_{50} value against wild-type virus
Data are expressed as the mean ± SD of 3 independent experiments.
a S-217622 fumaric acid, Remdesivir and PF-07321332: μmol/L, REGN-COV2: μg/mL
b In the evaluation of PF-07321332, CP-100356 monohydrochloride was added at the final concentration of 1 μmol/L.
c REGN-COV2: REGN antibody cocktail (REGN10933 and REGN10987)

strain	Time post-infection (hours)	Virus titers (log_{10} $TCID_{50}$/mL)	
		Mean	SD
rgSARS-CoV-2/Hu/DP/Kng/19-020 (wild-type)	0	1.50	0.00
	16	3.30	0.34
	24	5.67	0.29
	48	6.44	0.19
	72	5.56	0.10
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/D48G	0	1.50	0.00
	16	3.28	0.54
	24	4.72	0.10
	48	6.28	0.25
	72	5.56	0.10
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/P52S	0	1.50	0.00
	16	3.78	0.19
	24	5.06	0.10
	48	6.33	0.29
	72	5.85	0.36
Data represent the mean and SD of triplicate samples			
Study Number [Location in CTD]		S-217622-EB-116-N [4.2.1.1-13]	

2.6.3.2.14 抗 SARS-CoV-2 薬との併用による S-217622 フマル酸共結晶の SARS-CoV-2 感染細胞に対するウイルス増殖抑制試験

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with SARS-CoV-2 clinical isolate hCoV-19/Japan/TY11-927-P1/2021 (Pango Lineage: B.1.617.2)
Final concentration of test substances	Viral replication inhibition assay: S-217622 as free form: 0.0313, 0.0625, 0.125, 0.250, 0.500, 1.00, 2.00 μmol/L, which are equal to 0.0166, 0.0332, 0.0665, 0.133, 0.266, 0.532, 1.06 μg/mL Remdesivir: 0.0234, 0.0469, 0.0938, 0.188, 0.375,0.750, 1.50, 3.00, 6.00 μmol/L REGN-COV2: 0.00195, 0.00391, 0.00781, 0.0156, 0.0313, 0.0625, 0.125, 0.250, 0.500 μg/mL Cytotoxicity assay: S-217622 as free form: 0.0313, 0.0625, 0.125, 0.250, 0.500, 1.00, 2.00 μmol/L, which are equal to 0.0166, 0.0332, 0.0665, 0.133, 0.266, 0.532, 1.06 μg/mL Remdesivir: 0.0234, 0.0469, 0.0938, 0.188, 0.375,0.750, 1.50, 3.00, 6.00 μmol/L REGN-COV2: 0.00195, 0.00391, 0.00781, 0.0156, 0.0313, 0.0625, 0.125, 0.250, 0.500 μg/mL
Vehicle	0.5% DMSO for combinarion of S-217622 and remdesivir 0.25% DMSO and 0.25% DPBS for combination of S-217622 and REGN-COV2
Evaluation item	EC_{50}: The concentrations achieving 50% of inhibition against SARS-CoV-2 replication CI value: CI values under the condition that both substances (substance A and substance B) were added at the closest ratio of each EC_{50} CC_{50}: The concentrations achieving 50% of cytotoxicity
Evaluation Method	Viral replication inhibition assay: Twenty-five microliters/well of serially diluted S-217622 fumaric acid and remdesivir, or S-217622 fumaric acid and REGN-COV2 were added into each row or lane of 96-well plates respectively. Then, 50 μL/well of diluted SARS-CoV-2 virus solution was added into the plate containing test substances. Finally, 100 μL/well of VeroE6/TMPRSS2 cells which were adjusted to 1.5×10^5 cells/mL with the viral assay medium was added into the plate. The plates were mixed with a plate mixer and incubated at 37°C in a CO_2 incubator for 3 days. The infectious dose of virus was 1000 $TCID_{50}$/well. The inhibitory effects of the test substances on cytopathic effect induced by SARS-CoV-2 were measured by cell viability. Cytotoxicity assay: Twenty-five microliters/well of serially diluted S-217622 fumaric acid and remdesivir, or S-217622 fumaric acid and REGN-COV2 were added into each row or lane of 96-well plates respectively. Then, 50 μL/well of the viral assay medium was added into the plate containing test substances. Finally, 100 μL/well of VeroE6/TMPRSS2 cells which were adjusted to 1.5×10^5 cells/mL with the viral assay medium was added into the plate. The plates were mixed with a plate mixer and incubated at 37°C in a CO_2 incubator for 3 days. Cytotoxicity of the test substances in VeroE6/TMPRSS2 cells was measured by cell viability. Measurement of cell viability: After 3 days incubation of the plates prepared for the viral replication inhibition assay and cytotoxicity assay, 60 μL of CellTiter Glo® 2.0 Reagent was added into each well. After mixing with a plate mixer, plates were incubated at room temperature for approximately 30 minutes, and each 180 μL was dispensed on a new white 96-well plate. Luminescent signal was measured by a multiplate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).
Results	
D_A, D_B, $D_{A/A+B}$, $D_{B/A+B}$, CI values and combination effect of each combination	
(a) S-217622 fumaric acid and remdesivir (substance A: S-217622 fumaric acid, substance B: remdesivir)	

Exp. No.	D_A (μmol/L)	D_B (μmol/L)	$D_{A/A+B}$ (μmol/L)	$D_{B/A+B}$ (μmol/L)	CI	combination effect
1	0.510	2.40	0.203	1.22	1.11	additive
2	0.415	2.36	0.175	1.05	1.06	additive
3	0.469	2.35	0.181	1.09	1.03	additive

(b) S-217622 fumaric acid and REGN-COV2 (substance A:S-217622 fumaric acid, substance B: REGN-COV2)

Exp. No.	D_A (μg/mL)	D_B (μg/mL)	$D_{A/A+B}$ (μg/mL)	$D_{B/A+B}$ (μg/mL)	CI	combination effect
1	0.279	0.0420	0.120	0.0141	0.913	additive
2	0.269	0.0389	0.0950	0.0112	0.742	synergy
3	0.309	0.0603	0.0884	0.0208	0.730	synergy

CI = combination index
D_A: the EC_{50} of substance A alone
D_B: the EC_{50} of substance B alone
$D_{A/A+B}$: the concentration of substance A giving 50% inhibition in combination with substance B at the closest ratio of each EC_{50} value
$D_{B/A+B}$: the concentration of substance B giving 50% inhibition in combination with substance A at the closest ratio of each EC_{50} value
CI values were calculated by the following formula: $CI = (D_{A/A+B})/D_A + (D_{B/A+B})/D_B + (D_{A/A+B} \times D_{B/A+B})/(D_A \times D_B)$
Combination effect was determined according to the criteria as listed below:
$CI \leq 0.8$: synergy
$0.8 < CI < 1.2$: additive
$1.2 \leq CI$: antagonism

CC_{50} values of each substance alone and combination of substance A and substance B at the closest ratio of each EC_{50} value

(a) S-217622 fumaric acid and remdesivir (substance A: S-217622 fumaric acid, substance B: remdesivir)

	CC_{50} (μmol/L)		
	Exp.1	Exp.2	Exp.3
substance A	>2.00	>2.00	>2.00
substance B	>6.00	>6.00	>6.00
substance A + B	>7.00	>7.00	>7.00

(b) S-217622 fumaric acid and REGN-COV2 (substance A:S-217622 fumaric acid, substance B: REGN-COV2)

	CC_{50} (μg/mL)		
	Exp.1	Exp.2	Exp.3
substance A	>1.06	>1.06	>1.06
substance B	>0.500	>0.500	>0.500
substance A + B	>1.19	>1.19	>1.31

Study number [Location in CTD] S-217622-EB-094-N [4.2.1.1-14]

2.6.3.2.15 抗 SARS-CoV-2 薬との併用による S-217622 フマル酸共結晶の SARS-CoV-2 感染細胞に対するウイルス増殖抑制試験 (2)

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with reverse genetics-derived SARS-CoV-2 (rgSARS-CoV-2/Hu/DP/Kng/19-020))
Final concentration of test substances	S-217622 as free form: 0.00500, 0.0100, 0.0200, 0.0400, 0.0800, 0.160, 0.320 µmol/L EIDD-1931: 0.00625, 0.0125, 0.0250, 0.0500, 0.100, 0.200, 0.400, 0.800, 1.60 µmol/L PF-07321332: 0.0250, 0.0500, 0.100, 0.200, 0.400, 0.800, 1.60, 3.20, 6.40 µmol/L
Vehicle	0.5% DMSO
Evaluation item	EC_{50}: The concentrations achieving 50% of inhibition against SARS-CoV-2 replication CI value: CI values under the condition that both substances (substance A and substance B) were added at the closest ratio of each EC_{50} CC_{50}: The concentrations achieving 50% of cytotoxicity
Evaluation Method	Viral replication inhibition assay: Twenty-five microliters/well of serially diluted S-217622 fumaric acid and EIDD-1931, or S-217622 fumaric acid and PF-07321332 were added into each row or column of 96-well plates respectively. Then, 50 µL/well of diluted SARS-CoV-2 virus solution was added into the plate containing test substances. Finally, 100 µL/well of VeroE6/TMPRSS2 cells which were adjusted to 1.5×10^5 cells/mL with the viral assay medium was added into the plate. The plates were mixed with a plate mixer and incubated at 37°C in a CO_2 incubator for 2 days. The infectious dose of virus was 1000 $TCID_{50}$/well. The assay was performed triplicate in three independent experiments and the inhibitory effects of the test substances on viral replication were measured by [redacted] reporter assay. [redacted]Reporter Assay: After 2 days incubation of the plates prepared in the antiviral assay, 20 µL of [redacted] was added into each well and incubated at room temperature for approximately 15 minutes. After incubation, luminescent signal was measured by a multimode plate reader (EnSpire™, PerkinElmer). Cytotoxicity assay: Twenty-five microliters/well of serially diluted S-217622 fumaric acid and EIDD-1931, or S-217622 fumaric acid and PF-07321332 were added into each row or column of 96-well plates respectively. Then, 50 µL/well of the viral assay medium was added into the plate containing test substances. Finally, 100 µL/well of VeroE6/TMPRSS2 cells which were adjusted to 1.5×10^5 cells/mL with the viral assay medium was added into the plate. The plates were mixed with a plate mixer and incubated at 37°C in a CO_2 incubator for 2 days. Cytotoxicity of the test substances in VeroE6/TMPRSS2 cells was measured by cell viability. Measurement of cell viability: After 2 days incubation of the plates prepared for the viral replication inhibition assay and cytotoxicity assay, 60 µL of CellTiter Glo® 2.0 Reagent was added into each well. After mixing with a plate mixer, plates were incubated at room temperature for approximately 30 minutes, and each 180 µL was dispensed on a new white 96-well plate. Luminescent signal was measured by a multiplate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).
Results	
D_A, D_B, $D_{A/A+B}$, $D_{B/A+B}$, CI values and combination effect of each combination	
(a) S-217622 fumaric acid and EIDD-1931 (substance A: S-217622 fumaric acid, substance B: EIDD-1931)	

Exp. No.	D_A (μmol/L)	D_B (μmol/L)	$D_{A/A+B}$ (μmol/L)	$D_{B/A+B}$ (μmol/L)	CI	combination effect
1	0.0581	0.518	0.0240	0.240	1.07	additive
2	0.0588	0.549	0.0273	0.273	1.19	additive
3	0.0816	0.520	0.0419	0.209	1.12	additive
(b) S-217622 fumaric acid and REGN-COV2 (substance A:S-217622 fumaric acid, substance B: PF-07321332)						
Exp. No.	D_A (μg/mL)	D_B (μg/mL)	$D_{A/A+B}$ (μg/mL)	$D_{B/A+B}$ (μg/mL)	CI	combination effect
1	0.0607	1.21	0.0269	0.538	1.08	additive
2	0.0631	1.11	0.0270	0.541	1.12	additive
3	0.0851	1.84	0.0413	0.826	1.15	additive

CI = combination index
D_A: the EC_{50} of substance A alone
D_B: the EC_{50} of substance B alone
$D_{A/A+B}$: the concentration of substance A giving 50% inhibition in combination with substance B at the closest ratio of each EC_{50} value
$D_{B/A+B}$: the concentration of substance B giving 50% inhibition in combination with substance A at the closest ratio of each EC_{50} value
CI values were calculated by the following formula: $CI = (D_{A/A+B})/D_A + (D_{B/A+B})/D_B + (D_{A/A+B} \times D_{B/A+B})/(D_A \times D_B)$
Combination effect was determined according to the criteria as listed below:
$CI \leq 0.8$: synergy
$0.8 < CI < 1.2$: additive
$1.2 \leq CI$: antagonism

CC_{50} values of each substance alone and combination of substance A and substance B at the closest ratio of each EC_{50} value

(a) S-217622 fumaric acid and EIDD-1931 (substance A: S-217622 fumaric acid, substance B: EIDD-1931)

	CC_{50} (μmol/L)		
	Exp.1	Exp.2	Exp.3
substance A	>0.320	>0.320	>0.320
substance B	>1.60	>1.60	>1.60
substance A + B	>1.76	>1.76	>1.92

(b) S-217622 fumaric acid and PF-07321332 (substance A:S-217622 fumaric acid, substance B: PF-07321332)

	CC_{50} (μg/mL)		
	Exp.1	Exp.2	Exp.3
substance A	>0.320	>0.320	>0.320
substance B	>6.40	>6.40	>6.40
substance A + B	>6.72	>6.72	>6.72

Study number [Location in CTD] S-217622-EB-136-N [4.2.1.1-15]

2.6.3.2.16 SARS-CoV-2 感染マウスモデルにおける S-217622 フマル酸共結晶の PK/PD 解析

Test Article: S-217622 fumaric acid

Species/Strain	Mouse/ BALB/cAJcl
Gender/Number of animals	Pharmacokinetics (PK): Female/4 per each sampling point Pharmacodynamics (PD): Female/5 or 10 per group
Virus	hCoV-19/Japan/TY7-501/2021
Vehicle/Formulation	0.5 w/v% MC
Method of Administration	PK: Oral administration once daily for 1 day under non-anesthesia PD: Oral administration once daily, twice daily (every 12 hours), or four times daily (every 6 hours) for 1 day under non-anesthesia
Dose as free form (mg/kg/shot)	PK: 2, 8, 16, 32 or 64 PD: 0.5, 1, 2, 4, 8, 16, 32, or 64
Treatment	Mice were intranasally inoculated with 1.00×10^4 $TCID_{50}$ (50 μL/mouse) of the virus suspensions under anesthesia. The first administration of S-217622 fumaric acid or vehicle was performed immediately after virus infection.
Evaluation items for PD study	Virus titers in lungs 24 hours after the first administration
Evaluation method for PD study	Virus titers were measured by standard $TCID_{50}$ method using VeroE6/TMPRSS2 cells.
Sample for PK study	Plasma (0.5, 1, 2, 4, 6, 12, 18 and 24 hours post dose)
Analyte for PK study	S-217622
Determination for PK study	LC/MS/MS
PK analysis	The PK parameters on the plasma concentrations of S-217622 were calculated by using Phoenix WinNonlin (Ver. 8.1, Certara, L.P.). The plasma concentrations of all dosing groups in the PD study were simulated by non-parametric analysis from plasma concentration data obtained in the PK study. The PK parameters (C_{max}, $AUC_{0\text{-}24hr}$, C_{24hr}, and $Time_{High}$) were calculated based on the non-compartmental method.
PK/PD analysis	The relationship between PK parameters simulated by non-parametric superposition and non-compartmental method based on the PK study and PD effect of S 217622 fumaric acid 24 hours after first administration.

Articles	Dosing schedule	Dose (mg/kg)	Number of mice	Virus titers (log_{10} $TCID_{50}$/mL)	P-value
Vehicle	bid	–	10	5.48 ± 0.43	-
S-217622 fumaric acid	qd	2	5	4.87 ± 0.37	0.0292
		4	5	4.53 ± 0.32	0.0003
		8	5	3.48 ± 0.35	<0.0001
		16	5	2.71 ± 0.50	<0.0001
		32	5	1.97 ± 0.37	<0.0001
		64	5	1.80 ± 0.00	<0.0001
	bid	1	5	5.08 ± 0.28	0.3191
		2	5	4.83 ± 0.51	0.0289
		4	5	4.55 ± 0.36	0.0008
		8	5	3.75 ± 0.57	<0.0001
		16	5	1.95 ± 0.33	<0.0001
		32	5	1.80 ± 0.00	<0.0001
	qid	0.5	5	5.63 ± 0.00	0.9387
		1	5	5.37 ± 0.28	0.9786
		2	5	5.07 ± 0.36	0.1301
		4	5	4.65 ± 0.25	0.0003
		8	5	2.41 ± 0.44	<0.0001
		16	5	1.80 ± 0.00	<0.0001

Articles	Dosing schedule	Dose (mg/kg)	PK parameters		
			C_{max} [a] (ng/mL)	$AUC_{0\text{-}24hr}$ (ng·hr/mL)	C_{24hr} (ng/mL)
S-217622 fumaric acid	qd	2	7470	39100	6.02
		4	14900	78300	12.0
		8	16700	113000	135
		16	38800	237000	75.8
		32	72400	510000	210
		64	159000	1330000	1290
	bid	1	3740	38300	324
		2	7470	76600	647
		4	14900	153000	1290
		8	16700	219000	1840
		16	38800	466000	3760
		32	72400	993000	11400
	qid	0.5	1870	36000	941
		1	3740	71900	1880
		2	7470	144000	3770
		4	14900	288000	7530
		8	16700	412000	10900
		16	38800	875000	22600

Articles	Dosing schedule	Dose (mg/kg)	PK parameters			
			C_{max} [a]/PA-EC_{50}	$AUC_{0\text{-}24hr}$/PA-EC_{50}	C_{24hr}/PA-EC_{50}	$Time_{High}$ (1 × PA-EC_{50}) (hr)
S-217622 fumaric acid	qd	2	3.57	18.7	0.00288	7.19
		4	7.13	37.5	0.00574	9.99
		8	7.99	54.1	0.0646	11.2
		16	18.6	113	0.0363	13.3
		32	34.6	244	0.100	15.8
		64	76.1	636	0.617	21.7
	bid	1	1.79	18.3	0.155	7.38
		2	3.57	36.7	0.310	14.5
		4	7.13	73.2	0.617	20.1
		8	7.99	105	0.880	22.7
		16	18.6	223	1.80	24.0
		32	34.6	475	5.45	24.0
	qid	0.5	0.895	17.2	0.450	3.26
		1	1.79	34.4	0.900	19.5
		2	3.57	68.9	1.80	23.7
		4	7.13	138	3.60	23.9
		8	7.99	197	5.22	23.9
		16	18.6	419	10.8	24.0

PK/PD parameter	Model parameter	Estimate	r^2
C_{max} [a]/PA-EC_{50}	A	−3.72	0.856
	B	0.00	
	C	8.27	
	D	−2.60	
$AUC_{0\text{-}24hr}$/PA-EC_{50}	A	−4.26	0.793
	B	0.00	
	C	126	
	D	−1.52	
C_{24hr}/PA-EC_{50}	A	−14.0	0.114
	B	0.00	
	C	43500	
	D	−0.171	
$Time_{High}$ (1 × PA-EC_{50}) (hr)	A	−2.27	0.266
	B	0.00	
	C	9.35	
	D	−5.20	

$AUC_{0\text{-}24hr}$ = area under the plasma concentration-time curve from time 0 to 24 hours post-dose; bid = twice daily; C_{24hr} = plasma concentration at 24 hours after first administration;

C_{max} = maximum plasma concentration; LC/MS/MS = liquid chromatography with tandem mass spectrometry; MC = methylcellulose aqueous solution; qd = once daily; qid = 4 times daily; PD = pharmacodynamics; PK = pharmacokinetics; r^2 = coefficients of determination; $Time_{High}$ = total time above the target plasma concentration. a maximum concentration after first administration. The p-values were calculated by Dunnett's method in order to compare the virus titers between each dose group of S-217622 fumaric acid and the vehicle group. The following analytical model was applied to PD data and PK/PD parameters of S-217622 fumaric acid groups by nonlinear regression using XLfit (Ver. 5.3.1.3, ID Business Solutions Ltd.). These data was fitted to a four parameter logistic equation (model 205); y = A + ((B−A)/(1 + ((C/x)^D))) Where; A: Minimum y. B: Maximum y. C: EC_{50} (the value of a PK/PD parameter which produces a 50 % maximum effect) D: Slope factor y: Difference of the log $TCID_{50}$ (/mL) between each dosing group and the vehicle group x: Value of each PK/PD parameter (C_{max}/PA-EC_{50}, $AUC_{0\text{-}24hr}$/PA-EC_{50}, C_{24hr}/PA-EC_{50}, $Time_{High}$ [1 × PA-EC_{50}]) Each PK parameter: average value PA-EC_{50}: Protein-adjusted EC_{50} extrapolated to 100% mouse serum, 3.93 μmol/L=2090 ng/mL The values of x-axis and PK parameter were calculated when target pharmacological effect was obtained.

Study Number [Location in CTD]	S-217622-EB-018-N [4.2.1.1-16]

2.6.3.2.17 SARS-CoV-2 感染マウスにおける S-217622 フマル酸共結晶遅延投与の PK/PD 解析

Test Article: S-217622 fumaric acid

Species/Strain	Mouse/ BALB/cAJcl
Gender/Number of animals	Female/5 per group
Virus	hCoV-19/Japan/TY7-501/2021
Vehicle/Formulation	0.5 w/v% MC
Method of Administration	Oral administration once daily, twice daily (every 12 hours), or three times daily (every 8 hours) for 1 day or 2 days under non-anesthesia
Dose (mg/kg/shot)	8, 16, 32, or 64
Treatment	Mice were intranasally inoculated with 1.00×10^4 $TCID_{50}$ (50 μL/mouse) of the virus suspensions under anesthesia. The first administration of S-217622 fumaric acid or vehicle was performed 24 hours after virus infection.
Evaluation items	Virus titers in lungs 24 and 48 hours after the first administration
Evaluation method	Virus titers were measured by standard $TCID_{50}$ method using VeroE6/TMPRSS2 cells.
PK analysis	The PK parameters on the plasma concentrations of S-217622 were calculated by using Phoenix WinNonlin (Ver. 8.1, Certara, L.P.). The plasma concentrations of all dosing groups in the PD study were simulated by non-parametric analysis from plasma concentration data obtained in another PK study (Study Number: S-217622-EB-018-N). The PK parameters (C_{max}, $AUC_{0\text{-}48hr}$, C_{48hr}, and $Time_{High}$) were calculated based on the non-compartmental method.
PK/PD analysis	The relationship between PK parameters simulated by non-parametric superposition and non-compartmental method based on the PK study and PD effect of S-217622 fumaric acid 48 hours after first administration

Articles	Sampling time point of lungs (days post-infection)	Dosing schedule	Dose (mg/kg)	Number of mice	Virus titers (log_{10} $TCID_{50}$/mL)	p-value
Untreated	1	–	–	5	4.65 ± 0.56	–
Vehicle	2	bid for 1 day	0	5	6.11 ± 0.56	–
S-217622 fumaric acid		qd for 1 day	16	5	5.87 ± 0.19	0.9474
			32	5	5.77 ± 0.14	0.7335
			64	5	5.23 ± 0.51	0.0104
		bid for 1 day	8	5	5.73 ± 0.47	0.6130
			16	5	5.53 ± 0.45	0.1718
			32	5	4.55 ± 0.43	<0.0001
			64	5	3.60 ± 0.45	<0.0001
		tid for 1 day	8	5	5.73 ± 0.25	0.6420
			16	5	4.80 ± 0.37	<0.0001
			32	5	3.60 ± 0.38	<0.0001
			64	5	3.33 ± 0.41	<0.0001

Articles	Sampling time point of lungs (days post-infection)	Dosing schedule	Dose (mg/kg)	Number of mice	Virus titers (log_{10} $TCID_{50}$/mL)	p-value
Vehicle	3	bid for 2 days	0	5	6.57 ± 0.35	–
S-217622 fumaric acid		qd for 1 day	32	5	6.03 ± 0.35	0.2519
			64	5	6.03 ± 0.49	0.2519
		qd for 2 days	16	5	5.67 ± 0.68	0.0065
			32	5	5.23 ± 0.28	<0.0001
			64	5	4.95 ± 0.41	<0.0001
		bid for 2 days	8	5	5.83 ± 0.14	0.0423
			16	5	5.49 ± 0.31	0.0007
			32	5	4.40 ± 0.37	<0.0001
			64	5	3.47 ± 0.42	<0.0001
		tid for 2 days	8	5	5.55 ± 0.28	0.0015
			16	5	4.66 ± 0.18	<0.0001
			32	5	2.85 ± 0.51	<0.0001
			64	5	2.40 ± 0.40	<0.0001

Articles	Dosing schedule	Dose (mg/kg)	PK parameters		
			C_{max} (ng/mL)	$AUC_{0\text{-}48hr}$ (ng·hr/mL)	C_{48hr} (ng/mL)
S-217622 fumaric acid	qd for 1 day	32	72400	510000	0.0591
		64	159000	1340000	1.11
	qd for 2 days	16	38800	475000	75.8
		32	72500	1020000	210
		64	160000	2670000	1290
	bid for 2 days	8	18100	446000	1840
		16	41300	941000	3760
		32	79700	2010000	11400
		64	188000	5230000	42000
	tid for 2 days	8	21500	657000	5740
		16	47600	1390000	11600
		32	96000	2960000	30000
		64	232000	7650000	88300

Articles	Dosing schedule	Dose (mg/kg)	PK/PD parameters						
			C_{max}/ PA-EC_{50}	$AUC_{0\text{-}48hr}$/ PA-EC_{50}	C_{48hr}/ PA-EC_{50}	$Time_{High}$ (1 × PA-EC_{50}) (hr)	$Time_{High}$ (3 × PA-EC_{50}) (hr)	$Time_{High}$ (5 × PA-EC_{50}) (hr)	$Time_{High}$ (10 × PA-EC_{50}) (hr)
S-217622 fumaric acid	qd for 1 day	32	34.6	244	0.0000283	15.8	13.3	12.1	9.09
		64	76.1	641	0.000531	21.7	17.1	15.7	13.8
	qd for 2 days	16	18.6	227	0.0363	26.7	19.9	16.0	10.1
		32	34.7	488	0.100	31.6	26.5	24.2	18.2
		64	76.6	1280	0.617	43.4	34.2	31.4	27.5
	bid for 2 days	8	8.66	213	0.880	45.7	28.7	20.1	0.00
		16	19.8	450	1.80	48.0	40.1	32.3	20.6
		32	38.1	962	5.45	48.0	47.9	47.9	36.9
		64	90.0	2500	20.1	48.0	48.0	48.0	47.9
	tid for 2 days	8	10.3	314	2.75	47.9	45.2	32.7	2.13
		16	22.8	665	5.55	48.0	47.9	47.9	33.5
		32	45.9	1420	14.4	48.0	47.9	47.9	47.8
		64	111	3660	42.2	48.0	48.0	48.0	47.9

PK/PD parameter	Model parameter	Estimate	r^2
C_{max}/PA-EC_{50}	A	-4.77	0.311
	B	0	
	C	70.1	
	D	-0.859	
$AUC_{0\text{-}48hr}$/PA-EC_{50}	A	-4.77	0.735
	B	0	
	C	1180	
	D	-1.22	
C_{48hr}/PA-EC_{50}	A	-4.77	0.734
	B	0	
	C	6.35	
	D	-0.629	
$Time_{High}$ (1 × PA-EC_{50}) (hr)	A	-4.77	0.310
	B	0	
	C	50.6	
	D	-2.34	
$Time_{High}$ (3 × PA-EC_{50}) (hr)	A	-4.77	0.499
	B	0	
	C	44.9	
	D	-2.60	

PK/PD parameter	Model parameter	Estimate	r^2
$Time_{High}$ (5 × PA-EC_{50}) (hr)	A	-4.77	0.647
	B	0	
	C	40.8	
	D	-2.55	
$Time_{High}$ (10 × PA-EC_{50}) (hr)	A	-4.77	0.775
	B	0	
	C	33.3	
	D	-2.22	

Virus titer reduction	C_{48hr}/PA-EC_{50}	Mouse PA-EC_{50} (ng/mL)	Mouse C_{48hr} (ng/mL)	Human PA-EC_{50} (ng/mL)	Human C_{48hr} (ng/mL)
1 log_{10} $TCID_{50}$/mL	0.769	2090	1610	1610	1240
2 log_{10} $TCID_{50}$/mL	3.78		7900		6090
3 log_{10} $TCID_{50}$/mL	14.7		30700		23700

$AUC_{0\text{-}48hr}$ = area under the plasma concentration-time curve from 0 to 48 hours post-dose; bid = twice daily; C_{48hr} = plasma concentration at 48 hours after first administration;
tid = three times daily
The p-values were calculated by Dunnett's method in order to compare the virus titers between each dose group of S-217622 fumaric acid and the vehicle group at each sampling time point.
The following analytical model was applied to PD data and PK/PD parameters of S-217622 fumaric acid treatment groups by nonlinear regression using XLfit (Ver. 5.3.1.3, ID Business Solutions Ltd.). These data was fitted to a four parameter logistic equation (model 205);
y = A + ((B−A)/(1 + ((C/x)^D)))
Where;
A: Minimum y. (locked to -4.77)
The lower limit of quantification of virus titer was 1.80 log_{10} $TCID_{50}$/mL and the mean value of vehicle virus titer was 6.57 log_{10} $TCID_{50}$/mL.
B: Maximum y. (locked to 0)
C: EC_{50} (the value of a PK/PD parameter which produces a 50 % maximum effect)
D: Slope factor
y: Difference of the log $TCID_{50}$ (/mL) between each dosing group and the vehicle group
x: Value of each PK/PD parameter [C_{max}/PA-EC_{50}, $AUC_{0\text{-}48hr}$/PA-EC_{50}, C_{48hr}/PA-EC_{50}, $Time_{High}$/ (1 × PA-EC_{50}), $Time_{High}$/ (3 × PA-EC_{50}), $Time_{High}$/ (5 × PA-EC_{50}), $Time_{High}$/ (10 × PA-EC_{50})]
Each PK parameter: average value
PA-EC_{50}: Protein-adjusted EC_{50} extrapolated to 100% mouse serum, 3.93 μmol/L=2090 ng/mL
The values of x-axis and PK parameter were calculated when target pharmacological effect was obtained.

Study Number [Location in CTD]	S-217622-EB-081-N [4.2.1.1-17]

2.6.3.2.18 SARS-CoV-2 感染マウスにおける S-217622 フマル酸共結晶遅延投与による治療効果

Test Article: S-217622 fumaric acid

Species/Strain	Mouse/ BALB/cAJcl						
Gender/Number of animals	Female/4, 8, or 12 per group						
Virus	SARS-CoV-2 MA-P10 (mouse-adapted hCoV 19/Japan/TY/WK-521/2020)						
Vehicle/Formulation	0.5 w/v% MC						
Method of Administration	Oral administration twice daily (every 12 hours) under non-anesthesia for 5 days						
Dose as free form (mg/kg/shot)	0, 4, 8, 16, or 32						
Treatment	Mice were intranasally inoculated with 1.00×10^3 $TCID_{50}$ (50 µL/mouse) of the virus suspensions under anesthesia. The first administration of S-217622 fumaric acid or vehicle was performed after 24 hours virus infection.						
Evaluation Item	The survival and the body weight of mice were examined for 14 days after inoculation.						
Evaluation method	Mice were examined daily for survival through 14 days after infection. Mice were euthanized and regarded as dead when their body weights in the morning were less than 80% of the initial body weights (in the morning on the day of virus infection) according to humane endpoints.						
Results							
Inoculum		Dulbecco's Phosphate-buffered Saline (Uninfected control)	SARS-CoV-2 MA-P10				
Articles		Vehicle	Vehicle	S-217622 fumaric acid			
Dose (mg/kg)		0	0	4	8	16	32
Animal Number (Survived/Total)	Day 0	4/4	8/8	12/12	12/12	12/12	12/12
	Day 1	4/4	8/8	12/12	12/12	12/12	12/12
	Day 2	4/4	8/8	12/12	12/12	12/12	12/12
	Day 3	4/4	8/8	12/12	12/12	12/12	12/12
	Day 4	4/4	8/8	12/12	12/12	12/12	12/12
	Day 5	4/4	6/8	11/12	12/12	12/12	12/12
	Day 6	4/4	5/8	11/12	12/12	12/12	12/12
	Day 7	4/4	3/8	11/12	12/12	12/12	12/12
	Day 8	4/4	3/8	11/12	12/12	12/12	12/12
	Day 9	4/4	3/8	11/12	12/12	12/12	12/12
	Day 10	4/4	3/8	11/12	12/12	12/12	12/12
	Day 11	4/4	3/8	11/12	12/12	12/12	12/12
	Day 12	4/4	3/8	11/12	12/12	12/12	12/12
	Day 13	4/4	3/8	11/12	12/12	12/12	12/12
	Day 14	4/4	3/8	11/12	12/12	12/12	12/12
Survival rate (%)		100	37.5	91.7	100	100	100
p-value vs vehicle (infected)		–	–	0.0124	0.0015	0.0015	0.0015

Mice were orally administered (10 mL/kg) with S-217622 fumaric acid or vehicle (0.5 w/v% methylcellulose solution).
The first administration was performed 24 hours after virus infection.
Day 0, The day of virus inoculation
The p-values were calculated by the log-rank test (multiplicity was adjusted by fixed-sequence procedure).

Inoculum		Dulbecco's Phosphate-buffered Saline (Uninfected control)	SARS-CoV-2 MA-P10				
Articles		Vehicle	Vehicle	S-217622 fumaric acid			
Dose (mg/kg)		0	0	4	8	16	32
Body Weight Change (% of Initial Body Weight)	Day 2	0.0 ± 3.4	−2.8 ± 3.1	−2.4 ± 2.8	−4.6 ± 4.4	−3.1 ± 5.5	−2.2 ± 3.4
	Day 3	-1.4 ± 3.4	−9.3 ± 6.8	−8.8 ± 3.8	−6.1 ± 5.7	−2.6 ± 5.9**	0.0 ± 4.3***
	Day 4	1.3 ± 3.1	−9.8 ± 9.3	−7.7 ± 6.5	−3.3 ± 4.5*	1.0 ± 4.9***	1.5 ± 4.9***
	Day 5	1.9 ± 3.3	−12.1 ± 9.9	−7.4 ± 7.5	−1.5 ± 4.5***	2.3 ± 3.8***	2.0 ± 5.6***
Mice were orally administered (10 mL/kg) with S-217622 fumaric acid or vehicle (0.5 w/v% methylcellulose solution). The first administration was performed 24 hours after virus infection. *, ** and *** denote respectively P < 0.05, P < 0.01, and P < 0.001 vs vehicle, using ANOVA-test (multiplicity was adjusted by fixed-sequence procedure). Values are mean body weight change ± SD from the living mice at each day (AM).							
Study Number [Location in CTD]		S-217622-EB-099-N [4.2.1.1-18]					

2.6.3.2.19 S-217622 低感受性に関わるアミノ酸残基の SARS-CoV-2 3CL プロテアーゼの多型解析

Evaluation Item	GISAID data meeting the following criteria was included in the analysis. The first criterion was that the amino acid sequence of nsp5 did not include any ambiguity codes. The second criterion was that the length of amino acid sequence of nsp5 was longer than 299 amino acids. The third criterion was that isolates were from human. A total of 10,018,171 amino acid sequences met these criteria.
Evaluation method	The prevalence of polymorphisms for amino acids associated with S-217622 fumaric acid low susceptibility were calculated as the ratio of each amino acid to all the sequences met criteria (10,018,171 sequences).

Results			
3C-like protease (nsp5) amino acid position	Residue	Prevalence (total: n = 10,018,171)	
48	Aspartic acid	99.995%	n = 10,017,695
	Asparagine	0.002%	n = 219
	Glycine	0.001%	n = 109
	Tyrosine	0.001%	n = 101
	Glutamic acid	< 0.001%	n = 29
	Histidine	< 0.001%	n = 10
	Alanine	< 0.001%	n = 5
	Valine	< 0.001%	n = 2
	deletion	< 0.001%	n = 1
49	Methionine	99.979%	n = 10,016,079
	Isoleucine	0.019%	n = 1931
	Threonine	0.001%	n = 73
	Leucine	< 0.001%	n = 43
	Valine	< 0.001%	n = 37
	Lysine	< 0.001%	n = 7
	Tryptophan	< 0.001%	n = 1
52	Proline	99.999%	n = 10,018,098
	Serine	< 0.001%	n = 37
	Leucine	< 0.001%	n = 23
	deletion	< 0.001%	n = 7
	Histidine	< 0.001%	n = 4
	Threonine	< 0.001%	n = 2
144	Serine	> 99.999%	n = 10,018,139
	Leucine	< 0.001%	n = 14
	Alanine	< 0.001%	n = 9
	Glutamic acid	< 0.001%	n = 3
	Threonine	< 0.001%	n = 2
	Proline	< 0.001%	n = 2
	deletion	< 0.001%	n = 2

3C-like protease (nsp5) amino acid position	Original amino acid	Amino acid replacement	Prevalence (total: n = 10,018,171)	
48	Aspartic acid	Glycine	0.001%	n = 109
49	Methionine	Leucine	< 0.001%	n = 43
52	Proline	Serine	< 0.001%	n = 37
144	Serine	Alanine	< 0.001%	n = 9
49 / 144	Methionine / Serine	Leucine / Alanine	0%	n = 0
Study Number [Location in CTD]	S-217622-EB-172-N [4.2.1.1-19]			

2.6.3.2.20 SARS-CoV-2 変異 3CL プロテアーゼ活性に対する阻害効果

Test Article: S-217622 fumaric acid

Enzyme	Recombinant SARS-CoV-2 3CL protease mutants G15S, T21I, L89F, K90R, P108S, and P132H (expressed in *Escherichia coli*)	
Concentration as free form (nmol/L)	0.127, 0.381, 1.14, 3.43, 10.3, 30.9, 92.6, 278, 833, 2500	
Reference substance (Final concentration)	N/A	
Vehicle	DMSO	
Evaluation Items	Inhibitory effect of S-217622 fumaric acid on mutants of SARS-CoV-2 3CL protease activity.	
Evaluation Method	As for the three mutants G15S, K90R, and P132H, 3 nmol/L of each recombinant SARS-CoV-2 3CL protease mutant was incubated with 4 μmol/L substrate and S-217622 fumaric acid. As for the three mutants T21I, L89F, and P108S, 6 nmol/L of each recombinant SARS CoV-2 3CL protease mutant was incubated with 2 μmol/L substrate and S-217622 fumaric acid. Dabcyl KTSAVLQ as a reaction product was quantified by mass spectroscopy (6550 iFunnel Q-TOF, Agilent technologies) interfaced with liquid chromatography (RapidFireTM system, Agilent technologies). IC_{50} of test substance was calculated by using software XLfit 5.3.1.3. The fold change against each SARS-CoV-2 3CL protease mutant was calculated as relative IC_{50} to wild-type.	
Results		
SARS-CoV-2 3CL protease mutants	IC_{50} (nmol/L)	Fold change in IC_{50}
G15S	8.0 ± 1.1	0.60 ± 0.08
T21I	14.3 ± 0.8	1.08 ± 0.06
L89F	15.0 ± 1.2	1.13 ± 0.09
K90R	9.7 ± 1.1	0.73 ± 0.09
P108S	13.2 ± 1.0	1.00 ± 0.07
P132H	14.4 ± 2.2	1.09 ± 0.16
G15S = glycine-to-serine at amino acid position 15, K90R = lysine-to-arginine at amino acid position 90, L89F = leucine-to-phenylalanine at amino acid position 89, P108S = proline-to-serine at amino acid position 108, P132H = proline-to-histidine at amino acid position 132, T21I = threonine-to-isoleucine at amino acid position 21 Data are expressed as the mean ± SD in 3 independent experiments.		
Study Number [Location in CTD]	S-217622-EB-159-N [4.2.1.1-20]	

2.6.3.2.21 SARS-CoV-2 感染細胞に対する細胞変性抑制効果 (5)

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with SARS-CoV-2 clinical isolates (hCoV-19/Japan/TY38-871/2021 [Pango Lineage: B.1.1.529/BA.1.1] and hCoV-19/Japan/TY40-385/2022 [Pango Lineage: B.1.1.529/BA.2])
Final concentration as free form (μmol/L)	0.0046, 0.014, 0.041, 0.12, 0.37, 1.1, 3.3, 10
Reference substance (Final concentration [μmol/L])	Remdesivir Viral replication inhibition assay: 0.0091, 0.027, 0.082, 0.25, 0.74, 2.2, 6.7, 20
Vehicle	0.5% DMSO
Evaluation item	EC_{50}: The concentration achieving 50% of inhibition on CPE induced by SARS-CoV-2
Evaluation Method	Viral replication inhibition assay: One hundred microliters of serially diluted substance solutions were added to a 96-well plate. SARS-CoV-2 virus stock solutions were diluted to appropriate concentrations in advance, and each 50 μL/well was dispensed on the 96-well plate containing a test or reference substance. The infectious dose of each virus was as follows: 3000 $TCID_{50}$/well for hCoV-19/Japan/TY38-871/2021, 300 $TCID_{50}$/well for hCoV-19/Japan/TY40-385/2022. Then, each 50 μL/well of VeroE6/TMPRSS2 cells which were adjusted to 3.0×10^5 cells/mL was dispensed on the plate. The plates were mixed with a plate mixer and were incubated at 37°C in a CO_2 incubator for 4 days. The inhibitory effects of the test substance on CPE induced by SARS-CoV-2 virus were measured by cell viability. Measurement of cell viability: After 4 days incubation of the plates prepared in the viral replication inhibition assay, 60 μL of CellTiter-Glo® 2.0 Reagent was added into each well. After mixing with a plate mixer, plates were incubated at room temperature for approximately 30 minutes, and each 200 μL was dispensed on a new white 96-well plate. Luminescent signal was measured by a multiplate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).

Results

Strains	Pango Lineage	EC_{50} (μmol/L)	
		S-217622 fumaric acid	Remdesivir
hCoV-19/Japan/TY38-871/2021	B.1.1.529/BA.1.1	0.36 ± 0.077	1.0 ± 0.052
hCoV-19/Japan/TY40-385/2022	B.1.1.529/BA.2	0.52 ± 0.091	1.0 ± 0.23
Data are expressed as the mean ± SD in 3 independent experiments.			
Study Number [Location in CTD]		S-217622-EB-168-N [4.2.1.1-21]	

2.6.3.2.22 SARS-CoV-2 感染細胞に対する細胞変性抑制効果 (6)

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with SARS-CoV-2 clinical isolates (hCoV-19/Japan/26-717/2021 [Pango Lineage: B.1.621], hCoV-19/Japan/TY28-444/2021 [Pango Lineage: P.3], hCoV-19/Japan/TY33-456/2021 [Pango Lineage: C.37], and hCoV-19/Japan/TY41-686/2022 [Pango Lineage: XE])		
Final concentration as free form (μmol/L)	Viral replication inhibition assay: 0.0046, 0.014, 0.041, 0.12, 0.37, 1.1, 3.3, 10		
Reference substance (Final concentration [μmol/L])	Remdesivir Viral replication inhibition assay: 0.0091, 0.027, 0.082, 0.25, 0.74, 2.2, 6.7, 20		
Vehicle	0.5% DMSO		
Evaluation Items	EC_{50}: The concentration achieving 50% of inhibition on CPE induced by SARS-CoV-2		
Evaluation Method	Viral replication inhibition assay: One hundred microliters of serially diluted substance solutions were added to a 96-well plate. SARS-CoV-2 virus stock solutions were diluted to appropriate concentrations in advance, and each 50 μL/well was dispensed on the 96-well plate containing a test or reference substance. The infectious dose of each virus was as follows: 3000 $TCID_{50}$/well for hCoV-19/Japan/TY26-717/2021 strain and hCoV-19/Japan/TY33-456/2021 strain (multiplicity of infection of 0.20), 300 $TCID_{50}$/well for hCoV-19/Japan/TY28-444/2021 strain and hCoV-19/Japan/TY41-686/2022 strain (multiplicity of infection of 0.020).Then, each 50 μL/well of VeroE6/TMPRSS2 cells which were adjusted to 3.0×10^5cells/mL was dispensed on the plate. The plate was mixed with a plate mixer and was incubated at 37°C in a 5%CO_2 incubator for 3 or 4 days. The inhibitory effects of the test substance on CPE induced by SARS-CoV-2 were measured by cell viability. Measurement of cell viability: After 3 or 4 days incubation of the plate prepared in the viral replication inhibition assay, 60 μL of CellTiter-Glo® 2.0 Reagent was added into each well. After mixing with a plate mixer, plate was incubated at room temperature for approximately 30 minutes, and each 200 μL of mixture was dispensed on a new white 96-well plate. Luminescent signal was measured by a multiplate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).		
Results			

Strains	Pango Lineage	EC_{50} (μmol/L)	
		S-217622 fumaric acid	Remdesivir
hCoV-19/Japan/TY26-717/2021	B.1.621	0.43 ± 0.069	3.9 ± 0.41
hCoV-19/Japan/TY28-444/2021	P.3	0.29 ± 0.028	0.98 ± 0.10
hCoV-19/Japan/TY33-456/2021	C.37	0.27 ± 0.048	3.2 ± 0.61
hCoV-19/Japan/TY41-686/2022	XE	0.44 ± 0.037	1.1 ± 0.36

CPE = cytopathic effect; EC_{50} = 50% effective concentration; $TCID_{50}$ = 50% tissue culture infectious dose; TMPRSS2 = transmembrane protease, serine 2

Data are expressed as the mean ± SD in 3 independent experiments.

Study Number [Location in CTD]	S-217622-EB-174-N [4.2.1.1-22]

2.6.3.2.23 SARS-CoV-2 感染細胞に対する細胞変性抑制効果 (7)

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with SARS-CoV-2 clinical isolates (hCoV-19/Japan/TY41-703/2022 [Pango Lineage: BA.4], hCoV-19/Japan/TY41-702/2022 [Pango Lineage: BA.5])		
Final concentration as free form (μmol/L)	Viral replication inhibition assay: 0.0046, 0.014, 0.041, 0.12, 0.37, 1.1, 3.3, 10		
Reference substance (Final concentration [μmol/L])	Remdesivir Viral replication inhibition assay: 0.0091, 0.027, 0.082, 0.25, 0.74, 2.2, 6.7, 20		
Vehicle	0.5% DMSO		
Evaluation Items	EC_{50}: The concentration achieving 50% of inhibition on CPE induced by SARS-CoV-2		
Evaluation Method	Viral replication inhibition assay: One hundred microliters of serially diluted substance solutions were added to a 96-well plate. SARS-CoV-2 virus stock solutions were diluted to appropriate concentrations in advance, and each 50 μL/well was dispensed on the 96-well plate containing a test or reference substance. The infectious dose of each virus was as follows: 300 $TCID_{50}$/well. Then, each 50 μL/well of VeroE6/TMPRSS2 cells which were adjusted to 3.0×10^5cells/mL was dispensed on the plate. The plate was mixed with a plate mixer and was incubated at 37°C in a 5%CO_2 incubator for 4 days. The inhibitory effects of the test substance on CPE induced by SARS-CoV-2 were measured by cell viability. Measurement of cell viability: After 4 days incubation of the plate prepared in the viral replication inhibition assay, 60 μL of CellTiter-Glo® 2.0 Reagent was added into each well. After mixing with a plate mixer, plate was incubated at room temperature for approximately 30 minutes, and each 200 μL of mixture was dispensed on a new white 96-well plate. Luminescent signal was measured by a multiplate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).		
Results			
Strains	Pango Lineage	EC_{50} (μmol/L)	
		S-217622 fumaric acid	Remdesivir
hCoV-19/Japan/TY41-703/2022	BA.4	0.22 ± 0.072	0.65 ± 0.19
hCoV-19/Japan/TY41-702/2022	BA.5	0.40 ± 0.082	1.3 ± 0.54
CPE = cytopathic effect; EC_{50} = 50% effective concentration; $TCID_{50}$ = 50% tissue culture infectious dose; TMPRSS2 = transmembrane protease, serine 2 Data are expressed as the mean ± SD in 3 independent experiments.			
Study Number [Location in CTD]	S-217622-EB-177-N [4.2.1.1-23]		

2.6.3.2.24 SARS-CoV-2 感染細胞に対する細胞変性抑制効果 (8)

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with SARS-CoV-2 clinical isolates (hCoV-19/Japan/TY41-716/2022 [Pango Lineage: BA.2.75])		
Final concentration as free form (μmol/L)	Viral replication inhibition assay: 0.0046, 0.014, 0.041, 0.12, 0.37, 1.1, 3.3, 10		
Reference substance (Final concentration [μmol/L])	Remdesivir Viral replication inhibition assay: 0.0091, 0.027, 0.082, 0.25, 0.74, 2.2, 6.7, 20		
Vehicle	0.5% DMSO		
Evaluation Items	EC_{50}: The concentration achieving 50% of inhibition on CPE induced by SARS-CoV-2		
Evaluation Method	Viral replication inhibition assay: One hundred microliters of serially diluted substance solutions were added to a 96-well plate. SARS-CoV-2 virus stock solutions were diluted to appropriate concentrations in advance, and each 50 μL/well was dispensed on the 96-well plate containing a test or reference substance. The infectious dose of each virus was as follows: 300 $TCID_{50}$/well. Then, each 50 μL/well of VeroE6/TMPRSS2 cells which were adjusted to 3.0×10^5cells/mL was dispensed on the plate. The plate was mixed with a plate mixer and was incubated at 37°C in a 5%CO_2 incubator for 4 days. The inhibitory effects of the test substance on CPE induced by SARS-CoV-2 were measured by cell viability. Measurement of cell viability: After 4 days incubation of the plate prepared in the viral replication inhibition assay, 60 μL of CellTiter-Glo® 2.0 Reagent was added into each well. After mixing with a plate mixer, plate was incubated at room temperature for approximately 30 minutes, and each 200 μL of mixture was dispensed on a new white 96-well plate. Luminescent signal was measured by a multiplate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).		
Results			
Strains	Pango Lineage	EC_{50} (μmol/L)	
		S-217622 fumaric acid	Remdesivir
hCoV-19/Japan/TY41-716/2022	BA.2.75	0.30 ± 0.032	0.91 ± 0.080
CPE = cytopathic effect; EC_{50} = 50% effective concentration; $TCID_{50}$ = 50% tissue culture infectious dose; TMPRSS2 = transmembrane protease, serine 2 Data are expressed as the mean ± SD in 3 independent experiments.			
Study Number [Location in CTD]	S-217622-EB-184-N [4.2.1.1-24]		

2.6.3.2.25 S-217622 フマル酸共結晶に対するリバースジェネティクス由来 SARS-CoV-2 変異体の薬剤感受性試験

Test Article: S-217622 fumaric acid

Species/Biological material	VeroE6/TMPRSS2 cells infected with SARS-CoV-2, generated by reverse genetics (rgSARS-CoV-2/Hu/DP/Kng/19-020 [wild-type], rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/L87F, rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/T198I , rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/A234S, rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/H246Y)
Final concentration as free form (μmol/L)	Viral replication inhibition assay: 0.0046, 0.014, 0.041, 0.12, 0.37, 1.1, 3.3, 10
Reference substance (Final concentration [μmol/L])	Remdesivir Viral replication inhibition assay: 0.023, 0.069, 0.21, 0.62, 1.9, 5.6, 17, 50
Vehicle	0.5% DMSO
Evaluation Items	EC_{50}: The concentration achieving 50% of inhibition on CPE induced by SARS-CoV-2 FC: The relative EC_{50} to wild-type virus.
Evaluation Method	Viral replication inhibition assay: One hundred microliters of serially diluted substance solutions were added to a 96-well plate. SARS-CoV-2 virus stock solutions were diluted to appropriate concentrations in advance, and each 50 μL/well was dispensed on the 96-well plate containing a test or reference substance. The infectious dose of each virus was 1000 $TCID_{50}$/well (multiplicity of infection of 0.067). Then, each 50 μL/well of VeroE6/TMPRSS2 cells which were adjusted to 3.0×10^5 cells/mL was dispensed on the plate. The plate was mixed with a plate mixer and was incubated at 37°C in a 5%CO_2 incubator for 3 days. The inhibitory effects of the test substance on CPE induced by SARS-CoV-2 were measured by cell viability. Measurement of cell viability: After 3 days incubation of the plate prepared in the viral replication inhibition assay, 60 μL of CellTiter-Glo® 2.0 Reagent was added into each well. After mixing with a plate mixer, plate was incubated at room temperature for approximately 30 minutes, and each 200 μL of mixture was dispensed on a new white 96-well plate. Luminescent signal was measured by a multiplate reader EnSpire™ (PerkinElmer Japan Co., Ltd.).

Results

Strains	S-217622 fumaric acid		Remdesivir	
	EC_{50} (μmol/L)	FC	EC_{50} (μmol/L)	FC
rgSARS-CoV-2/Hu/DP/Kng/19-020 (wild-type)	0.21 ± 0.044	N/A	2.2 ± 0.12	N/A
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/L87F	0.19 ± 0.025	0.92	1.0 ± 0.031	0.47
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/T198I	0.31 ± 0.080	1.5	1.5 ± 0.032	0.72
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/A234S	0.36 ± 0.086	1.7	2.5 ± 0.085	1.2
rgSARS-CoV-2/Hu/DP/Kng/19-020-nsp5/H246Y	0.19 ± 0.030	0.90	2.0 ± 0.041	0.93

CPE = cytopathic effect; EC_{50} = 50% effective concentration; FC = fold change; $TCID_{50}$ = 50% tissue culture infectious dose; TMPRSS2 = transmembrane protease, serine 2

N/A = not applicable.

Data are expressed as the mean ± SD in 3 independent experiments.

Study Number [Location in CTD]	S-217622-EB-178-N [4.2.1.1-25]

2.6.3.3 副次的薬理試験

2.6.3.3.1 各種受容体，イオンチャネル及びトランスポーター結合並びに酵素アッセイ

Test Article:S-217622 fumaric acid

Type of Study	Test System	Method of Administration	Dose or Concentration	Noteworthy Findings	Testing Facilities	GLP status	Study Number [Location in CTD]
The specific interaction of S-217622 fumaric acid on various receptors and enzymes	108 Various receptors, channels, transporters and enzymes	In vitro	100 µmol/L in duplicate	From 108 targets tested with S-217622 fumaric acid at the high concentration of 100 µmol/L PDE4A1A, PDE4B1, PDE4C1, PDE4D2, PDE7A, PDE7B, CCK2, nicotinic acetylcholine α1 subunit, and adenosine transporter showed inhibition > 50% in specific radioligand binding or enzimatic activity.	[redacted]	NR	S-217622-EF-046-N [4.2.1.2-01]
Additional follow-up concenrtration response test	19 Various receptors, channels, transporters and enzymes which selected based on primary study results		0.03, 0.1, 0.3, 1, 3, 10, 30, 100 µmol/L in duplicate	The > 50% inhibition effect was not detected on the enzymatic assay of PDE7A, PDE7B, [^{125}I] alpha-Bungarotoxin binding of nicotinic acetylcholine α1 subunit, and CCK2 receptor IP1 functional assay. No significant effect of S-217622 fumaric acid on 10 peptidases (angiotensin converting enzyme, caspase 2, chymotrypsin, cathepsin B, cathepsin D, cathepsin G, cathepsin L, neutrophil elastase 2, HIV-1 protease, and thrombin) was observed for additional follow-up study. Results showed the inhibitory effects of S-217622 fumaric acid with IC_{50} values (µmol/L) of 63.2, 69.1, 75.7, and 73.4 for phosphodiesterase isozymes PDE4A1A, PDE4B1, PDE4C1, and PDE4D2 respectively, and inhibitory effect with IC_{50} value of 1.43 µmol/L on adenosine uptake functional assay.			
CCK = cholecystokinin; HIV = human immunodeficiency virus; IP = inositol phosphate; NR = not required; PDE = phosphodiesterase							

2.6.3.3.2 ヒト初代培養細胞に対する細胞毒性

Test Article:S-217622 fumaric acid

Species/Biological material	Human primary cells: Normal human mixed renal epithelial cells, Human hepatocytes, Human peripheral blood CD4+ T cells	
Concentration as free form (μg/mL)	0.29, 0.88, 2.6, 8.0, 23.9, 71.7	
Reference substance (concentration)	Tamoxifen, Papaverine (0.1, 1, 3, 10, 30, 100 μM)	
Vehicle	0.3% DMSO	
Evaluation item	CC_{50}: the concentration producing a half-maximal inhibition of control response	
Evaluation Method	All cell lines were seeed at 2 different densities; proliferative condition and near confluent condition (non-proliferative condition) in assay plates. The assay plates were incubated at 37°C in a CO_2 incubator for 48 hours. The cellular ATP amounts were measured to evaluate the cytotoxicity. The entire testing was performed in duplicate.	
Results		
Test Item: S-217622 fumaric acid		
Cellular condition	Proliferative condition	Non-proliferative condition
Human primary cells	CC_{50} (μg/mL)	CC_{50} (μg/mL)
Normal human mixed renal epithelial cells	> 72	NC
Human hepatocytes	NC	NC
Human peripheral blood $CD4^+$ T cells	NC	NC
Test Item: Tamoxifen		
Cellular condition	Proliferative condition	Non-proliferative condition
Human primary cells:	CC_{50} (mol/L)	CC_{50} (mol/L)
Normal human mixed renal epithelial cells	1.00E-05	1.20E-05
Human hepatocytes	1.20E-05	2.40E-05
Human peripheral blood $CD4^+$ T cells	1.20E-05	2.40E-05
Test Item: Papaverine		
Proliferating condition	Proliferative condition	Non-proliferative condition
Human primary cells:	CC_{50} (mol/L)	CC_{50} (mol/L)
Normal human mixed renal epithelial cells	6.30E-06	5.30E-06
Human hepatocytes	1.10E-05	1.70E-05
Human peripheral blood CD4+ T cells	> 1.00E-04	> 1.00E-04
NC = not calculated The CC_{50} values are determined by non-linear regression analysis of the concentration-response curves generated with mean replicate values using Hill equation curve fitting $Y=D+(A-D)/[1+(C/C_{50})^{nH}]$ where Y = response, A = left asymptote of the curve, D = right asymptote of the curve, C = compound concentration, C_{50} = EC_{50}, nH = slope factor.		
Study Number [Location in CTD]	S-217622-EF-084-N [4.2.1.2-02]	

2.6.3.3.3 ヒト各種組織由来株化細胞に対する細胞毒性

Test Article:S-217622 fumaric acid

Species/Biological material	Human cell lines: Hepatocellular carcinoma (HepG2), Renal glomeruli (HK-2), Embryonic kidney (HEK293), Neuroblastoma (SH-SY-5Y), Neuroblastoma (SK-N-SH), T cell leukemia (Jurkat), Prostate carcinoma (E006AA-hT), Vascular endothelial (HUVEC) Lung fibroblast (MRC-5)	
Concentration as free form (μg/mL)	0.29, 0.88, 2.6, 8.0, 23.9, 71.7	
Reference substance (concentration)	Tamoxifen, Papaverine (0.1, 1, 3, 10, 30, 100 μM)	
Vehicle	0.3% DMSO	
Evaluation item	CC_{50}: the concentration producing a half-maximal inhibition of control response	
Evaluation Method	All cell lines were seeed at 2 different densities; proliferative condition and near confluent condition (non-proliferative condition) in assay plates. The assay plates were incubated at 37°C in a CO_2 incubator for 48 hours. The cellular ATP amounts were measured to evaluate the cytotoxicity. The entire testing was performed in duplicate.	
Results		
Test Item: S-217622 fumaric acid		
Cellular condition	Proliferative condition	Non-proliferative condition
Cell lines	CC_{50} (μg/mL)	CC_{50} (μg/mL)
HepG2	NC	NC
HK-2	NC	NC
HEK293	NC	NC
SH-SY-5Y	NC	NC
SK-N-SH	NC	NC
Jurkat	> 72	> 72
E006AA-hT	NC	NC
HUVEC	NC	NC
MRC-5	NC	NC

Test Item: Tamoxifen		
Cellular condition	Proliferative condition	Non-proliferative condition
Cell lines	CC_{50} (mol/L)	CC_{50} (mol/L)
HepG2	1.60E-05	2.00E-05
HK-2	1.10E-05	1.70E-05
HEK293	2.70E-05	3.10E-05
SH-SY-5Y	1.80E-05	2.00E-05
SK-N-SH	1.70E-05	3.10E-05
Jurkat	1.30E-05	1.50E-05
E006AA-hT	1.70E-05	5.00E-05
HUVEC	4.20E-06	1.30E-05
MRC-5	1.70E-05	2.20E-05
Test Item: Papaverine		
Proliferating condition	Proliferative condition	Non-proliferative condition
Cell lines	CC_{50} (mol/L)	CC_{50} (mol/L)
HepG2	1.20E-05	1.70E-05
HK-2	2.60E-06	1.90E-06
HEK293	> 1.00E-04	8.60E-05
SH-SY-5Y	> 1.00E-04	> 1.00E-04
SK-N-SH	1.60E-05	5.50E-06
Jurkat	2.00E-05	2.20E-06
E006AA-hT	> 1.00E-04	> 1.00E-04
HUVEC	NC	> 1.00E-04
MRC-5	> 1.00E-04	> 1.00E-04
The CC_{50} values are determined by non-linear regression analysis of the concentration-response curves generated with mean replicate values using Hill equation curve fitting $Y=D+(A-D)/[1+(C/C_{50})^{nH}]$ where Y = response, A = left asymptote of the curve, D = right asymptote of the curve, C = compound concentration, $C_{50} = EC_{50}$, nH = slope factor.		
Study Number [Location in CTD]	S-217622-EF-085-N [4.2.1.2-03]	

2.6.3.3.4 ヒト細胞に対するミトコンドリア毒性

Test Article: S-217622 fumaric acid

Species/Biological material			HepG2 cells				
Final concentration as free form (μg/mL)			0.096, 0.48, 2.4, 12, 60, 300				
Vehicle			DMSO				
Reference substance			Positive control: oligomycin, chloramphenicol				
Exposure period			2 days, 14 days				
Analyte			ATP				
Measurement			Plate reader				
Mitochondrial Toxicity			None				
Evaluation items			CC_{50}: calculated from the relationship between the concentration and the ATP amount ratio (%) Fold change: CC_{50} glucose/ CC_{50} galactose				
Result							
Exposure period	Test substance or positive control		DMEM-Glucose		DMEM-Galactose		Fold change
	Compound	Concentration	ATP amount ratio (%)	CC_{50}	ATP amount ratio (%)	CC_{50}	
	None	0	100.0	–	100.0	–	–
2 days	S-217622 fumaric acid (μg/mL as free form)	0.096	97.2	287	110.1	294	0.976
		0.48	96.5		108.4		
		2.4	96.8		108.8		
		12	96.3		106.2		
		60	94.1		105.2		
		300	37.0		45.5		
	Oligomycin (nmol/L)	0.1	103.8	8.52	101.4	1.67	5.10
		0.3	100.7		99.4		
		1	99.7		74.3		
		3	61.3		22.9		
		10	41.4		1.0		
		30	33.5		0.5		

Exposure period	Test substance or positive control		DMEM-Glucose		DMEM-Galactose		Fold change
	Compound	Concentration	ATP amount ratio (%)	CC_{50}	ATP amount ratio (%)	CC_{50}	
	None	0	100.0	–	100.0	–	–
14 days	S-217622 fumaric acid (μg/mL as free form)	0.096	97.9	100	105.4	97.9	1.02
		0.48	97.6		102.7		
		2.4	95.6		103.3		
		12	92.1		95.6		
		60	81.9		83.6		
		300	3.5		2.2		
	Chloramphenicol (μmol/L)	10	96.9	134	98.4	40.5	3.31
		30	103.2		53.4		
		100	48.5		22.9		
		300	29.0		12.6		
		1000	14.0		4.0		
		3000	0.6		0.3		
Study Number [Location in CTD]	S-217622-EF-077-N [4.2.1.2-04]						

ATP = adenosine triphosphate; DMEM = Dulbecco's modified eagle medium

2.6.3.4 安全性薬理試験

Test Article: S-217622 fumaric acid

Organ Systems Evaluated	**Test System**	**Method of Administration (Vehicle/ Formulation)**	**Doses [a] (mg/kg)**	**Gender and No. per Group**	**Noteworthy Findings**	**GLP Compliance**	**Study Number/ Location in CTD**
Central nervous system (modified FOB method)	Rat/ Crl:CD (Sprague Dawley)	Oral gavage (0.5% MC/ Suspension)	20, 100, 1000	6M	Central nervous system: None Other: 1000 mg/kg: Decrease in urine quantity [b] Decrease in the number of fecal pellets [c]	Yes	S-217622-SF-035-L/ 4.2.1.3-01
Cardiovascular system	hERG transfected CHO cells	Perfusion	10, 30, 100 µmol/L	5	10, 30, and 100 µmol/L: Decreased hERG currents at compensated suppression rates of 8.92%, 16.16%, and 38.98%, respectively. IC_{50} was estimated to be greater than 100 µmol/L.	Yes	S-217622-SF-034-L/ 4.2.1.3-02
Cardiovascular and respiratory systems (by telemetry and blood gas analysis)[d]	Cynomolgus monkey	Oral gavage (0.5% MC/ Suspension)	10 [e], 50, 150	4M [f]	Cardiovascular system: 10 mg/kg: None 50 mg/kg: Increase in HR [3/4] [g] 150 mg/kg: Increase in HR [3/4] Respiratory system: None Toxicokinetics (n = 4, mean value)	Yes	S-217622-SF-033-L/ 4.2.1.3-03

Dose (mg/kg)	10	50	150
C_{max} (µg/mL)	43.8	141	326
$AUC_{0\text{-}24hr}$ (µg·hr/mL)	732	2760	6190
T_{max} (hr)	3.5	4.0	5.5

FOB = functional observation battery; GLP = Good Laboratory Practice; HR = heart rate; M = male; T_{max} = time to maximum plasma concentration

a Unless otherwise specified. As free from.

b Between immediately (0 hours) and 1 hour after dosing and between 0 and 8 hours after dosing.

c Between 0 and 8 hours after dosing.

d Blood pressure (systolic, diastolic, and mean), HR, electrocardiogram (PR interval, QRS duration, QT interval, QTc interval [individual animal-corrected QT interval], and incidence of abnormal waveform), respiratory rate, and blood gas parameters (arterial blood pH, arterial oxygen tension, arterial carbon dioxide tension, and hemoglobin oxygen saturation).

e Respiratory system was not evaluated at 10 mg/kg.

f The same animals were dosed repeatedly at 7-day intervals. At 10 mg/kg, blood pressure was evaluated in 3 animals.

g Numerals in parentheses represent the number of animals showing sign/total number of animals.

2.6.3.5 薬力学的薬物相互作用試験

該当する試験なし.