1 **Metalloproteinase-dependent and TMPRSS2-independnt cell surface entry**

2 **pathway of SARS-CoV-2 requires the furin-cleavage site and the S2 domain of spike**

3 **protein**

4

5

6 **Mizuki Yamamoto[1], Jin Gohda[1], Ayako Kobayashi[1], Keiko Tomita[1], Youko**

7 **Hirayama[1], Naohiko Koshikawa[2], Motoharu Seiki[3], Kentaro Semba[4], Tetsu**

8 **Akiyama[5], Yasushi Kawaguchi[1,6] and Jun-ichiro Inoue[7]***

9 [1]Research Center for Asian Infectious Diseases, The Institute of Medical Science, The

10 University of Tokyo, Tokyo 108-8639, Japan

11 [2]Department of Life Science and Technology, Tokyo Institute of Technology, Yokohama

12 226-8501 Japan

13 [3]Division of Cancer Cell Research, The Institute of Medical Science, The University of

14 Tokyo, Tokyo 108-8639, Japan

15 [4]Department of Life Science and Medical Bio-Science, Waseda University, Shinjuku-ku,

16 Tokyo, 162-8480, Japan

17 [5]Laboratory of Molecular and Genetic Information, Institute for Quantitative Biosciences,

18 The University of Tokyo, Tokyo 113-0032, Japan

19 [6]Division of Molecular Virology, Department of Microbiology and Immunology, The

20 Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

21 [7]Research Platform Office, The Institute of Medical Science, The University of Tokyo,

22 Tokyo 108-8639, Japan

23

24 * Corresponding author:

25 E-mail: jun-i@ims.u-tokyo.ac.jp (JI)

26

Abstract

28   The ongoing global vaccination program to prevent SARS-CoV-2 infection, the causative

29   agent of COVID-19, has had significant success. However, recently virus variants have

30   emerged that can evade the immunity in a host achieved through vaccination.

31   Consequently, new therapeutic agents that can efficiently prevent infection from these

32   new variants, and hence COVID-19 spread are urgently required. To achieve this,

33   extensive characterization of virus-host cell interactions to identify effective therapeutic

34   targets is warranted. Here, we report a cell surface entry pathway of SARS-CoV-2 that

35   exists in a cell type-dependent manner is TMPRSS2-independent but sensitive to various

36   broad-spectrum metalloproteinase inhibitors such as marimastat and prinomastat.

37   Experiments with selective metalloproteinase inhibitors and gene-specific siRNAs

38   revealed that a disintegrin and metalloproteinase 10 (ADAM10) is partially involved in

39   the metalloproteinase pathway. Consistent with our finding that the pathway is unique to

40   SARS-CoV-2 among highly pathogenic human coronaviruses, both the furin cleavage

41   motif in the S1/S2 boundary and the S2 domain of SARS-CoV-2 spike protein are

42   essential for metalloproteinase-dependent entry. In contrast, the two elements of SARS-

43   CoV-2 independently contributed to TMPRSS2-dependent S2 priming. The

44   metalloproteinase pathway is involved in SARS-CoV-2-induced syncytia formation and

45 cytopathicity, leading us to theorize that it is also involved in the rapid spread of SARS-

46 CoV-2 and the pathogenesis of COVID-19. Thus, targeting the metalloproteinase

47 pathway in addition to the TMPRSS2 and endosome pathways could be an effective

48 strategy by which to cure COVID-19 in the future.

49

50 **Author Summary**

51 To develop effective therapeutics against COVID-19, it is necessary to elucidate in detail

52 the infection mechanism of the causative agent, SARS-CoV-2, including recently

53 emerging variants. SARS-CoV-2 binds to the cell surface receptor ACE2 via the Spike

54 protein, and then the Spike protein is cleaved by host proteases to enable entry. Selection

55 of target cells by expression of these tissue-specific proteases contributes to pathogenesis.

56 Here, we found that the metalloproteinase-mediated pathway is important for SARS-

57 CoV-2 infection, variants included. This pathway requires both the prior cleavage of

58 Spike into two domains and a specific sequence in the second domain S2, conditions met

59 by SARS-CoV-2 but lacking in the related human coronavirus SARS-CoV. The

60 contribution of several proteases, including metalloproteinases, to SARS-CoV-2

61 infection was cell type dependent, especially in cells derived from kidney, ovary, and

62 endometrium, in which SARS-CoV-2 infection was metalloproteinase-dependent. In

3

63   these cells, inhibition of metalloproteinases by treatment with marimastat or prinomastat,

64   whose safety was previously confirmed in clinical trials, was important in preventing cell

65   death. Our study provides new insights into the complex pathogenesis unique to COVID-

66   19 and relevant to the development of effective therapies.

Introduction

68 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of

69 coronavirus disease 2019 (COVID-19), was first recognized in late 2019 and led to the

70 development of a global pandemic in 2020[1]. Two other human coronaviruses, SARS-

71 CoV[2, 3] and Middle East respiratory syndrome coronavirus (MERS-CoV)[4], are also

72 capable of inducing lethal pneumonia and systemic symptoms. However, SARS-COV-2

73 has been found to also exhibit enhanced pathogenicity and transmissibility[5, 6].

74 Effective vaccines have been developed, and ongoing global vaccination programs have

75 significantly curbed the spread of infection[7, 8]. However, current vaccinations may

76 provide imperfect protection as new variants of the virus that can spread more easily and

77 evade the host immunity achieved through vaccination have been reported[7, 9-11].

78 Furthermore, although several drugs that may provide effective treatments for COVID-

79 19 are currently under clinical trial and awaiting approval[12, 13], it is currently unclear

80 if daily life around the world will ever return to that of pre-COVID-19 times.

81 Consequently, further extensive characterization of the virus and its interactions with host

82 cells are required to develop vaccines and therapeutic agents that efficiently prevent

83 infection from the new emerging highly infective variants so as to limit further worsening

84 of COVID-19.

85   The initiation of SARS-CoV-2 entry requires two steps after its spike (S) protein

86   is cleaved into S1 and S2 by furin-like proteases expressed in virus-producing cells prior

87   to viral release[14-16]. First, the S protein binds to its receptor angiotensin converting

88   enzyme 2 (ACE2) in the plasma membrane through its receptor-binding domain

89   (RBD)[17, 18]. Second, the S2 protein is cleaved to generate S2′ by either cell surface

90   transmembrane serine protease 2 (TMPRSS2)[19] or endosomal protease cathepsin-

91   B/L[19, 20]. This cleavage is called priming, and exposes the fusion peptide within S2′,

92   allowing it to stick into the plasma or endosomal membrane, resulting in fusion between

93   the viral envelope and the cellular membrane (envelope fusion). This fusion allows viral

94   RNA to enter the cytoplasm where it replicates. Whether SARS-CoV-2 viruses use the

95   plasma membrane, the endosome pathway, or both is dependent on the cell type[19, 21,

96   22]. Furin-mediated cleavage at the S1/S2 boundary leads to efficient viral entry into

97   airway cells[15, 16], where the TMPRSS2-dependent surface entry route dominates

98   endosomal entry[19, 23].

99   In this study, we screened for inhibitors of SARS-CoV-2 infection and identified

100   a   cell surface entry pathway of SARS-CoV-2 that is TMPRSS2-independent but

101   sensitive to various metalloproteinase inhibitors. Interestingly, the metalloproteinase-

102   dependent pathway requires both the furin cleavage motif in the S1/S2 boundary and the

6

103 S2 domain of SARS-CoV-2, which is unique to SARS-CoV-2. These results suggest that

104 co-operation between furin and some metalloproteinases could be crucial for SARS-CoV-

105 2 spread and disease development *in vivo*. Consequently, targeting the metalloproteinase-

106 pathway in addition to the TMPRSS2 and cathepsin-B/L pathways could be an effective

107 strategy to cure COVID-19.

108

109 **Results**

110 **TMPRSS2-independent membrane fusion induced by the S protein of SARS-CoV-2**

111 **is blocked by metalloproteinase inhibitors**

112 In this investigation, the screening system used to detect effective inhibitors of

113 coronavirus infection included a quantitative cell fusion assay between effector cells

114 expressing S protein and target cells expressing either ACE2 (for SARS-CoV and SARS-

115 CoV-2)[24] or CD26 (for MERS-CoV)[25], with or without TMPRSS2 (S1a,b Fig).

116 Quantitation was accomplished using the dual split chimeric reporter proteins (DSP)1-7

117 and DSP8-11, which contain both *Renilla* luciferase (RL) and green fluorescent protein

118 (GFP) variants[26]. The DSP assay quantifies the degree of membrane fusion between

119 the effector cells expressing DSP1-7 and the target cells expressing DSP8-11 based on

120 the RL activity (S1c Fig). During analysis with the DSP cell fusion assay, a significant

121   amount of ACE2-dependent but TMPRSS2-independent cell-cell fusion was induced by

122   the S protein of SARS-CoV-2, but not by that of SARS- or MERS-CoV (Fig 1a,b).

123   Consistent with this finding, the cell fusion with TMPRSS2 in the target cells induced by

124   the S protein of SARS-CoV and MERS-CoV was completely blocked when TMPRSS2

125   was inhibited with 1 µM nafamostat, while approximately 20% of the fusion by the

126   SARS-CoV-2 S protein remained, even in the presence of 10 µM nafamostat (Fig 1c).

127   This amount of residual fusion was almost equal to that induced by the SARS-CoV-2 S

128   protein in the absence of TMPRSS2 (Fig 1d).

129   To explore the mechanism of TMPRSS2-independent membrane fusion, we

130   screened the Validated Compound Library (1,630 clinically approved compounds and

131   1,885 pharmacologically active compounds) obtained from the Drug Discovery Initiative

132   (The University of Tokyo). We aimed to identify compounds that preferentially inhibited

133   SARS-CoV-2 S protein-induced TMPRSS2-independent fusion and not TMPRSS2-

134   dependent fusion. We compared the relative fusion values of the target cells expressing

135   both TMPRSS2 and ACE2 (X-axis of Fig 2a) with those of the target cells expressing

136   ACE2 alone (Y-axis of Fig 2a), and chose for further validation compounds that limited

137   the fusion without TMPRSS2 by less than 60% and allowed the fusion with TMPRSS2

138   by more than 70% (Fig 2a). The compounds selected included two metalloproteinase

139   inhibitors (ilomastat, CTS-1027), three tyrosine kinase inhibitors (sunitinib, PD-166285,

140   PD-173952), two checkpoint kinase inhibitors (PF-477736, AZD-7762), a protein kinase

141   C inhibitor (midostaurin), and a hormonal contraceptive (algestone). Ilomastat and CTS-

142   1027 preferentially inhibited the TMPRSS2-independent fusion in a dose-dependent

143   manner without affecting TMPRSS2-dependent fusion (Fig 2b). Furthermore, the

144   luciferase activities of the preformed DSP1-7/DSP8-11 complex were not affected,

145   confirming the specificity of the DSP assay (S2a,b Fig). However, other compounds

146   inhibited both the TMPRSS2-dependent and -independent fusions to similar degrees (S3

147   Fig). These data suggest that the metalloproteinase-dependent cell surface entry pathway

148   (the metalloproteinase pathway) may be unique to SARS-CoV-2 among human

149   pathogenic coronaviruses with high mortality rates. Considering that metalloproteinase

150   inhibitors could thus possibly be used as prophylactic or therapeutic agents for COVID-

151   19, we further demonstrated that marimastat[27] and prinomastat[28] (whose safety was

152   previously confirmed in clinical trials to investigate their use as anticancer agents, such

153   as CTS-1027[29]) can preferentially block the TMPRSS2-independent fusion induced by

154   the SARS-CoV-2 S protein (Fig 2b and S2b Fig).

155

156   **The metalloproteinase pathway is SARS-CoV-2 specific and cell type-dependent**

157   We investigated whether the metalloproteinase pathway exists in SARS-CoV-2 S-bearing

158   vesicular stomatitis virus (VSV) pseudovirus. The pseudovirus entry into the A704 cells

159   (human kidney) was entirely blocked by 1 μM marimastat (Fig 3a). This indicates that

160   the metalloproteinase pathway is involved in the entry of the virus, and that 1 μM

161   marimastat could be used to determine if the pathway exists in other cells. Similarly, all

162   entry pathways in OVISE cells (human ovary) were blocked by 25 μM E-64d (Fig 3a),

163   indicating that it could be used to investigate the existence of cathepsin-B/L-dependent

164   endosome pathways in other cells. Furthermore, the entry pathways in Calu-3 cells

165   (human lung) were entirely blocked by 0.1 μM nafamostat (Fig 3a), which indicates that

166   more than 0.1 μM nafamostat may be used to investigate the existence of the TMPRSS2-

167   dependent surface entry pathways in other cells. Consequently, 1 μM marimastat, 25 μM

168   E-64d, and 10 μM nafamostat were used to elucidate the patterns of the entry pathways

169   in various cells. Marimastat significantly inhibited the pseudovirus entry into VeroE6

170   (African green monkey kidney), HEC50B (human endometrium), OVTOKO (human

171   ovary), and A704 cells (Fig 3b). In addition to the metalloproteinase pathway, virus entry

172   was partially inhibited by E-64d, and the combination of marimastat and E-64d showed

173   additive effects in VeroE6, HEC50B, and OVTOKO cells (Fig 3b). These results suggest

174   that the metalloproteinase and endosomal pathways are mutually independent. Neither

10

175 marimastat nor nafamostat alone significantly inhibited the entry pathways of IGROV1

176 (human ovary), OUMS-23 (human colon), or OVISE, whereas E-64d significantly

177 inhibited the entry pathways of IGROV1 and OUMS-23 cells and the overall entry

178 pathway into the OVISE cells (Fig 3c). Interestingly, E-64d resistant entry in IGROV1

179 cells was inhibited by the combination of marimastat with E-64d while the E-64d resistant

180 entry into OUMS-23 cells was inhibited by the combination of nafamostat and E-64d.

181 These results indicate that the endosome entry pathway dominates these cells, while

182 coexisting with either the metalloproteinase or TMPRSS2 surface pathway. Nafamostat

183 inhibited the overall entry pathways into the Calu-3 and Caco-2 (human colon) cells,

184 while marimastat and E-64d showed no inhibitory effects. (Fig 3d). Together, these

185 findings show that the metalloproteinase-dependent cell surface entry pathway exists in

186 a cell type-dependent manner and coexists with the endosome pathway in some cell lines.

187 As we could not find cell lines with both metalloproteinase- and TMPRSS2-dependent

188 cell surface entry pathways, we generated HEC50B cell lines ectopically expressing

189 TMPRSS2 (HEC50B-TMPRSS2). In the HEC50B-TMPRSS2 cells, approximately 80%

190 of the entry pathways were TMPRSS2-dependent, while the rest were predominantly

191 metalloproteinase-dependent (Fig 3e). This indicates that the metalloproteinase pathway

11

192   independently coexists with the TMPRSS2 dependent pathway. These results also

193   suggest that there could be cells *in vivo* that naturally have both surface entry pathways.

194

195   **The metalloproteinase pathway requires both the furin-cleavage site and S2 region**

196   **of the SARS-CoV-2 S protein**

197   Our results showed that metalloproteinase-dependent and TMPRSS2-independent cell-

198   cell fusion was induced by the S protein of SARS-CoV-2 but not by that of SARS-CoV

199   or MERS-CoV (Fig 1a,b). In line with these results, metalloproteinase-dependent entry

200   was observed only when the pseudovirus bearing the S protein of SARS-CoV-2, but not

201   SARS-CoV or MERS-CoV, was used in HEC50B (Fig 4a), A704 (S4a Fig), and VeroE6

202   cells (S4b Fig). Furthermore, pseudoviruses bearing the S protein of HCoV-NL63 and

203   WIV1-CoV, which like SARS-CoV-2 use ACE2 as their receptor, cannot utilize the

204   metalloproteinase pathway in HEC50B cells (Fig 4b). While SARS-CoV-2 S uses both

205   the metalloproteinase and endosome pathways, SARS-CoV, MERS-CoV, HCoV-NL63,

206   and WIV1-CoV S exclusively use the endosome pathway, and none of these S proteins

207   can use the metalloproteinase or TMPRSS2 pathway in HEC50B (Fig 4a,b), A704 (S4a

208   Fig), and VeroE6 cells (S4b Fig). The ability to use the metalloproteinase pathway and

209   sensitivities against various protease inhibitors are conserved among the variants of

210   SARS-CoV-2 we tested (S5 Fig).

211   The S proteins of SARS-CoV-2 and MERS-CoV have furin cleavage sites (Arg-

212   X-X-Arg) in their S1/S2 boundary area, and they were efficiently cleaved during the

213   preparation of the pseudovirus (Fig 4c,d). In contrast, the S proteins of SARS-CoV,

214   HCoV-NL63, and WIV1-CoV, which do not use the metalloproteinase pathway, do not

215   have furin cleavage site and are not cleaved to any notable degree (Fig 4c,d and S6 Fig).

216   Given that the MERS-CoV S protein does not use the metalloproteinase pathway (Fig 4a)

217   even though it harbors a furin cleavage site and was efficiently cleaved, we speculated

218   that furin-catalyzed S protein cleavage is a prerequisite but not sufficient for using the

219   metalloproteinase pathway. To test this hypothesis, we generated pseudoviruses bearing

220   chimeric S proteins in which the S1, S1/S2 boundary, and S2 domains were derived from

221   either SARS-CoV or SARS-CoV-2 (Fig 4c,d). As expected, the S2 fragment of the C-

222   terminal Flag-tagged S protein was mainly detected with anti-Flag antibody when

223   pseudoviruses bearing S proteins with the furin-cleavage site (S121, S122, SARS-CoV-

224   2 S (222), and S221) were analyzed (Fig 4d). In contrast, uncleaved S protein (S0) was

225   mainly detected when S proteins without the furin-cleavage site (SARS-CoV S (111),

226   S112, S212, and S211) were used (Fig 4d). When only section, the S1/S2 boundary or the

13

227 S2 domain, was replaced with the corresponding domain of SARS-CoV-2 in the SARS-

228 CoV S protein (S121, S112), the metalloproteinase pathway did not appear (Fig 4e).

229 However, when both the S1/S2 and S2 domains were replaced with the corresponding

230 domains of SARS-CoV-2 (S122), the metalloproteinase pathway appeared in addition to

231 the endosome pathway (Fig 4e), similar to the pattern observed for bona fide SARS-CoV-

232 2 S (S222) (Fig 4f). Furthermore, when either the S1/S2 or S2 domain was replaced with

233 the corresponding domain of SARS-CoV in the SARS-CoV-2 S protein (S212, S221), the

234 metalloproteinase pathway disappeared (Fig 4f). Similar requirements for the S1/S2

235 boundary and S2 domains for SARS-CoV-2 to use the metalloproteinase pathway were

236 also observed in VeroE6 cells (S4c,d Fig). These results indicate that both the S1/S2

237 boundary and S2 domain of SARS-CoV-2 are strictly required for the virus to utilize the

238 metalloproteinase pathway when the endosome pathway coexists.

239   To compare the structural requirements needed for the S protein to use the

240 metalloproteinase pathway with those needed to use the TMPRSS2 pathway in cells with

241 the endosome pathway as an alternative, we used HEC50B-TMPRSS2 and VeroE6 cells

242 ectopically expressing TMPRSS2 (VeroE6-TMPRSS2). While both these cell types

243 exhibit the TMPRSS2 and endosome pathways depending on the source of S protein (Fig

244 4g and S4e Fig), HEC50B-TMPRSS2 cells in addition maintained a significant amount

14

245   of the metalloproteinase pathway (approximately 20% of the total entry pathway) (Fig

246   3e), whereas most of the metalloproteinase pathway disappeared in the VeroE6-

247   TMPRSS2 cells when compared with their parental line (Fig 3b and S4e Fig). Given that

248   the metalloproteinase pathway is dependent on the structural features of the S protein, its

249   blockade by marimastat will promote protein structural requirements for S protein to use

250   the TMPRSS2 pathway in HEC50B-TMPRSS2 (Fig 4g-i) but not in VeroE6-TMPRSS2

251   cells (S4e-g Fig). In both cell types, SARS-CoV-2 predominantly used the TMPRSS2

252   entry pathway, while SARS-CoV used the endosome pathway (Fig 4g and S4e Fig).

253   However, nafamostat partially, but more efficiently, inhibited the entry of S112 and S121

254   pseudoviruses when compared with SARS-CoV (S111), while it inhibited S122 virus

255   entry almost completely (Fig 4h and S4f Fig). Furthermore, when compared with SARS-

256   CoV-2 (S222), nafamostat only partially inhibited S221 or S212 virus entry, while it

257   scarcely inhibited S211 virus entry (Fig 4i and S4g Fig). These results indicate that the

258   S1/S2 boundary and S2 domain of SARS-CoV-2 additively contribute to the ability of

259   the virus to use the TMPRSS2 pathway. Together, these results show that although both

260   the TMPRSS2 and the metalloproteinase pathways undergo priming of the S protein at

261   the cell surface, the structural requirements of the S protein for efficient priming differ

262   between the metalloproteinase and TMPRSS2 pathways.

15

263

Possible involvement of ADAM-10 in the metalloproteinase-dependent entry of

SARS-CoV-2

In addition to marimastat, other metalloproteinase inhibitors, including prinomastat,

ilomastat, and CTS-1027, which block the TMPRSS2-independent cell-cell fusion

induced by SARS-CoV-2 S (Fig 2b), inhibited the metalloproteinase-dependent entry of

SARS-CoV-2 pseudovirus in VeroE6, HEC50B, and A704 cells (Fig 5a). Since these

inhibitors exhibited broad specificity[30-33], selective inhibitors were then used to

narrow down the metalloproteinases involved in the metalloproteinase-dependent entry

pathway. The VeroE6 and HEC50B cells had significantly E-64d sensitive endosome

pathways but the A704 cells did not (Fig 3b). Consequently, selective metalloproteinase

inhibitors were tested in the presence of E-64d so that the reduction in the

metalloproteinase pathway could be easily recognized in VeroE6 and HEC50B cells (Fig

5a). Similar inhibitory patterns were observed in all three cell lines tested (Fig 5a), and

their viabilities were not affected by any of the metalloproteinase inhibitors at the

concentrations used in the experiment (S7a-d Fig). These results suggest that the

metalloproteinases involved in the pathway are likely to be common to all three cell lines.

GW280264X[34] (ADAM10/17 inhibitor) and GI1254023X[34, 35] (MMP9/ADAM10

16

281   inhibitor) significantly inhibited the metalloproteinase pathway, whereas TAPI2[36]

282   (ADAM-17 inhibitor) and BK-1361[37] (ADAM8 inhibitor) did not (Fig 5a). This

283   suggests that ADAM10 may be involved in the ADAM family. MMP408[30]

284   (MMP3/12/13 inhibitor) and MMP2/9 inhibitor I[38] scarcely affected virus entry,

285   whereas UK370106[39] (MMP3/12 inhibitor) and MMP9 inhibitor I[40] were

286   significantly inhibitory (Fig5a), suggesting that MMP3/9/12/13 may not be crucial, but

287   that the unidentified metalloproteinase, which could be inhibited by UK370106 or MMP9

288   inhibitor I, may be involved in the pathway in cooperation with ADAM10. MLN-

289   4760[41] (ACE2 inhibitor) did not inhibit virus entry (Fig 5a), indicating that the catalytic

290   activity of ACE2, to which the S protein directly binds as a receptor, is not involved. To

291   further confirm the involvement of ADAM10 in the metalloproteinase-dependent

292   pathway, ADAM10 was depleted by siRNA in HEC50B cells. Three independent siRNAs

293   effectively suppressed the expression of both the precursor and active forms of ADAM10

294   (Fig 5b). An ADAM10 knockdown significantly inhibited SARS-CoV-2 pseudovirus

295   entry, while the entry of SARS-CoV, MERS-CoV, and VSVG pseudoviruses were not

296   affected (Fig 5c), indicating that ADAM10 plays a role unique to SARS-CoV-2 in viral

297   entry. Furthermore, we examined the effects of the ADAM10 knockdown on the entry

298   pathway patterns by treating siRNA-transfected cells with either E-64d, marimastat, or a

17

299   combination of both. The combination treatment with E-64d and marimastat led to an

300   additive effect for the single treatments, resulting in the complete inhibition of viral entry

301   in both cells with normal ADAM10 expression and those with reduced ADAM10

302   expression (Fig 5d). These results indicated that E-64d-resistant viral entry is a

303   metalloproteinase-dependent pathway, while marimastat-resistant viral entry is

304   dependent on the endosome pathway. The ADAM10-knockdown significantly inhibited

305   the metalloproteinase pathway (Fig 5e, E64-d treatment）while the ADAM10-knockdown

306   had only a modest effect on the endosome pathway (Fig 5e, marimastat-treatment),

307   indicating that ADAM10 is involved in the metalloproteinase-dependent entry pathway

308   of SARS-CoV-2.

309   Recently, it was reported that ACE2 shedding by ADAM17 promotes SARS-

310   CoV-2 infection[42]. While a CRISPR/Cas9-mediated knockout of ADAM17 enhanced

311   the accumulation of cellular ACE2 in HEC50B cells due to the inhibition of ACE2

312   shedding (S8a Fig), SARS-CoV-2 S pseudovirus entry was increased, and this was

313   probably because of the enhanced binding of virus to the cell surface ACE2 (S8b Fig).

314   However, the patterns of the metalloproteinase and endosome pathways were similar

315   between the wild-type and ADAM17 knockout cells (S8c Fig), suggesting that ADAM17

316   may not be involved in metalloproteinase-dependent virus entry in HEC50B cells.

317

The metalloproteinase-dependent entry pathway of authentic SARS-CoV-2 is

involved in syncytia formation and cytopathicity

To confirm the involvement of the metalloproteinase pathway in authentic SARS-CoV-2

entry, we first evaluated the effects of marimastat and prinomastat on the amount of

cytoplasmic viral RNA transcribed from the N gene after infection. Both inhibitors

significantly suppressed SARS-CoV-2 infection (Fig 6a). The IC_{50} values of the

marimastat and prinomastat were 160 nM and 130 nM in HEC50B cells, and 150 nM and

250 nM in the A704 cells, respectively. The IC_{50} value of the marimastat in the VeroE6

cells was 340 nM. Consistent with the results from the pseudoviruses experiments,

nafamostat showed a marked inhibitory effect on the Calu-3 cells but not on the HEC50B,

A704, or VeroE6 cells (S9a Fig). In contrast, 25 µM E-64d and 10 mM NH_4Cl, which

inhibits endosome-lysosome system acidification[43], significantly suppressed SARS-

CoV-2 infection in the HEC50B, A704, and VeroE6 cells (S9b,c Fig). This indicated that

the endosome pathway coexists with the metalloproteinase pathway to contribute to

authentic SARS-CoV-2 infection in these cells. Combination treatments with E-

64d/marimastat or NH_4Cl/marimastat showed much stronger inhibitory effects than the

treatment with each drug alone (Fig 6b). Similarly, combination treatments with

19

335 nafamostat and marimastat or nafamostat and E-64d showed stronger inhibitory effects

336 than the nafamostat treatment alone in the HEC50B-TMPRSS2 cells (Fig 6c).

337 Furthermore, when all three drugs were combined, they had a much stronger inhibitory

338 effect on viral infection when compared with the two-drug combinations (Fig 6c). These

339 results strongly suggest that drugs that block the metalloproteinase pathway are effective

340 for COVID-19 treatment.

341   Next, we examined whether ADAM10 is involved in SARS-CoV-2 infection.

342 GW280264X[34] (ADAM10/17 inhibitor) and GI1254023X[34, 35] (MMP9/ADAM10

343 inhibitor) significantly suppressed SARS-CoV-2 infection, whereas TAPI2[36] (ADAM-

344 17 inhibitor) did not (Fig 6d). Moreover, the ADAM10 knockdown by siRNA suppressed

345 SARS-CoV-2 infection by approximately 40% (Fig 6e), indicating that ADAM10 is

346 partially involved. This effect was smaller than that for the various metalloproteinase

347 inhibitors (Fig 6a,d), suggesting that metalloproteinases other than ADAM10 are also

348 involved in this pathway.

349   The ability of SARS-CoV-2 to form syncytia and induce cytopathicity is thought

350 to be related to its pathogenesis[44, 45]. To determine whether the metalloproteinase-

351 dependent pathway is involved in syncytia formation, we first used HEC50B cells as a

352 representative for cells that predominantly use the metalloproteinase and endosome

20

353   pathways. Interestingly, the SARS-CoV-2-induced syncytia formation in HEC50B cells

354   24 h after infection was significantly blocked by 500 nM of marimastat and prinomastat

355   but not notably affected by 25 μM E-64d (Fig 6f and S10 Fig). These results indicate that

356   the metalloproteinase-dependent pathway, but not the endosome pathway, is crucial for

357   syncytium formation, although both pathways similarly reduce viral infection (Fig 6b).

358   Given that the metalloproteinases are normally localized at the cell surface, we used

359   HEC50B-TMPRSS2 cells, which have cell surface TMPRSS2 and metalloproteinase

360   pathways, to investigate their involvement in syncytia formation when they coexist. The

361   SARS-CoV-2-induced syncytia formation in the HEC50B-TMPRSS2 cells was not

362   significantly inhibited by marimastat or nafamostat alone, but was clearly inhibited by

363   the combined treatment (Fig 6g). These results suggest that the metalloproteinase and

364   TMPRSS2 pathways cooperate to form syncytia. Next, we addressed the role of the

365   metalloproteinase pathway in SARS-CoV-2-induced cytotoxicity. SARS-CoV-2-induced

366   cytopathicity of HEC50B cells 3 d after infection was not inhibited by either E-64d,

367   marimastat, or prinomastat alone, but was significantly blocked when cells were treated

368   with E-64d in combination with either marimastat or prinomastat (Fig 6h). In addition,

369   SARS-CoV-2-induced cytopathicity of the HEC50B-TMPRSS2 cells was not inhibited

370   by either E-64d, nafamostat, or marimastat alone, but was significantly blocked when

21

371   cells were treated with a combination of all three drugs (Fig 6i). These results strongly

372   suggest that the inhibition of the metalloproteinase pathway is crucial to block syncytia

373   formation and cytopathicity *in vivo*, and consequently, that the metalloproteinase pathway

374   is likely to be involved in the pathogenesis of COVID-19.

375

376   **Discussion**

377   Previous studies of the S proteins found in SARS-CoV-2, SARS-CoV, and MERS-CoV

378   have shown that the priming of the S2 domain, catalyzed by either TMPRSS2 on the

379   plasma membrane or cathepsin-B/L in endosomes, results in the fusion peptide protruding

380   to induce envelope fusion, thereby establishing viral entry through the plasma membrane

381   or endosomal membrane, respectively[14, 19, 20]. In this study, we have demonstrated

382   that SARS-CoV-2, unlike SARS-CoV or MERS-CoV, has a unique TMPRSS2-

383   independent cell surface entry pathway, which is sensitive to various metalloproteinase

384   inhibitors including ilomastat, CTS-1027, marimastat, and prinomastat, but resistant to

385   previously known inhibitors of SARS-CoV-2 entry, such as nafamostat[24, 46] and E-

386   64d[19]. As a representative of these broad-spectrum metalloproteinase inhibitors, we

387   chose marimastat to investigate the cell type-dependent distribution of the

388   metalloproteinase pathway by measuring pseudovirus infection. A significant proportion

389 of the entry pathway is metalloproteinase-dependent in A704 (kidney), HEC50B

390 (endometrium), OVTOKO (ovary), and VeroE6 (kidney) cells. Only a small proportion

391 was metalloproteinase-dependent in IGROV1 (ovary) cells, while the metalloproteinase

392 pathway was not detected in OMUS-23 (colon), OVISE (ovary), Calu-3 (lung), and Caco-

393 2 (colon) cells. These results indicate that the metalloproteinase pathway of SARS-CoV-

394 2 is cell-type specific and independently coexists with other entry pathways, including

395 the TMPRSS2-dependent surface pathway and the endosome pathway. The kidney[47,

396 48], ovary[49, 50], and endometrium[51] are known to express ACE2. Furthermore,

397 SARS-CoV-2 can infect the kidney[48, 52] and induce acute kidney injury[53] in

398 COVID-19 patients. Although SARS-CoV-2 infection of the ovary or endometrium has

399 not previously been reported, the metalloproteinase-dependent infection pathway may

400 contribute to the pathogenesis of COVID-19, especially multiple organ failure. The

401 metalloproteinase pathway is thus a potential target for future COVID-19 therapies.

402   The S1/S2 boundary of SARS-CoV-2 contains the furin cleavage motif (Arg-X-

403 X-Arg), while that of SARS-CoV contains only a single Arg. It has been reported that the

404 motif greatly increases the efficiency of S1/S2 cleavage[15, 16], leading to enhanced viral

405 transmission both *in vitro[15, 16, 23]* and *in vivo[54, 55].* This may be partially due to

406 the enhanced availability of S2 to TMPRSS2, due to the dissociation of S1[56, 57]. We

407   have shown that the furin cleavage motif is required for the metalloproteinase pathway,

408   and we propose that the induction of metalloproteinase-induced S2 priming is another

409   role of furin-mediated S1/S2 cleavage in enhanced viral transmission. Therefore, the

410   metalloproteinase-dependent entry pathway, which is unique among highly pathogenic

411   coronaviruses, is likely to be associated with the rapid spread of SARS-CoV-2.

412   Interestingly, experiments using pseudoviruses bearing chimeric S proteins between

413   SARS-CoV (without the metalloproteinase pathway) and SARS-CoV-2 (with the

414   metalloproteinase pathway) revealed that both the S1/S2 boundary of SARS-CoV-2 and

415   the S2 domain of SARS-CoV-2 S are essential for metalloproteinase-dependent entry. In

416   contrast, the two domains of SARS-CoV-2 independently contributed to TMPRSS2-

417   dependent S2 priming. This discrepancy may be partially due to the difference in the

418   substrate recognition properties of the priming proteases in the two pathways. The S112

419   pseudovirus (a VSV pseudovirus bearing SARS-CoV S mutant, in which the S2 region

420   was replaced with the corresponding domain of SARS-CoV-2) can use the TMPRSS2

421   pathway more efficiently than the SARS-CoV S pseudovirus, which suggests that

422   TMPRSS2 may be partially accessible to the priming site in SARS-CoV-2 (C-terminal of

423   Arg815) but not to that in SARS-CoV (C-terminal of Arg797) without S1 dissociation.

424   In contrast, the putative priming protease in the metalloproteinase pathway, which may

24

425   not be a metalloproteinase but a protease activated by metalloproteinases, can access the

426   priming site only when the site occurs within the contextual characteristics of SARS-

427   CoV-2 S2, and S1/S2 is cleaved to allow S1 dissociation. Determination of the priming

428   site in the metalloproteinase pathway and identification of the critical amino acid residues

429   generating the structural characteristics of SARS-CoV-2 S2 that allow metalloproteinase-

430   dependent priming are required to understand its molecular mechanisms for the two

431   distinct surface entry pathways. From an evolutionary perspective, SARS-CoV-2

432   acquired the metalloproteinase pathway by introducing mutations into the S2 region,

433   which may have contributed to the SARS-CoV-2 pandemic. The function of point

434   mutations in the S2 domain have not yet been fully analyzed in comparison to those in

435   the S1 domain, which cause escape from neutralizing antibodies[58]. However, various

436   point mutations in the S2 domain may play important roles in increasing the efficiency of

437   infection and disease progression and the generation of highly infectious variants.

438   Using selective metalloproteinase inhibitors and ADAM10 knockdowns

439   generated using specific siRNAs, we have demonstrated that ADAM10 plays an

440   important role in the metalloproteinase pathway. ADAM10 is ubiquitously expressed in

441   various tissues[59] and cell lines[60], and functionally regulates cell differentiation and

442   proliferation by cleaving ligands and receptors such as epidermal growth factor (EGF),

443   heparin-binding EGF-like growth factor (HB-EGF), and Notch[61]. ADAM10 is thus

444   likely to contribute to SARS-CoV-2 infection in various organs. ADAM17, similar to

445   ADAM10, is also known as a metalloproteinase that induces the cleavage of receptors

446   and ligands, and both can cleave common substrates such as Notch and HB-EGF[61]. It

447   has been reported that ACE2 shedding by ADAM17 promotes SARS-CoV-2

448   infection[42]. Although we observed that ADAM17 depletion resulted in the cellular

449   accumulation of ACE2, which is indicative of reduced ACE2 shedding, total SARS-CoV-

450   2 pseudovirus infection was unexpectedly augmented and the relative contribution of the

451   metalloproteinase pathway was not affected. ADAM10 and ADAM17 thus both play

452   crucial but distinct roles in SARS-CoV-2 infection. It has recently been reported that

453   ADAM9 inhibition decreases SARS-CoV-2 infection *in vitro[62]*. Although the

454   involvement of ADAM9 in viral entry is not clear, the results suggest that a group of

455   metalloproteinases cooperate in the metalloproteinase pathway. This may indicate that

456   the observed ADAM10 depletion-induced inhibition was a part of the maximum

457   inhibition by various metalloproteinase inhibitors. A recent report also showed that

458   MMP12 knockouts inhibited SARS-CoV-2 infection *in vitro[63]*. However,

459   MMP408[30], an inhibitor of MMP12, did not prevent SARS-CoV-2 infection in various

460   cell lines in this investigation, suggesting that metalloproteinases involved in the

461 metalloproteinase pathway may differ in a cell type-dependent manner. Further studies

462 are required to identify the functional metalloproteinases that are involved in the

463 metalloproteinase pathway.

464   Various compounds are reported to inhibit SARS-CoV-2 infection by inhibiting

465 envelope fusion *in vitro*. However, camostat[64], an inhibitor of the TMPRSS2-

466 dependent surface entry, and hydroxychloroquine[65, 66], an inhibitor of the endosomal

467 pathway, have failed to show sufficient therapeutic efficacy in clinical trials. Our entry

468 pathway analysis revealed that the metalloproteinase surface pathway coexists with the

469 TMPRSS2 pathway and/or the endosome pathway in various cell types. Furthermore, in

470 HEC50B and HEC50B-TMPRSS2 cells, cell death could not be inhibited unless all entry

471 pathways in each cell were inhibited using inhibitor co-treatments for each pathway.

472 Therefore, future clinical trials on virus entry in which the TMPRSS2, metalloproteinase,

473 and endosome pathways are all efficiently blocked, need to be conducted. We propose

474 that to address this challenge both marimastat and prinomastat should be utilized in

475 clinical trials. The mean maximum plasma concentration (C_{max}) at a reasonably well-

476 tolerated dose was 590 nM for marimastat[27] and 680 nM for prinomastat[28].

477 Furthermore, we demonstrated that these two drugs significantly inhibited SARS-CoV-2

478 infection at concentrations lower than their C_{max} values. These metalloprotease inhibitors,

27

479   in combination with other protease inhibitors targeting the TMPRSS2 and endosome

480   pathways, may effectively inhibit SARS-CoV-2 infection in various tissues and cure

481   COVID-19. Recently, there has been concern about the spread of SARS-CoV-2 variants,

482   such as the delta strain, as they reduce the effectiveness of the neutralizing antibodies

483   produced by vaccination[7, 9, 10]. Since sensitivities against marimastat, nafamostat, and

484   E-64d have been conserved in the various variants investigated so far, the strategies to

485   use protease inhibitors for COVID-19 treatment are likely to be significantly effective

486   against these variants. The results of this study may contribute to the development of

487   COVID-19 treatments targeting viral entry pathways.

488

489   **Materials and Methods**

490   **Cell lines, viruses, and reagents**

491   VeroE6 (CRL-1586), 293T (CRL-3216), A704 (HTB-45) and Calu-3 (HTB-55) cells

492   were obtained from the American Type Culture Collection (Rockville, MD, USA).

493   OVTOKO (JCRB1048), OVISE (JCRB1043), HEC50B (JCRB1145), VeroE6-

494   TMPRSS2 (JCRB1819)[67], and OUMS-23 (JCRB1022) cells were obtained from the

495   Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). IGROV1 cells

496   (SCC203) were purchased from Merck (Darmstadt, Germany) and Caco-2 cells

28

497   (RCB0988) were obtained from the RIKEN BioResource Research Center (Tsukuba,

498   Japan). A704, Calu-3, VeroE6, HEC50B, and Caco-2 cells were maintained in Eagle's

499   minimum essential medium (EMEM; 055-08975, FUJIFILM Wako Pure Chemical,

500   Osaka, Japan) containing 15% fetal bovine serum (FBS). OUMS-23, IGROV1, and 293T

501   cells were maintained in Dulbecco's modified Eagle's medium (DMEM; 041-30081,

502   FUJIFILM Wako Pure Chemical) containing 10% FBS. VeroE6-TMPRSS2 (JCRB1819)

503   cells were cultured in DMEM containing 10% FBS and 1 mg/mL G418. OVISE and

504   OVTOKO cells were maintained in Roswell Park Memorial Institute (RPMI)-1640

505   medium (189-02025, FUJIFILM Wako Pure Chemical) containing 10% FBS. A pair of

506   previously described 293FT-based reporter cell lines that stably express individual split

507   reporters (DSP1-7 and DSP8-11 proteins)[68] were maintained in DMEM containing

508   10% FBS and 1 μg/mL puromycin. To establish stable cell lines expressing the S protein

509   of SARS-CoV, SARS-CoV-2, or MERS-CoV, recombinant pseudotype lentiviruses were

510   produced in 293T cells with psPAX2 packaging plasmid, vesicular stomatitis virus

511   (VSV)-G-expressing plasmid and lentiviral transfer plasmid expressing S protein. To

512   establish stable cell lines expressing ACE2 or CD26 with TMPRSS2, recombinant

513   pseudotype lentiviruses expressing one of the proteins were produced using 293T cells

514   with psPAX2 packaging plasmid and VSV-G-expressing plasmid. The 293FT-derived

515   reporter cells infected with the pseudotype viruses were selected with 1 μg/mL puromycin,

516   10 μg/mL blasticidin, and 300 μg/mL hygromycin for at least 1 week. These bulk-selected

517   cells were used for fusion assays. To establish HEC50B cells expressing TMPRSS2

518   (HEC50B-TMPRSS2), recombinant pseudotype lentivirus expressing TMPRSS2 was

519   produced using 293T cells with psPAX2 packaging plasmid and VSV-G-expressing

520   plasmid. HEC50B cells infected with pseudotype viruses were selected with 300 μg/mL

521   hygromycin   for   at   least   1   week.   The   SARS-CoV-2   isolate   (UT-

522   NCGM02/Human/2020/Tokyo)[69] was propagated in VeroE6-TMPRSS2 (JCRB1819)

523   cells in DMEM containing 5% FBS. Titers were determined with plaque assays using

524   VeroE6/TMPRSS2 (JCRB1819) cells. Negative control No.1 siRNA (4390843), negative

525   control No.2 siRNA (4390846), and three distanced ADAM10-specific siRNAs were

526   purchased from Thermo Fisher Scientific (MA, USA) The siRNA sequences used were

527   5′-UCA CCU UGU UCU ACC AUU CCA (S1004, ADAM10#1); 5′-UAA CCU CUA

528   AAA UCG UUG CAA (S1005, ADAM10#2); and 5′-UAC GGA UUC CGG AGA AGU

529   CTG (S1006, ADAM10#3) for the ADAM10 knockdown. Cell viability was analyzed

530   using the CellTiter-Glo luminescent cell viability assay (G7570, Promega, WI, USA)

531   according to the manufacturer's protocol.

532

30

Protease inhibitors and compound libraries

533

534 Nafamostat mesylate (N0959, Tokyo Chemical Industry, Tokyo, Japan), pepstatin A

535 (4397, Peptide institute, Osaka, Japan), bestatin (027-14101, FUJIFILM Wako Pure

536 Chemical), leupeptin (4041, Peptide institute), E-64d (4321-v, Peptide institute), furin

537 inhibitor II (344931, Merck), ilomastat (HY-15768, MedChemExpress, NJ, USA), CTS-

538 1027 (HY-10398, MedChemExpress), marimastat (HY-12169, MedChemExpress),

539 prinomastat hydrochloride (PZ0198, Sigma-Aldrich, MO, USA), UK370106 (2900,

540 Tocris, MN, USA), GW280264X (31388, Cayman, MI, USA), GI254023X (SML0789,

541 Sigma-Aldrich), TAPI-2 (14695, Cayman), MLN-4760 (530616, Merck), BK-1361 (PC-

542 60981, ProbeChem, Shanghai, China), MMP408 (444291, Millipore, MA, USA),

543 MMP2/9 inhibitor I (ab145190, Abcam, Cambridge, UK), and MMP9 inhibitor I (444278,

544 Millipore) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM.

545 Validated Compound Library (1,630 clinically approved compounds and 1,885

546 pharmacologically active compounds) obtained from the Drug Discovery Initiative (The

547 University of Tokyo) was used for compound screening.

548

Expression vector construction

549

550   To construct expression vectors for ACE2, CD26, and TMPRSS2, genes were cloned into

551   a lentiviral transfer plasmid (CD500B-1, SBI, Palo Alto, CA, USA). Synthetic DNA

552   corresponding to the codon-optimized S gene of SARS-CoV-2 (Wuhan-Hu-1, RefSeq:

553   NC_045512.2), SARS-CoV-2 variants (B.1.1.7, GISAID: EPI_ISL_601443, B.1.351,

554   GenBank: MZ747297.1, B.1.617.1, GISAID: EPI_ISL_1704611, B.1.617.2, GISAID:

555   EPI_ISL_3189054), SARS-CoV (Tor2, RefSeq: NC_004718.3), bat SARS-like

556   coronavirus WIV1 (GenBank: KF367457.1), human coronavirus NL63 (RefSeq:

557   NC_005831.2), and the chimeric S gene (S1, S1/S2 boundary, and S2 domains were

558   derived from either SARS-CoV Tor2 or SARS-Cov-2 Wuhan-Hu-1), and the DNA

559   sequence corresponding to the Flag-tag 5′-GGA GGC <u>GAT TAC AAG GAT GAC GAT</u>

560   <u>GAC AAG</u> TAA-3' (underline, Flag-tag) at the 3′ end were all generated by Integrated

561   DNA Technologies (IA, USA). Previously described synthetic DNA corresponding to the

562   codon-optimized S gene of a MERS-CoV (EMC 2012, RefSeq: NC_019843.3)[25] with

563   a DNA sequence corresponding to the Flag-tag 5′-GGA GGC GAT TAC AAG GAT

564   GAC GAT GAC AAG TAA-3' at the 3′ end was used in this study. To construct

565   expression vectors for the S protein, the coding regions were cloned into a lentiviral

566   transfer plasmid (CD500B-1, SBI).

567

DSP assay to monitor membrane fusion

DSP1-7 has the structure RL_{1-155}-Ser-Gly-Gly-Gly-Gly-GFP_{1-156}, while DSP8-11 has the structure Met-$GFP_{157-231}$-Gly-Gly-Gly-Gly-Ser- $RL_{156-311}$. RL and GFP become active only when DSP1-7 associates with DSP-8-11 (S1c Fig). For the DSP assay using 293FT cells, DSP8-11 expressing effector cells expressing S protein and DSP1-7 expressing target cells expressing CD26 or ACE2 alone or together with TMPRSS2 were seeded in 10 cm cell culture plates (4×10^6 cells/10 mL) one day prior to the assay (S1a,b Fig). Cells were treated with 6 μM EnduRen (Promega), a substrate for Renilla luciferase (RL), for 2 h to activate EnduRen. For compound library screening, 0.25 μL of each compound dissolved in DMSO were added to the 384-well plates (Greiner Bioscience, Frickenhausen, Germany). To test the effects of the selected inhibitors, 1 μL of each inhibitor dissolved in DMSO was added to the 384-well plates (Greiner Bioscience). Next, 50 μL of each single cell suspension (effector and target cells) was added to the 384-well plates using a Multidrop dispenser (Thermo Fisher Scientific, MA, USA). After incubation at 37 °C in 5% CO_2 for 4 h, the RL activity was measured using a Centro xS960 luminometer (Berthold, Bad Wildbad, Germany).

Western blotting

33

586 Western blot analysis was performed as described previously[70]. The primary antibodies

587 used were rabbit anti-ACE2 (1:1000, ab15348, Abcam), rabbit anti-TACE (1:1000,

588 3976S, Cell Signaling Technology, MA, USA), rabbit anti-ADAM10 (1:1000, 14194S,

589 Cell Signaling Technology), rabbit anti-Flag-tag (1:1000, PM020, MBL, MA, USA),

590 mouse anti-tubulin (1:1000, CP06, Millipore), and mouse anti-VSVM (1:1000, 23H12,

591 Absolute antibody). The Secondly antibodies used were HRP-linked donkey anti-rabbit

592 IgG antibody (NA934; GE Healthcare, Piscataway, NJ, USA) and HRP-linked donkey

593 anti-mouse IgG antibody (NA931V; GE Healthcare). Cell supernatants containing the

594 pseudotype viral particles were centrifuged at 109,000 g or 35 min at 4 °C using a

595 TLA100.3 rotor with an Optima TLX ultracentrifuge (Beckman Coulter, CA, USA), and

596 the pellet was then lysed for western blotting analysis.

597

598 **Preparation of pseudotype VSV viral particles and infection experiments**

599 To prepare pseudotype VSV viral particles 293T cells were transfected with an expression

600 plasmid for S, VSV G, or a control expression plasmid using calcium phosphate

601 precipitation. At 16 h post-transfection, the cells were inoculated with a replication-

602 deficient VSV, Δ VSV-Luci, which lacks the VSV G gene and encodes firefly luciferase,

603 at a multiplicity of infection (MOI) of 1, as was described previously[71]. After

34

604   incubation at 37 °C in 5% CO_2 for 2 h, the cells were washed with DMEM and further

605   incubated at 37 °C in 5% CO_2 for 16 h before the supernatants containing the pseudotype

606   viral particles were harvested. Cellular debris was removed from the supernatants using

607   a syringe filter with a 0.45 μm size pore (Millipore). For the infection assay, target cells

608   were seeded in 96-well plates (2×10^4 cells/well) and incubated overnight at 37 °C with

609   5% CO_2. The cells were pre-treated with inhibitors for 1 h before infection. Pseudotype

610   viral particles were added to the cells in the presence of the inhibitors. Luciferase activity

611   was measured 16 h post-infection using the Bright-Glo Luciferase Assay System or ONE-

612   Glo Luciferase Assay System (Promega) and Centro xS960 luminometer (Berthold).

613

614   **Transfection**

615   siRNA transfection was performed using Lipofectamine RNAiMAX (Thermo Fisher

616   Scientific) according to the manufacturer's protocol. Cells were seeded in 6-well plates

617   (1.6×10^6 cells/well) with siRNA and Lipofectamine RNAiMAX. Then 24 h after

618   transfection, the cells were seeded in 96-well plates (2×10^4 cells/well), and 48 h after

619   transfection the cells were used for the infection experiments.

620

621   **Quantification of intracellular SARS-CoV-2 RNA**

622 Cells were seeded at 5×10^4 cells per well in a 96-well cell culture plate. After an

623 overnight incubation at 37 °C in 5% CO_2, cells were treated with protease inhibitors for

624 1 h and added with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.01 for HEC50B

625 and HEC50B-TMPRSS2 cells, and MOI of 0.1 for VeroE6, Calu-3, and A704 cells. After

626 24 h of incubation at 37 °C in 5% CO_2, the cells were washed three times with PBS. Cell

627 lysis and cDNA synthesis were performed using SuperPrep II Cell Lysis & RT Kit for

628 qPCR (SCQ-401, TOYOBO, Osaka, Japan) following the manufacturer's instructions.

629 SARS-CoV-2 RNA was detected using a primer set targeting the SARS-CoV-2 N gene.

630 Quantitative real-time RT-PCR was performed using THUNDERBIRD SYBR qPCR

631 Mix (TOYOBO) at 95 °C for 3 min, followed by 50 cycles of 95 °C for 10 s and 60 °C

632 for 1 min. Fluorescence was detected during the thermal cycling process, and

633 quantification studies were performed using the CFX ConnectTM Real-Time PCR

634 detection system (Bio-Rad, CA, USA). The level of ribosomal protein L13a (*Rpl13a*)

635 mRNA expression in each sample was used to standardize the data. The primer sequences

636 used were 5′-AAA TTT TGG GGA CCA GGA AC-3' (forward primer) and 5′-TGG CAG

637 CTG TGT AGG TCA AC-3' (reverse primer) for the SARS-CoV-2 N gene; 5′-TGT TTG

638 ACG GCA TCC CAC-3' (forward primer) and 5′-CTG TCA CTG CCT GGT ACT TC-

639 3' (reverse primer) for human *Rpl13a* gene; and 5′-CTC AAG GTT GTG CGT CTG AA-

36

640 3' (forward primer) and 5′-CTG TCA CTG CCT GGT ACT TCC A-3' (reverse primer)

641 for the African green monkey *Rpl13a* gene.

642

643 **Immunofluorescence Staining**

644 HEC50B and HEC50B-TMPRSS2 cells were seeded at 1.5×10^5 cells per well in a 24-

645 well cell culture plate. After an overnight incubation at 37 °C in 5% CO_2, cells were

646 treated with protease inhibitors for 1 h and then with SARS-CoV-2 at an MOI of 1. After

647 24 h of incubation at 37 °C in 5% CO_2, cells were fixed with 4% paraformaldehyde in

648 PBS(-) for 10 min at RT and then permeabilized with 0.1% Triton X-100 in PBS(-) for

649 10 min at RT. Cells were then incubated with anti-SARS-CoV-2 nucleocapsid (1:1000,

650 GTX135357, GeneTex, CA, USA) primary antibody for 16 h at 4 °C and detected with

651 anti-rabbit-Alexa488 (1:200, A11008, Invitrogen, CA, USA) secondary antibodies for 40

652 min at RT. Cell nuclei were stained with 1 μg/mL Hoechst 33342 (#080-09981,

653 FUJIFILM Wako Pure Chemical). Fluorescent signals were detected using a BZ-X810

654 fluorescent microscope (Keyence, Osaka, Japan).

655

656 **Cytopathicity assay**

657 HEC50B and HEC50B-TMPRSS2 cells were seeded at 1.5×10^5 cells per well in a 24-

658 well cell culture plate. After an overnight incubation at 37 °C in 5% CO_2, cells were

659 treated with protease inhibitors for 1 h and then with SARS-CoV-2 at an MOI of 1. To

660 maintain the drug concentration, half of the culture supernatant was replaced daily with

661 fresh medium that contained drugs. After incubation at 37 °C in 5% CO_2 for 3 d, the cells

662 were fixed with 4% paraformaldehyde in PBS for 10 min at 25 °C and stained with 0.2%

663 crystal violet solution for 5 min. After washing four times with water, the wells were air-

664 dried at 25 °C. Ethanol was added to each well to dissolve crystal violet. The absorbance

665 was measured at 595 nm using an iMarkTM Microplate Reader (Bio-Rad).

666

667 **Statistical analysis**

668 Statistical analyses were performed in Microsoft Excel 2016 (Microsoft, Redmond, WA,

669 USA) and GraphPad Prism 8 (GraphPad Software, San Diego, CA). Statistically

670 significant differences between the mean values were determined using a two-tailed

671 Student's t-test. Dunnett's test and Tukey's test were used for multiple comparisons. All

672 data represent three independent experiments, and values represent the mean ± standard

673 deviation (s.d.), with a $p < 0.05$, considered statistically significant.

674

Acknowledgements

We thank Yoshihiro Kawaoka for providing the SARS-CoV-2 isolate (UT-NCGM/Human/2020/Tokyo), Robert Whittier for critical reading of our manuscript and Kinuyo Miyazaki for her secretarial assistance.

Author contributions

M.Y., J.G., and J.I. designed the study; M.Y., J.G., A.K., K.T., and Y.H. performed the experiments; M.Y., J.G., N.K., M.S., K.S., T.A., Y.K., and J.I. analyzed and interpreted the data; and M.Y. and J.I. wrote the manuscript.

References

1.   Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020;579(7798):270-3. Epub 2020/02/03. doi: 10.1038/s41586-020-2012-7. PubMed PMID: 32015507; PubMed Central PMCID: PMCPMC7095418.

2.   Zhong NS, Zheng BJ, Li YM, Poon, Xie ZH, Chan KH, et al. Epidemiology and cause of severe acute respiratory syndrome (SARS) in Guangdong, People's Republic of China, in February, 2003. Lancet. 2003;362(9393):1353-8. doi: 10.1016/s0140-6736(03)14630-2.

693   PubMed PMID: 14585636; PubMed Central PMCID: PMCPMC7112415.

694   3.   Drosten C, Günther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al.

695   Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N

696   Engl J Med. 2003;348(20):1967-76. Epub 20030410. doi: 10.1056/NEJMoa030747. PubMed

697   PMID: 12690091.

698   4.   Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation

699   of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med.

700   2012;367(19):1814-20. doi: 10.1056/NEJMoa1211721. PubMed PMID: 23075143.

701   5.   Harrison AG, Lin T, Wang P. Mechanisms of SARS-CoV-2 Transmission and

702   Pathogenesis.   Trends   Immunol.   2020;41(12):1100-15.   Epub   20201014.   doi:

703   10.1016/j.it.2020.10.004.   PubMed   PMID: 33132005;   PubMed   Central   PMCID:

704   PMCPMC7556779.

705   6.   Hu B, Guo H, Zhou P, Shi ZL. Characteristics of SARS-CoV-2 and COVID-19. Nat

706   Rev Microbiol. 2021;19(3):141-54. Epub 20201006. doi: 10.1038/s41579-020-00459-7. PubMed

707   PMID: 33024307; PubMed Central PMCID: PMCPMC7537588.

708   7.   Tregoning JS, Flight KE, Higham SL, Wang Z, Pierce BF. Progress of the COVID-

709   19 vaccine effort: viruses, vaccines and variants versus efficacy, effectiveness and escape. Nat

710   Rev Immunol. 2021;21(10):626-36. Epub 20210809. doi: 10.1038/s41577-021-00592-1.

711   PubMed PMID: 34373623; PubMed Central PMCID: PMCPMC8351583.

712   8.   Pritchard E, Matthews PC, Stoesser N, Eyre DW, Gethings O, Vihta KD, et al.

713   Impact of vaccination on new SARS-CoV-2 infections in the United Kingdom. Nat Med.

714   2021;27(8):1370-8. Epub 20210609. doi: 10.1038/s41591-021-01410-w. PubMed PMID:

715   34108716; PubMed Central PMCID: PMCPMC8363500.

716   9.   Planas D, Veyer D, Baidaliuk A, Staropoli I, Guivel-Benhassine F, Rajah MM, et al.

717   Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. Nature.

718   2021;596(7871):276-80. Epub 20210708. doi: 10.1038/s41586-021-03777-9. PubMed PMID:

719   34237773.

720   10.   Tao K, Tzou PL, Nouhin J, Gupta RK, de Oliveira T, Kosakovsky Pond SL, et al. The

721   biological and clinical significance of emerging SARS-CoV-2 variants. Nat Rev Genet. 2021.

722   Epub 20210917. doi: 10.1038/s41576-021-00408-x. PubMed PMID: 34535792; PubMed

723   Central PMCID: PMCPMC8447121.

724   11.   Uriu K, Kimura I, Shirakawa K, Takaori-Kondo A, Nakada TA, Kaneda A, et al.

725   Neutralization of the SARS-CoV-2 Mu Variant by Convalescent and Vaccine Serum. N Engl

726   J Med. 2021. Epub 20211103. doi: 10.1056/NEJMc2114706. PubMed PMID: 34731554.

727   12.   Mei M, Tan X. Current Strategies of Antiviral Drug Discovery for COVID-19. Front

728   Mol Biosci. 2021;8:671263. Epub 20210513. doi: 10.3389/fmolb.2021.671263. PubMed PMID:

729   34055887; PubMed Central PMCID: PMCPMC8155633.

730   13.   Rando HM, Wellhausen N, Ghosh S, Lee AJ, Dattoli AA, Hu F, et al. Identification

731   and Development of Therapeutics for COVID-19. mSystems. 2021:e0023321. Epub 20211102.

732   doi: 10.1128/mSystems.00233-21. PubMed PMID: 34726496; PubMed Central PMCID:

733   PMCPMC8562484.

734   14.   Jackson CB, Farzan M, Chen B, Choe H. Mechanisms of SARS-CoV-2 entry into

735   cells. Nat Rev Mol Cell Biol. 2021. Epub 20211005. doi: 10.1038/s41580-021-00418-x. PubMed

736   PMID: 34611326; PubMed Central PMCID: PMCPMC8491763.

737   15.   Hoffmann M, Kleine-Weber H, Pöhlmann S. A Multibasic Cleavage Site in the Spike

738   Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. Mol Cell.

739   2020;78(4):779-84.e5. Epub 20200501. doi: 10.1016/j.molcel.2020.04.022. PubMed PMID:

740   32362314; PubMed Central PMCID: PMCPMC7194065.

741   16.   Shang J, Wan Y, Luo C, Ye G, Geng Q, Auerbach A, et al. Cell entry mechanisms of

742   SARS-CoV-2. Proc Natl Acad Sci U S A. 2020;117(21):11727-34. Epub 2020/05/06. doi:

743   10.1073/pnas.2003138117. PubMed PMID: 32376634.

744   17.   Yan R, Zhang Y, Li Y, Xia L, Guo Y, Zhou Q. Structural basis for the recognition of

745   SARS-CoV-2 by full-length human ACE2. Science. 2020;367(6485):1444-8. Epub 20200304.

746   doi: 10.1126/science.abb2762. PubMed PMID: 32132184; PubMed Central PMCID:

747   PMCPMC7164635.

748   18.   Lan J, Ge J, Yu J, Shan S, Zhou H, Fan S, et al. Structure of the SARS-CoV-2 spike

749   receptor-binding domain bound to the ACE2 receptor. Nature. 2020;581(7807):215-20. Epub

750   20200330. doi: 10.1038/s41586-020-2180-5. PubMed PMID: 32225176.

751   19.   Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al.

752   SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically

753   Proven   Protease   Inhibitor.   Cell.   2020;181(2):271-80.e8.   Epub   2020/03/05.   doi:

754   10.1016/j.cell.2020.02.052.   PubMed   PMID: 32142651;   PubMed   Central   PMCID:

755   PMCPMC7102627.

756   20.   Zhao MM, Yang WL, Yang FY, Zhang L, Huang WJ, Hou W, et al. Cathepsin L plays

757   a key role in SARS-CoV-2 infection in humans and humanized mice and is a promising target

758   for new drug development. Signal Transduct Target Ther. 2021;6(1):134. Epub 20210327. doi:

759   10.1038/s41392-021-00558-8.   PubMed   PMID: 33774649;   PubMed   Central   PMCID:

760   PMCPMC7997800.

761   21.   Murgolo N, Therien AG, Howell B, Klein D, Koeplinger K, Lieberman LA, et al.

762   SARS-CoV-2 tropism, entry, replication, and propagation: Considerations for drug discovery

763   and   development.   PLoS   Pathog.   2021;17(2):e1009225.   Epub   2021/02/17.   doi:

764   10.1371/journal.ppat.1009225.   PubMed   PMID: 33596266;   PubMed   Central   PMCID:

765   PMCPMC7888651.

766   22.   Koch J, Uckeley ZM, Doldan P, Stanifer M, Boulant S, Lozach PY. TMPRSS2

767   expression dictates the entry route used by SARS-CoV-2 to infect host cells. EMBO J.

768   2021;40(16):e107821. Epub 20210713. doi: 10.15252/embj.2021107821. PubMed PMID:

769   34159616; PubMed Central PMCID: PMCPMC8365257.

770   23.   Bestle D, Heindl MR, Limburg H, Van Lam van T, Pilgram O, Moulton H, et al.

771   TMPRSS2 and furin are both essential for proteolytic activation of SARS-CoV-2 in human

772   airway cells. Life Sci Alliance. 2020;3(9). Epub 20200723. doi: 10.26508/lsa.202000786.

773   PubMed PMID: 32703818; PubMed Central PMCID: PMCPMC7383062.

774   24.   Yamamoto M, Kiso M, Sakai-Tagawa Y, Iwatsuki-Horimoto K, Imai M, Takeda M,

775   et al. The Anticoagulant Nafamostat Potently Inhibits SARS-CoV-2 S Protein-Mediated

776   Fusion in a Cell Fusion Assay System and Viral Infection In Vitro in a Cell-Type-Dependent

777   Manner. Viruses. 2020;12(6). Epub 2020/06/10. doi: 10.3390/v12060629. PubMed PMID:

778   32532094.

779   25.   Yamamoto M, Matsuyama S, Li X, Takeda M, Kawaguchi Y, Inoue JI, et al.

780   Identification of Nafamostat as a Potent Inhibitor of Middle East Respiratory Syndrome

781   Coronavirus S Protein-Mediated Membrane Fusion Using the Split-Protein-Based Cell-Cell

782   Fusion Assay. Antimicrob Agents Chemother. 2016;60(11):6532-9. Epub 2016/08/24. doi:

783   10.1128/aac.01043-16. PubMed PMID: 27550352; PubMed Central PMCID:

784   PMCPMC5075056.

785   26.   Ishikawa H, Meng F, Kondo N, Iwamoto A, Matsuda Z. Generation of a dual-

786   functional split-reporter protein for monitoring membrane fusion using self-associating split

787   GFP. Protein Eng Des Sel. 2012;25(12):813-20. doi: 10.1093/protein/gzs051. PubMed PMID:

788   22942393.

789   27.   Wojtowicz-Praga S, Torri J, Johnson M, Steen V, Marshall J, Ness E, et al. Phase I

790   trial of Marimastat, a novel matrix metalloproteinase inhibitor, administered orally to

791   patients with advanced lung cancer. J Clin Oncol. 1998;16(6):2150-6. doi:

792   10.1200/JCO.1998.16.6.2150. PubMed PMID: 9626215.

793   28.   Hande KR, Collier M, Paradiso L, Stuart-Smith J, Dixon M, Clendeninn N, et al.

794   Phase I and pharmacokinetic study of prinomastat, a matrix metalloprotease inhibitor. Clin

795   Cancer Res. 2004;10(3):909-15. doi: 10.1158/1078-0432.ccr-0981-3. PubMed PMID: 14871966.

796   29.   Standard of Care (SOC) With or Without CTS-1027 in Hepatitis C (HCV) Null-

797   Responders https://clinicaltrials.gov/ct2/show/results/NCT01273064.

798   30.   Vandenbroucke RE, Dejonckheere E, Libert C. A therapeutic role for matrix

799   metalloproteinase inhibitors in lung diseases? Eur Respir J. 2011;38(5):1200-14. Epub

800   20110609. doi: 10.1183/09031936.00027411. PubMed PMID: 21659416.

801   31.   Jacobsen JA, Major Jourden JL, Miller MT, Cohen SM. To bind zinc or not to bind

802   zinc: an examination of innovative approaches to improved metalloproteinase inhibition.

803   Biochim   Biophys   Acta.   2010;1803(1):72-94.   Epub   20090825.   doi:

804   10.1016/j.bbamcr.2009.08.006. PubMed PMID: 19712708.

805   32.   Madoux F, Dreymuller D, Pettiloud JP, Santos R, Becker-Pauly C, Ludwig A, et al.

806   Discovery of an enzyme and substrate selective inhibitor of ADAM10 using an exosite-

807   binding glycosylated substrate. Sci Rep. 2016;6(1):11. Epub 20161205. doi: 10.1038/s41598-

808   016-0013-4. PubMed PMID: 28442704; PubMed Central PMCID: PMCPMC5431342.

809   33.   Liechti FD, Bächtold F, Grandgirard D, Leppert D, Leib SL. The matrix

810   metalloproteinase   inhibitor   RS-130830   attenuates   brain   injury   in   experimental

811   pneumococcal   meningitis.   J   Neuroinflammation.   2015;12:43.   Epub   20150304.   doi:

812   10.1186/s12974-015-0257-0.   PubMed   PMID: 25890041;   PubMed   Central   PMCID:

813   PMCPMC4352253.

814   34.   Hundhausen C, Misztela D, Berkhout TA, Broadway N, Saftig P, Reiss K, et al. The

815   disintegrin-like metalloproteinase ADAM10 is involved in constitutive cleavage of CX3CL1

816   (fractalkine) and regulates CX3CL1-mediated cell-cell adhesion. Blood. 2003;102(4):1186-95.

817   Epub 20030424. doi: 10.1182/blood-2002-12-3775. PubMed PMID: 12714508.

818   35.   Ludwig A, Hundhausen C, Lambert MH, Broadway N, Andrews RC, Bickett DM, et

819   al. Metalloproteinase inhibitors for the disintegrin-like metalloproteinases ADAM10 and

820   ADAM17 that differentially block constitutive and phorbol ester-inducible shedding of cell

821   surface molecules. Comb Chem High Throughput Screen. 2005;8(2):161-71. doi:

822   10.2174/1386207053258488. PubMed PMID: 15777180.

823   36.   Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, et al. A

824   metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature.

825   1997;385(6618):729-33. doi: 10.1038/385729a0. PubMed PMID: 9034190.

826   37.   Schlomann U, Koller G, Conrad C, Ferdous T, Golfi P, Garcia AM, et al. ADAM8 as

827   a drug target in pancreatic cancer. Nat Commun. 2015;6:6175. Epub 20150128. doi:

828   10.1038/ncomms7175.   PubMed   PMID: 25629724;   PubMed   Central   PMCID:

829   PMCPMC5014123.

830   38.   Tamura Y, Watanabe F, Nakatani T, Yasui K, Fuji M, Komurasaki T, et al. Highly

831   selective and orally active inhibitors of type IV collagenase (MMP-9 and MMP-2): N-

832   sulfonylamino acid derivatives. J Med Chem. 1998;41(4):640-9. doi: 10.1021/jm9707582.

833   PubMed PMID: 9484512.

834   39.   Fray MJ, Dickinson RP, Huggins JP, Occleston NL. A potent, selective inhibitor of

835   matrix metalloproteinase-3 for the topical treatment of chronic dermal ulcers. J Med Chem.

836   2003;46(16):3514-25. doi: 10.1021/jm0308038. PubMed PMID: 12877590.

837 40.   Levin JI, Chen J, Du M, Hogan M, Kincaid S, Nelson FC, et al. The discovery of

838 anthranilic acid-based MMP inhibitors. Part 2: SAR of the 5-position and P1(1) groups.

839 Bioorg Med Chem Lett. 2001;11(16):2189-92. doi: 10.1016/s0960-894x(01)00419-x. PubMed

840 PMID: 11514167.

841 41.   Dales NA, Gould AE, Brown JA, Calderwood EF, Guan B, Minor CA, et al.

842 Substrate-based design of the first class of angiotensin-converting enzyme-related

843 carboxypeptidase (ACE2) inhibitors. J Am Chem Soc. 2002;124(40):11852-3. doi:

844 10.1021/ja0277226. PubMed PMID: 12358520.

845 42.   Yeung ML, Teng JLL, Jia L, Zhang C, Huang C, Cai JP, et al. Soluble ACE2-

846 mediated cell entry of SARS-CoV-2 via interaction with proteins related to the renin-

847 angiotensin   system.   Cell.   2021;184(8):2212-28.e12.   Epub   20210302.   doi:

848 10.1016/j.cell.2021.02.053.   PubMed   PMID: 33713620;   PubMed   Central   PMCID:

849 PMCPMC7923941.

850 43.   Rodríguez E, Everitt E. Adenovirus uncoating and nuclear establishment are not

851 affected by weak base amines. J Virol. 1996;70(6):3470-7. doi: 10.1128/JVI.70.6.3470-

852 3477.1996. PubMed PMID: 8648679; PubMed Central PMCID: PMCPMC190220.

853 44.   Zhang Z, Zheng Y, Niu Z, Zhang B, Wang C, Yao X, et al. SARS-CoV-2 spike protein

854 dictates syncytium-mediated lymphocyte elimination. Cell Death Differ. 2021;28(9):2765-77.

855   Epub 20210420. doi: 10.1038/s41418-021-00782-3. PubMed PMID: 33879858; PubMed

856   Central PMCID: PMCPMC8056997.

857   45.   Bussani R, Schneider E, Zentilin L, Collesi C, Ali H, Braga L, et al. Persistence of

858   viral RNA, pneumocyte syncytia and thrombosis are hallmarks of advanced COVID-19

859   pathology. EBioMedicine. 2020;61:103104. Epub 20201103. doi: 10.1016/j.ebiom.2020.103104.

860   PubMed PMID: 33158808; PubMed Central PMCID: PMCPMC7677597.

861   46.   Hoffmann M, Schroeder S, Kleine-Weber H, Müller MA, Drosten C, Pöhlmann S.

862   Nafamostat mesylate blocks activation of SARS-CoV-2: New treatment option for COVID-19.

863   Antimicrob Agents Chemother. 2020. Epub 2020/04/20. doi: 10.1128/AAC.00754-20. PubMed

864   PMID: 32312781.

865   47.   Fan C, Lu W, Li K, Ding Y, Wang J. ACE2 Expression in Kidney and Testis May

866   Cause Kidney and Testis Infection in COVID-19 Patients. Front Med (Lausanne).

867   2020;7:563893. Epub 20210113. doi: 10.3389/fmed.2020.563893. PubMed PMID: 33521006;

868   PubMed Central PMCID: PMCPMC7838217.

869   48.   Liu J, Li Y, Liu Q, Yao Q, Wang X, Zhang H, et al. SARS-CoV-2 cell tropism and

870   multiorgan infection. Cell Discov. 2021;7(1):17. Epub 20210323. doi: 10.1038/s41421-021-

871   00249-2. PubMed PMID: 33758165; PubMed Central PMCID: PMCPMC7987126.

872   49.   Reis FM, Bouissou DR, Pereira VM, Camargos AF, dos Reis AM, Santos RA.

873   Angiotensin-(1-7), its receptor Mas, and the angiotensin-converting enzyme type 2 are

874   expressed in the human ovary. Fertil Steril. 2011;95(1):176-81. Epub 20100801. doi:

875   10.1016/j.fertnstert.2010.06.060. PubMed PMID: 20674894.

876   50.   Jing Y, Run-Qian L, Hao-Ran W, Hao-Ran C, Ya-Bin L, Yang G, et al. Potential

877   influence of COVID-19/ACE2 on the female reproductive system. Mol Hum Reprod.

878   2020;26(6):367-73. doi: 10.1093/molehr/gaaa030. PubMed PMID: 32365180; PubMed Central

879   PMCID: PMCPMC7239105.

880   51.   Henarejos-Castillo I, Sebastian-Leon P, Devesa-Peiro A, Pellicer A, Diaz-Gimeno P.

881   SARS-CoV-2 infection risk assessment in the endometrium: viral infection-related gene

882   expression across the menstrual cycle. Fertil Steril. 2020;114(2):223-32. Epub 20200617. doi:

883   10.1016/j.fertnstert.2020.06.026. PubMed PMID: 32641214; PubMed Central PMCID:

884   PMCPMC7298504.

885   52.   Puelles VG, Lütgehetmann M, Lindenmeyer MT, Sperhake JP, Wong MN, Allweiss

886   L, et al. Multiorgan and Renal Tropism of SARS-CoV-2. N Engl J Med. 2020;383(6):590-2.

887   Epub 2020/05/13. doi: 10.1056/NEJMc2011400. PubMed PMID: 32402155; PubMed Central

888   PMCID: PMCPMC7240771.

889   53.   Diao B, Wang C, Wang R, Feng Z, Zhang J, Yang H, et al. Human kidney is a target

890   for novel severe acute respiratory syndrome coronavirus 2 infection. Nat Commun.

50

891   2021;12(1):2506. Epub 20210504. doi: 10.1038/s41467-021-22781-1. PubMed PMID:

892   33947851; PubMed Central PMCID: PMCPMC8096808.

893   54.   Johnson BA, Xie X, Bailey AL, Kalveram B, Lokugamage KG, Muruato A, et al. Loss

894   of furin cleavage site attenuates SARS-CoV-2 pathogenesis. Nature. 2021;591(7849):293-9.

895   Epub 20210125. doi: 10.1038/s41586-021-03237-4. PubMed PMID: 33494095; PubMed

896   Central PMCID: PMCPMC8175039.

897   55.   Peacock TP, Goldhill DH, Zhou J, Baillon L, Frise R, Swann OC, et al. The furin

898   cleavage site in the SARS-CoV-2 spike protein is required for transmission in ferrets. Nat

899   Microbiol. 2021;6(7):899-909. Epub 20210427. doi: 10.1038/s41564-021-00908-w. PubMed

900   PMID: 33907312.

901   56.   Benton DJ, Wrobel AG, Xu P, Roustan C, Martin SR, Rosenthal PB, et al. Receptor

902   binding and priming of the spike protein of SARS-CoV-2 for membrane fusion. Nature.

903   2020;588(7837):327-30. Epub 20200917. doi: 10.1038/s41586-020-2772-0. PubMed PMID:

904   32942285; PubMed Central PMCID: PMCPMC7116727.

905   57.   Stevens CS, Oguntuyo KY, Lee B. Proteases and variants: context matters for

906   SARS-CoV-2 entry assays. Curr Opin Virol. 2021;50:49-58. Epub 20210724. doi:

907   10.1016/j.coviro.2021.07.004. PubMed PMID: 34365113; PubMed Central PMCID:

908   PMCPMC8302850.

909 58.   Harvey WT, Carabelli AM, Jackson B, Gupta RK, Thomson EC, Harrison EM, et al.

910 SARS-CoV-2 variants, spike mutations and immune escape. Nat Rev Microbiol.

911 2021;19(7):409-24. Epub 20210601. doi: 10.1038/s41579-021-00573-0. PubMed PMID:

912 34075212; PubMed Central PMCID: PMCPMC8167834.

913 59.   The human protein atlas - Tissue expression of

914 ADAM10 https://www.proteinatlas.org/ENSG00000137845-ADAM10/tissue.

915 60.   The human protein atlas - Cell type atlas

916 https://www.proteinatlas.org/ENSG00000137845-ADAM10/celltype.

917 61.   Caescu CI, Jeschke GR, Turk BE. Active-site determinants of substrate recognition

918 by the metalloproteinases TACE and ADAM10. Biochem J. 2009;424(1):79-88. Epub

919 20091023. doi: 10.1042/BJ20090549. PubMed PMID: 19715556; PubMed Central PMCID:

920 PMCPMC2774824.

921 62.   Carapito R, Li R, Helms J, Carapito C, Gujja S, Rolli V, et al. Identification of driver

922 genes for critical forms of COVID-19 in a deeply phenotyped young patient cohort. Sci Transl

923 Med. 2021:eabj7521. Epub 20211026. doi: 10.1126/scitranslmed.abj7521. PubMed PMID:

924 34698500.

925 63.   Daniloski Z, Jordan TX, Wessels HH, Hoagland DA, Kasela S, Legut M, et al.

926 Identification of Required Host Factors for SARS-CoV-2 Infection in Human Cells. Cell.

52

927   2021;184(1):92-105.e16. Epub 20201024. doi: 10.1016/j.cell.2020.10.030. PubMed PMID:

928   33147445; PubMed Central PMCID: PMCPMC7584921.

929   64.   Gunst JD, Staerke NB, Pahus MH, Kristensen LH, Bodilsen J, Lohse N, et al.

930   Efficacy of the TMPRSS2 inhibitor camostat mesilate in patients hospitalized with Covid-19-

931   a double-blind randomized controlled trial. EClinicalMedicine. 2021;35:100849. Epub

932   20210422. doi: 10.1016/j.eclinm.2021.100849. PubMed PMID: 33903855; PubMed Central

933   PMCID: PMCPMC8060682.

934   65.   Boulware DR, Pullen MF, Bangdiwala AS, Pastick KA, Lofgren SM, Okafor EC, et

935   al. A Randomized Trial of Hydroxychloroquine as Postexposure Prophylaxis for Covid-19. N

936   Engl J Med. 2020;383(6):517-25. Epub 20200603. doi: 10.1056/NEJMoa2016638. PubMed

937   PMID: 32492293; PubMed Central PMCID: PMCPMC7289276.

938   66.   Self WH, Semler MW, Leither LM, Casey JD, Angus DC, Brower RG, et al. Effect of

939   Hydroxychloroquine on Clinical Status at 14 Days in Hospitalized Patients With COVID-19:

940   A Randomized Clinical Trial. JAMA. 2020;324(21):2165-76. doi: 10.1001/jama.2020.22240.

941   PubMed PMID: 33165621; PubMed Central PMCID: PMCPMC7653542.

942   67.   Matsuyama S, Nao N, Shirato K, Kawase M, Saito S, Takayama I, et al. Enhanced

943   isolation of SARS-CoV-2 by TMPRSS2-expressing cells. Proc Natl Acad Sci U S A.

944   2020;117(13):7001-3. Epub 2020/03/12. doi: 10.1073/pnas.2002589117. PubMed PMID:

945   32165541; PubMed Central PMCID: PMCPMC7132130.

946   68.   Wang H, Li X, Nakane S, Liu S, Ishikawa H, Iwamoto A, et al. Co-expression of

947   foreign proteins tethered to HIV-1 envelope glycoprotein on the cell surface by introducing

948   an intervening second membrane-spanning domain. PLoS One. 2014;9(5):e96790. doi:

949   10.1371/journal.pone.0096790. PubMed PMID: 24804933; PubMed Central PMCID:

950   PMCPMC4013048.

951   69.   Imai M, Iwatsuki-Horimoto K, Hatta M, Loeber S, Halfmann PJ, Nakajima N, et al.

952   Syrian hamsters as a small animal model for SARS-CoV-2 infection and countermeasure

953   development. Proc Natl Acad Sci U S A. 2020;117(28):16587-95. Epub 20200622. doi:

954   10.1073/pnas.2009799117. PubMed PMID: 32571934; PubMed Central PMCID:

955   PMCPMC7368255.

956   70.   Yamamoto M, Abe C, Wakinaga S, Sakane K, Yumiketa Y, Taguchi Y, et al. TRAF6

957   maintains mammary stem cells and promotes pregnancy-induced mammary epithelial cell

958   expansion. Commun Biol. 2019;2:292. Epub 2019/08/06. doi: 10.1038/s42003-019-0547-7.

959   PubMed PMID: 31396572; PubMed Central PMCID: PMCPMC6684589.

960   71.   Tani H, Shiokawa M, Kaname Y, Kambara H, Mori Y, Abe T, et al. Involvement of

961   ceramide in the propagation of Japanese encephalitis virus. J Virol. 2010;84(6):2798-807.

962   Epub 20100106. doi: 10.1128/JVI.02499-09. PubMed PMID: 20053738; PubMed Central

963   PMCID: PMCPMC2826033.

964

965   **Additional information**

966   **Financial Disclosure Statement**

978

979   **Competing interests:** The authors declare no competing interests.

980

981 **Figure Captions**

982 **Fig 1. ACE2-dependent but TMPRSS2-independent membrane fusion activity of the**

983 **SARS-CoV-2 S protein**

984 (a) Cell fusion kinetics induced by the S proteins from SARS-CoV, SARS-CoV-2, and

985 MERS-CoV were determined using the DSP assay. Target cells expressing ACE2 alone

986 or together with TMPRSS2 were used for co-culturing with effector cells expressing

987 SARS-CoV S and SARS-CoV-2 S, and cells expressing CD26 alone or together with

988 TMPRSS2 were used for co-culturing with effector cells expressing MERS-CoV- S.

989 Relative cell-fusion values were calculated by normalizing the RL activity of each co-

990 culture to that of the co-culture with cells expressing both receptor and TMPRSS2 at 240

991 min, which was set to 100%. Values are means $\pm$ SD ($n = 3$/group). ** $p < 0.01$. (b) Phase

992 contrast images of S protein-mediated cell fusion 16 h after co-culture. Red arrowheads

993 indicate syncytia formation Scale bars, 100 μm. (c) Effect of nafamostat on the

994 TMPRSS2-dependent cell fusion. Target cells expressing ACE2 with TMPRSS2 were

995 used for co-culturing with effector cells expressing SARS-CoV S and SARS-CoV-2 S,

996 and cells expressing CD26 with TMPRSS2 were used for co-culturing with effector cells

997 expressing MERS-CoV- S. Relative cell-fusion values were calculated by normalizing

998 the RL activity for each co-culture to that of the co-culture with cells expressing both

56

999 receptor and TMPRSS2 in the presence of DMSO, which was set to 100%. Values are

1000 means ± SD (n = 3/group). ** p < 0.01. (**d**) Effects of the nafamostat on the TMPRSS2-

1001 independent or -dependent cell fusion. Target cells expressing ACE2 alone or together

1002 with TMPRSS2 were used for co-culturing with effector cells expressing SARS-CoV-2

1003 S. Relative cell-fusion value was calculated by normalizing the RL activity for each co-

1004 culture to that of the co-culture with cells expressing both ACE2 and TMPRSS2 in the

1005 presence of DMSO, which was set to 100%. Values are means ± SD (n = 3/group). nafamo,

1006 nafamostat.

1007

1008 **Fig 2. TMPRSS2-independent membrane fusion induced by the S protein of SARS-**

1009 **CoV-2 is blocked by various metalloproteinase inhibitors.**

1010 (**a**) High-throughput screening of the Validated Compound Library (1,630 clinically

1011 approved compounds and 1,885 pharmacologically active compounds) in the DSP assay

1012 using the SARS-CoV-2 S protein. The x-axis shows the relative cell-fusion value using

1013 cells expressing both TMPRSS2 and ACE2 in the presence of each compound (1 µM in

1014 DMSO), n = 1. The y-axis shows the relative cell-fusion value using cells expressing

1015 ACE2 alone in the presence of each compound (1 µM in DMSO), n = 1. The relative cell-

1016 fusion value was calculated by normalizing the RL activity for each compound to that of

1017   the control assay (DMSO alone; set to 100%). Each dot represents an individual

1018   compound. Dots in the red-dashed box indicate compounds that preferentially inhibit

1019   TMPRSS2-independent membrane fusion. ($< 30\%$ inhibition of the relative cell fusion

1020   value using the target cells expressing both TMPRSS2 and ACE2 and $> 40\%$ inhibition

1021   of the relative cell-fusion value using the target cells expressing ACE2 alone. The

1022   compound names for the candidates are indicated. (**b**) Effects of the metalloproteinase

1023   inhibitors on cell fusion in the co-cultures of the cells expressing SARS-CoV-2 S protein

1024   with those expressing ACE2 alone or in combination with TMPRSS2. Relative cell-

1025   fusion values were calculated by normalizing the RL activity for each co-culture to that

1026   of the co-culture with cells expressing both ACE2 and TMPRSS2 in the presence of

1027   DMSO, which was set to 100%. Values are means $\pm$ SD ($n = 3$/group). ** $p < 0.01$.

1028

1029   **Fig 3. The metalloproteinase-dependent viral entry pathway is cell type-dependent.**

1030   Effects of drugs on the entry of SARS-CoV-2 S-bearing vesicular stomatitis virus (VSV)

1031   pseudotype virus. The relative pseudovirus entry was calculated by normalizing the FL

1032   activity for each condition to the FL activity of cells infected with SARS-CoV-2 S-

1033   bearing pseudovirus in the presence of DMSO alone, which was set to 100%. Values are

1034   means $\pm$ SD ($n = 3$/group). * $p < 0.05$, ** $p < 0.01$. Cont: cells infected with pseudovirus

1035   without S protein; SARS-CoV-2: cells infected with SARS-CoV-2 S-bearing pseudovirus.

1036   E-64d: 25 μM E-64d, nafamo: 10 μM nafamostat, marima: 1 μM marimastat. (**a**) Effects

1037   of marimastat, E-64d, or nafamostat on the pseudovirus entry in A704, OVISE, and Calu-

1038   3 cells, respectively. (**b-e**) Effects of a single drug treatment or a combination treatment

1039   on the pseudovirus entry in VeroE6, HEC50B, OVTOKO and A704 cells (b), IGROV1,

1040   OUMS-23 and OVISE cells (c), Calu-3 and Caco-2 (d), and HEC50B-TMPRSS2 cells

1041   (e).

1042

1043   **Fig 4. The metalloproteinase-dependent entry pathway requires both the furin-**

1044   **cleavage site and S2 region of the SARS-CoV-2 S protein.**

1045   Effects of drugs on the entry of S protein-bearing vesicular stomatitis virus (VSV)

1046   pseudotype virus. The relative pseudovirus entry was calculated by normalizing the FL

1047   activity for each condition to the FL activity of cells infected with pseudovirus in the

1048   presence of DMSO alone, which was set to 100%. Values are means $\pm$ SD ($n = 3$/group).

1049   ** $p < 0.01$. Cont: cells infected with pseudovirus without S protein. E-64d: 25 μM E-

1050   64d, marima: 1 μM marimastat, nafamo: 10 μM nafamostat. (**a**) Effects of E-64d and

1051   marimastat on the entry of pseudoviruses bearing SARS-CoV S, SARS-CoV-2 S, MERS-

1052   CoV S, or VSV G in HEC50B cells. **b**, Effects of E-64d and marimastat on the entry of

1053   HCoV-NL63 S and WIV1-CoV S pseudovirus in HEC50B cells. **c**, Schematic illustration

1054   of C-terminally Flag-tagged chimeric S proteins in which the S1, S1/S2 boundary, and

1055   S2 domain from SARS-CoV S (red) or SARS-CoV-2 S (yellow) are indicated (top).

1056   Amino acid sequences of the residues around the S1/S2 boundary of the coronaviruses

1057   (bottom). Numbers refer to the amino acid residues. F: Flag-tag. Arginine residues in the

1058   S1/S2 cleavage site and furin cleavage motif are highlighted in red. (**d**) Expression of

1059   chimeric S protein in pseudoviruses. S proteins were detected using an anti-Flag-tag

1060   antibody that binds to a Flag-tag on the C-terminus of the S proteins (top). Detection of

1061   the vesicular stomatitis virus matrix protein (VSV M) served as the control (bottom). S0:

1062   uncleaved S protein; S2: cleaved S2 domain of the S protein. (**e, f**) Effects of E-64d and

1063   marimastat on the entry of pseudoviruses bearing chimeric S proteins in HEC50B cells.

1064   (**g**) Effects of E-64d and nafamostat on the entry of pseudoviruses bearing SARS-CoV S,

1065   SARS-CoV-2 S, MERS-CoV S, or VSV G in HEC50B-TMPRSS2 cells in the presence

1066   of marimastat. (**h, i**) Effects of E-64d and nafamostat on the entry of pseudoviruses

1067   bearing chimeric S proteins in HEC50B-TMPRSS2 cells in the presence of marimastat.

1068

1069   **Fig 5. Possible involvement of ADAM-10 in the metalloproteinase-dependent entry**

1070   **of SARS-CoV-2.**

1071   (**a**) Effects of metalloprotease inhibitors on the entry of pseudoviruses bearing SARS-

1072   CoV-2 S or VSV G in VeroE6 and HEC50B cells in the presence of E-64d, and A704

1073   cells in the absence of E-64d. The relative pseudovirus entry was calculated by

1074   normalizing the FL activity for each condition to the FL activity of cells infected with

1075   pseudovirus in the presence of DMSO alone, which was set to 100%. Values are means

1076 $\pm$ SD (n = 3/group). Data were compared with those obtained from cells infected

1077   pseudoviruses bearing SARS-CoV-2 S in the presence of E-64d for HEC50B and VeroE6,

1078   and in the presence of DMSO alone for A704. * $p < 0.05$, ** $p < 0.01$. Cont: cells infected

1079   with pseudovirus without S protein. marima: marimastat, prinoma: prinomastat, iloma:

1080   ilomastat, CTS: CTS-1027, UK: UK370106, GW: GW280264X, GI: GI254023X, MLN:

1081   MLN-4760, BK: BK-1361, MMP2/9i: MMP2/9 inhibitor I, MMP9i: MMP9 inhibitor I.

1082   (**b**) Effects of the ADAM10 knockdown on ACE2 (top), ADAM10 (middle), and tubulin

1083   (bottom) expression. HEC50B cells were transfected with two distinct control siRNAs or

1084   three distinct siRNAs against Adam10 for 48 h. (**c**) The effect of the ADAM10

1085   knockdown on the entry of pseudoviruses bearing SARS-CoV-2 S, SARS-CoV S,

1086   MERS-CoV S, or VSV G. HEC50B cells were transfected with siRNAs for 48 h and then

1087   infected with pseudoviruses. The relative pseudovirus entry was calculated by

1088   normalizing the FL activity for each condition to the FL activity of cells infected with

61

1089   pseudovirus in the absence of siRNA (mock), which was set to 100%. Values are means

1090   ± SD ($n = 3$/group). * p < 0.05, ** p < 0.01. (**d, e**) Effect of ADAM10 knockdown on the

1091   patterns of the entry pathways for SARS-CoV-2 S pseudovirus in HEC50B cells.

1092   HEC50B cells were transfected with siRNAs for 48 h and then infected with

1093   pseudoviruses in the presence of drugs. Values are means ± SD ($n = 3$/group). ** p < 0.01.

1094   E-64d: 25 μM E-64d, marima: 1 μM marimastat. Data are displayed as the conditions of

1095   siRNA treatment (d) and drug treatment (e).

1096

1097   **Fig 6. The metalloproteinase-dependent entry pathway of authentic SARS-CoV-2 is**

1098   **involved in syncytia formation and cytopathicity.**

1099   Effects of the drugs on the cytoplasmic viral RNA after SARS-CoV-2 infection. The

1100   relative amount of viral RNA in the cells was normalized to cellular *Rpl13a* mRNA

1101   expression. Values are means ± SD ($n = 3$/group in a-d, $n = 10$/group in e). * p < 0.05, **

1102   p < 0.01. (**a**) Effects of marimastat or prinomastat on SARS-CoV-2 infection in HEC50B,

1103   A704, and VeroE6 cells. (**b**) Effects of marimastat and the inhibitor of the endosome

1104   pathway on SARS-CoV-2 infection in HEC50B, A704, and VeroE6 cells. marima: 1 μM

1105   marimastat, E-64d: 25 μM E-64d, NH4Cl: 10 mM NH4Cl. (**c**) Effect of marimastat, E-

1106   64d, and nafamostat on SARS-CoV-2 infection in HEC50B-TMPRSS2 cells. marima: 1

62

1107 μM marimastat, E-64d: 25 μM E-64d, nafamo: 10 μM nafamostat. (**d**) Effects of selective

1108 metalloprotease inhibitors on SARS-CoV-2 infection in HEC50B cells. GW:

1109 GW280264X, GI: GI254023X. (**e**) Effect of ADAM10 knockdown on SARS-CoV-2

1110 infection in HEC50B cells. (**f, g**) Effects of drugs on SARS-CoV-2-induced syncytia

1111 formation in HEC50B (f) and HEC50B-TMPRSS2 (g) cells. Cells were stained with anti-

1112 SARS-CoV-2 N antibody (green) 24 h after infection. Nuclei were stained with Hoechst

1113 33342 (blue). Scale bars, 200 μm. (**h, i**) Effects of drugs on SARS-CoV-2-induced

1114 cytopathicity in HEC50B (h) and HEC50B-TMPRSS2 (i) cells. marima: marimastat,

1115 prinoma: prinomastat. E-64d: 25 μM E-64d, nafamo: 10 μM nafamostat. Values are

1116 means $\pm$ SD (n = 3/group). ** $p < 0.01$.

1117

1118 **Supporting information**

1119 **S1 Fig. Cell-based membrane-fusion assay for coronavirus S proteins using the DSP**

1120 **reporter.**

1121 (**a**) A method to monitor cell-cell membrane fusion mediated by the S protein of

1122 coronaviruses[24, 25]. Effector cells (293FT cells expressing DSP8-11 and S protein) and

1123 target cells (293FT cells expressing DSP1-7 and receptor protein with TMPRSS2 for "cell

1124 fusion with TMPRSS2" (top) or receptor protein without TMPRSS2 for "cell fusion

63

1125 without TMPRSS2" (bottom)) were co-cultured for 4 h. Both GFP (fluorescence) and RL

1126 (luminescence) signals were generated following DSP1-7 and DSP8-11 reassociation

1127 upon mixing of the cells during the assay. (**b**) Expression of S proteins in effector cells

1128 were detected using an anti-Flag-tag antibody that binds to a Flag-tag on the C-terminus

1129 of S proteins (top). Tubulin was used as a control (bottom panel). S0: uncleaved S protein;

1130 S2: cleaved S2 domain of the S protein. (**c**) Schematic diagram of split chimeric reporter

1131 proteins. DSP1-7 has the structure RL1–155-Ser-Gly-Gly-Gly-Gly-GFP1–156. DSP8-11

1132 has the Met- GFP157–231 -Gly-Gly-Gly-Gly-Ser- RL156–311. As GFP1–156 contains

1133 the first seven β sheets, and GFP157–231 contains the remaining four β sheets, the split

1134 proteins were called DSP1-7 and DSP8-11, respectively. DSP1-7 and DSP8-11

1135 reassociate efficiently, resulting in the reconstitution of functional RL and GFP to

1136 generate luminescent and fluorescent signals, respectively.

1137

1138 **S2 Fig. Control experiments for the DSP assay.**

1139 (**a**) A method to check whether compounds directly inhibit DSP activity without affecting

1140 cell-cell fusion[25]. 293FT cells expressing DSP1-7 and DSP8-11 were treated with

1141 compounds for 4 h. Measuring RL activities of the preformed DSP1-7/DSP8-11 complex

1142 to check whether the compounds directly inhibit RL activities without affecting cell-cell

1143   fusion. (**b**) Effect of metalloproteinase inhibitors on RL activity. Relative DSP activity

1144   was calculated by normalizing the RL activity for each condition to that of the control

1145   assay (DMSO alone; set to 100%). Values are means ± SD ($n = 3$/group).

1146

1147   **S3 Fig. Effects of candidate compounds on the cell-cell fusion and RL activities.**

1148   Effector cells expressing SARS-CoV-2 S were co-cultured with target cells expressing

1149   ACE2 alone for the TMPRSS2-independent cell-cell fusion assay (blue) or cells

1150   expressing ACE2 with TMPRSS2 for the TMPRSS2-dependent cell-cell fusion assay

1151   (red) in the presence of candidate compounds for 4 h. Cells expressing DSP1-7 and DSP8-

1152   11 in the presence of candidate compounds for 4 h to determine whether compounds

1153   directly inhibit RL activities (purple). Relative DSP activity was calculated by

1154   normalizing the RL activity for each condition to that of the control assay (DMSO alone;

1155   set to 100%). Values are means ± SD ($n = 3$/group).

1156

1157   **S4 Fig. The metalloproteinase-dependent entry pathway strictly requires both the**

1158   **furin-cleavage site and S2 region of S protein of SARS-CoV-2.**

1159   Effects of the drugs on the entry of S protein-bearing vesicular stomatitis virus (VSV)

1160   pseudotype virus. The relative pseudovirus entry was calculated by normalizing the FL

1161   activity for each condition to the FL activity of the cells infected with pseudovirus in the

1162   presence of DMSO alone, which was set to 100%. Values are means $\pm$ SD ($n = 3$/group).

1163   ** p < 0.01. Cont: cells infected with pseudovirus without S protein. E-64d: 25 μM E-

1164   64d, marima: 1 μM marimastat, nafamo: 10 μM nafamostat. (**a, b**) Effects of E-64d and

1165   marimastat on the entry of pseudoviruses bearing SARS-CoV S, SARS-CoV-2 S, MERS-

1166   CoV S, or VSV G in A704 (a) and VeroE6 (b) cells. (**c, d**) Effects of E-64d and

1167   marimastat on the entry of pseudoviruses bearing chimeric S proteins in VeroE6 cells. (**e**)

1168   Effects of E-64d and nafamostat on the entry of pseudoviruses bearing SARS-CoV S,

1169   SARS-CoV-2 S, MERS-CoV S, or VSV G in VeroE6-TMPRSS2 cells. (**f, g**) Effects of

1170   E-64d and nafamostat on the entry of pseudoviruses bearing chimeric S proteins in

1171   VeroE6-TMPRSS2 cells. To establish VeroE6 cells expressing TMPRSS2 (VeroE6-

1172   TMPRSS2), recombinant pseudotype lentivirus expressing TMPRSS2 was produced

1173   using 293T cells with a VSV-G-expressing plasmid. Cells infected with pseudotype

1174   viruses were selected with 300 μg/mL hygromycin for at least 1 week.

1175

1176   **S5 Fig. Patterns of entry pathways were conserved in various variants of SARS-**

1177   **CoV-2.**

66

1178   (**a**) Expression of WT or mutant SARS-CoV-2 S proteins with mutations precent in

1179   B.1.1.7, B.1.351, B.1.617.1 and B.1.617.2 variants in the pseudoviruses. S proteins were

1180   detected using an anti-Flag-tag antibody that binds to a Flag-tag on the C-terminus of S

1181   proteins (top). Detection of vesicular stomatitis virus matrix protein (VSV M) served as

1182   a control (bottom). S0: uncleaved S protein; S2: cleaved S2 domain of the S protein. (**b**)

1183   Effects of E-64d and marimastat on the entry of pseudoviruses bearing SARS-CoV-2 S

1184   in VeroE6 cells. E-64d: 25 μM E-64d, marima: 1 μM marimastat. (**c**) Effects of

1185   nafamostat on the entry of pseudovirus bearing SARS-CoV-2 S in VeroE6-TMPRSS2

1186   cells. (**d**) Effects of E-64d and marimastat on the entry of pseudoviruses bearing SARS-

1187   CoV-2 S in HEC50B cells. E-64d: 25 μM E-64d, marima: 1 μM marimastat. (**e**) Effects

1188   of marimastat on the entry of pseudoviruses bearing SARS-CoV-2 S in A704 cells. (**f**)

1189   Effects of nafamostat on the entry of pseudoviruses bearing SARS-CoV-2 S in Calu-3

1190   cells. The relative pseudovirus entry was calculated by normalizing the FL activity for

1191   each condition to the FL activity of cells infected with pseudovirus in the presence of

1192   DMSO alone, which was set to 100%. Values are means $\pm$ SD (n = 3/group in b-f). Data

1193   were compared with those obtained from cells infected pseudoviruses bearing variant

1194   SARS-CoV-2 S in the presence of DMSO alone. * $p < 0.05$, ** $p < 0.01$. Cont: cells

1195   infected with a pseudovirus without S protein in (**b-f**).

67

1196

1197 **S6 Fig. Expression of WIV1-CoV and HCoV-NL63 S protein in pseudoviruses.**

1198 (**a**) Schematic illustration of C-terminally FLAG-tagged S proteins of WIV1-CoV and

1199 HCoV-NL63 and amino acid sequences of the residues around the S1/S2 boundary of the

1200 coronaviruses (bottom). Numbers refer to amino acid residues. F: Flag tag. Arginine

1201 residues in the S1/S2 cleavage site and furin cleavage motif are highlighted in red. (**b**)

1202 Expression of S protein in pseudoviruses S proteins were detected using an anti-Flag-tag

1203 antibody that binds to a Flag-tag on the C-terminus of S proteins (top). The detection of

1204 VSV M served as a control (bottom). S0: uncleaved S protein; S2: cleaved S2 domain of

1205 the S protein.

1206

1207 **S7 Fig. Effects of drugs on cell viabilities.**

1208 (**a-c**) VeroE6 (a), HEC50B (b), and A704 (c) cells were treated with various drugs, and

1209 cell viability was analyzed using Celltiter-Glo 24 h after the treatment. The relative cell

1210 viability was calculated by normalizing the FL activity for each condition to the FL

1211 activity of the cells in the presence of DMSO alone, which was set to 100%. Values are

1212 means ± SD ($n = 3$/group). (**d, e**) HEC50B (d), and HEC50B-TMPRSS2 (e) cells were

1213 treated with various drugs for 3 days. Half of the culture supernatant was replaced daily

1214　　with fresh medium containing the drugs. The relative cell viability was calculated by

1215　　normalizing the FL activity for each condition to the FL activity of cells in the presence

1216　　of DMSO alone, which was set to 100%. Values are means ± SD ($n = 6$/group).

1217

1218　　**S8 Fig. Patterns of the entry pathways of the pseudovirus bearing SARS-CoV-2 S**

1219　　**were not affected by the ADAM17 knockout in the HEC50B cells.**

1220　　(**a**) Effect of the ADAM17 knockout on ACE2 (top), ADAM17 (middle), and tubulin

1221　　(bottom). (**b**) Effect of the ADAM17 knockout on the entry of the pseudoviruses bearing

1222　　SARS-CoV-2 S. Values are means ± SD ($n = 3$/group). ** $p < 0.01$. (**c**) Effect of the

1223　　ADAM17 knockout on the patterns of the entry pathways of SARS-CoV-2 S pseudovirus

1224　　in HEC50B cells. The relative pseudovirus entry was calculated by normalizing the FL

1225　　activity for each condition to the FL activity of cells infected with pseudovirus in the

1226　　presence of DMSO alone, which was set to 100%. Values are means ± SD ($n = 3$/group).

1227　　* $p < 0.05$, ** $p < 0.01$. E-64d: 25 μM E-64d, marima: 1 μM marimastat. To establish the

1228　　ADAM17-knockout HEC50B cells, lentiviruses were produced by transfecting the

1229　　lentiCRISPRv2 vector (#52961 Addgene, MA, USA) with the following gRNA

1230　　sequences. The gRNA sequences used were 5′-GCG AGG TAT TCG GCT CCG CG-3'

1231　　(Cont #1), 5′-GCT TTC ACG GAG GTT CGA CG-3' (Cont #2) and 5′-ATG TTG CAG

1232   TTC GGC TCG AT-3' (Cont #3) for the control experiments, and 5′-AAC GTT CAG

1233   TAC TTG ATG TC-3' (ADAM10 #1) and 5′-GGA CTT CTT CAC TGG ACA CG-3'

1234   (ADAM10 #2) and 5′-CTT AAG GTG AGC CTG ACT CT-3' (ADAM10 #3) for the

1235   establishment of ADAM17-knockout cells. Pooled HEC50B cells infected with

1236   pseudotype viruses were selected with 1 μg/mL puromycin for 1 week.

1237

1238   **S9 Fig. Effects of drugs on SARS-CoV-2 infection.**

1239   (**a**) Effects of the nafamostat on the SARS-CoV-2 infection in Calu-3, HEC50B, A704,

1240   and VeroE6 cells. Values are means ± SD ($n = 3$/group). ** $p < 0.01$. (**b**) Effects of the

1241   E-64d on the SARS-CoV-2 infection in HEC50B, A704, and VeroE6 cells. Values are

1242   means ± SD ($n = 3$/group). * $p < 0.05$, ** $p < 0.01$. (**c**) Effects of the NH_4Cl on the SARS-

1243   CoV-2 infection in HEC50B cells. Values are means ($n = 2$/group). The relative amount

1244   of viral RNA in the cells was normalized to cellular *Rpl13a* mRNA expression in (a-c).

1245

1246   **S10 Fig. The metalloproteinase-dependent entry pathway of authentic SARS-CoV-**

1247   **2 is involved in syncytia formation.**

1248   Phase contrast images of syncytia formation 24 h after SARS-CoV-2 infection in the

1249   presence of inhibitors. Red arrowheads indicate syncytia formation Scale bars, 100 μm.

1250

a

Effector cell

| SARS-CoV S | SARS-CoV-2 S | MERS-CoV S |

Target cell:
- Receptor + TMPRSS2
- Receptor
- None

b

c

d

a

ACE2 (% of DMSO) vs TMPRSS2 + ACE2 (% of DMSO)

Labeled points: nafamostat, camostat (green), algestone, AZD-7762, PF-477736, CTS-1027 (red), midostaurin, ilomastat (red), sunitinib, PD-166285, PD173952 (blue). Thresholds at 60% and 70%.

b

Target cell: ACE2 (blue), ACE2+TMPRSS2 (red)

Top left: Relative cell fusion (%) vs ilomastat (μM), concentrations DMSO, DMSO, 0.1, 1, 10. ** significance marked.
Effector: None | CoV-2 S
Target: ACE2 w/wo TMPRSS2

Top right: Relative cell fusion (%) vs CTS-1027 (μM), concentrations DMSO, DMSO, 0.1, 1, 10. ** significance marked.
Effector: None | CoV-2 S
Target: ACE2 w/wo TMPRSS2

Bottom left: Relative cell fusion (%) vs marimastat (μM), concentrations DMSO, DMSO, 0.1, 1, 10. ** significance marked.
Effector: None | CoV-2 S
Target: ACE2 w/wo TMPRSS2

Bottom right: Relative cell fusion (%) vs prinomastat (μM), concentrations DMSO, DMSO, 0.1, 1, 10. ** significance marked.
Effector: None | CoV-2 S
Target: ACE2 w/wo TMPRSS2

a

A704 — Pseudovirus entry (% of control); Cont DMSO / SARS-CoV-2 DMSO, marimastat 0.1, 1, 10 (µM); **

OVISE — Pseudovirus entry (% of control); Cont DMSO / SARS-CoV-2 DMSO, E-64d 0.25, 2.5, 25; **

Calu-3 — Pseudovirus entry (% of control); Cont DMSO / SARS-CoV-2 DMSO, nafamostat 0.001, 0.01, 0.1 (µM); **

b

VeroE6 — Pseudovirus entry (% of control); DMSO Cont / SARS-CoV-2 DMSO, E-64d, nafamo, marima, E-64d + marima; **, **, *

HEC50B — DMSO Cont / SARS-CoV-2 DMSO, E-64d, nafamo, marima, E-64d + marima; **, **, **

OVTOKO — DMSO Cont / SARS-CoV-2 DMSO, E-64d, nafamo, marima, E-64d + marima; **, **, **

A704 — DMSO Cont / SARS-CoV-2 DMSO, E-64d, nafamo, marima; **

c

IGROV1 — Pseudovirus entry (% of control); DMSO Cont / SARS-CoV-2 DMSO, E-64d, nafamo, marima, E-64d + nafamo, E-64d + marima; **, *

OUMS-23 — DMSO Cont / SARS-CoV-2 DMSO, E-64d, nafamo, marima, E-64d + nafamo, E-64d + marima; **, *

OVISE — DMSO Cont / SARS-CoV-2 DMSO, E-64d, nafamo, marima; **

d

Calu-3 — Pseudovirus entry (% of control); DMSO Cont / SARS-CoV-2 DMSO, E-64d, nafamo, marima; **

Caco-2 — DMSO Cont / SARS-CoV-2 DMSO, E-64d, nafamo, marima; **

e

HEC50B-TMPRSS2 — Pseudovirus entry (% of control); Cont / SARS-CoV-2 S

marima	−	−	+	−	+	−	+	−	+
E-64d	−	−	−	+	+	−	−	+	+
nafamo	−	−	−	−	−	+	+	+	+

**, *