

1 **SARS-CoV-2 and SARS-CoV spike-mediated cell-cell fusion differ in the requirements for**
2 **receptor expression and proteolytic activation**

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4 RUNNING TITLE: SARS-CoV-2 and SARS-CoV cell-cell fusion

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20 **ABSTRACT**

21 The SARS-Coronavirus-2 (SARS-CoV-2) infects cells through interaction of its spike protein
22 (SARS2-S) with the ACE2 receptor and activation by proteases, in particular TMPRSS2.
23 Viruses can also spread through fusion of infected with uninfected cells. We compared the
24 requirements of ACE2 expression, proteolytic activation, and the sensitivity to inhibitors for
25 SARS2-S-mediated and SARS1-S-mediated cell-cell fusion. SARS2-S-driven fusion was
26 moderately increased by TMPRSS2 and strongly by ACE2, while SARS1-S-driven fusion was
27 strongly dependent on activation by TMPRSS2 and less so on ACE2 expression. In contrast to
28 SARS1-S, SARS2-S-mediated cell-cell fusion was efficiently activated by Batimastat-sensitive
29 metalloproteases. Mutation of the S1/S2 proteolytic cleavage site reduced effector-target-
30 cell fusion when ACE2 or TMPRSS2 were limiting and rendered SARS2-S-driven cell-cell
31 fusion more dependent on TMPRSS2. When both ACE2 and TMPRSS2 were abundant, initial
32 target-effector-cell fusion was unaltered compared to wt SARS2-S, but syncytia remained
33 smaller over time. Mutation of the S2' site specifically abrogated activation by TMPRSS2 for
34 both cell-cell fusion and SARS2-S-driven pseudoparticle entry but still allowed for activation
35 by metalloproteases for cell-cell fusion and by cathepsins for particle entry. Finally, we found
36 that the TMPRSS2 inhibitor Bromhexine was unable to reduce TMPRSS2-activated cell-cell
37 fusion by SARS1-S and SARS2-S as opposed to the inhibitor Camostat. Paradoxically,
38 Bromhexine enhanced cell-cell fusion in the presence of TMPRSS2, while its main metabolite
39 Ambroxol exhibited weak inhibitory activity in some conditions. On Calu-3 lung cells,
40 Ambroxol weakly inhibited SARS2-S-driven lentiviral pseudoparticle entry, and both
41 substances exhibited a dose-dependent trend towards weak inhibition of authentic SARS-
42 CoV-2.

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44
45 **IMPORTANCE**

46 Cell-cell fusion allows the virus to infect neighboring cells without the need to produce free
47 virus and contributes to tissue damage by creating virus-infected syncytia. Our results
48 demonstrate that the S2' cleavage site is essential for activation by TMPRSS2 and unravel

49 important differences between SARS-CoV and SARS-CoV-2, among those greater
50 dependence of SARS-CoV-2 on receptor expression and activation by metalloproteases for
51 cell-cell fusion. Bromhexine, reportedly an inhibitor of the TMPRSS2 protease, is currently
52 tested in clinical trials against COVID-19. Our results indicate that Bromhexine enhances
53 fusion in some conditions. We therefore caution against use of Bromhexine in higher dosage
54 until its effects on SARS-CoV-2 spike activation are better understood. The related
55 compound Ambroxol, which similarly to Bromhexine is clinically used as an expectorant, did
56 not exhibit activating effects on cell-cell fusion. Both compounds exhibited weak inhibitory
57 activity against SARS-CoV-2 infection at high concentrations, which might be clinically
58 attainable for Ambroxol.

59

60 INTRODUCTION

61 The coronavirus disease 2019 (COVID-19) disease spectrum is caused by the severe acute
62 respiratory syndrome-related coronavirus 2 (SARS-CoV-2), which was first identified in
63 patients with pneumonia of unknown origin in the city of Wuhan, China (1). While first
64 characterized as a pneumonia, COVID-19 probably affects a number of organ systems (2–4).
65 SARS-CoV-2 was shown to use the ACE2 receptor, which was previously described as
66 receptor for the closely related SARS-CoV (5), for the infection of human cells (1, 6, 7). For
67 the proteolytic activation of the viral spike protein, a prerequisite for fusion activity of
68 coronaviruses (reviewed in (8)), the transmembrane protease serine subtype 2 (TMPRSS2)
69 (7, 9) as well as the related TMPRSS4 (2) were reported to be of critical importance. In
70 addition, TMPRSS2 was demonstrated to colocalize with the ACE2 receptor (10) and
71 therefore may be biologically particularly relevant. Depending on the cell type, SARS-CoV-2
72 spike-driven entry can also occur through endocytotic pathways where virus-cell fusion is
73 most likely activated by cathepsins (7). Another study reported that several members of the
74 TMPRSS family can activate SARS-CoV-2 spike-mediated membrane fusion (11). The
75 proposed mechanisms for spike priming and initiation of fusion therefore require further
76 clarification, e.g. whether serine protease activity is required under all circumstances, or
77 whether fusion can also occur without the action of serine proteases, where these proteases
78 act on the spike, and whether there are differences between cell-cell and cell-particle fusion.
79 It was recently discovered that the polybasic S1/S2 cleavage site of SARS-CoV-2 is required
80 for efficient infection of lung-derived cells (12) and promotes the formation of syncytia.
81 Understanding syncytium formation may be important as large syncytial elements are
82 reported to constitute a hallmark of COVID-19-associated pathology (13). Nevertheless, the
83 exact contribution of the two known proteolytic priming sites to cell-cell fusion and their
84 protease usage are not entirely clear. To address these questions, we mutated the S1/S2 site
85 as well as the S2' site, we assessed the effects of proteolytic activation by using inhibitors of
86 TMPRSS2 and other proteases, and we analyzed the effects of different levels of protease
87 and receptor expression on SARS1-S and SARS2-S fusion activity.

88 TMPRSS2, which is expressed in airway cells (14), may be amenable to specific inhibition by
89 Bromhexine (15), a molecule normally used as an expectorant that thins phlegm and eases
90 coughing and is widely known as a popular over-the-counter medication, which would make
91 its repurposing for COVID-19 particularly attractive. For these or additional reasons,
92 Bromhexine is now being tested in at least three clinical trials for efficacy against COVID-19
93 (NCT04355026, NCT04273763, NCT04340349). We therefore tested the effect of the specific
94 TMPRSS2 inhibitor Bromhexine on spike protein-mediated cell-cell fusion and SARS2-S
95 driven cell entry and compared its potency to the serine protease inhibitor Camostat. We
96 also included Ambroxol, an active metabolite of Bromhexine in our studies (16). Ambroxol

97 has often replaced Bromhexine as an over-the-counter medication, and the structural
98 similarity to Bromhexine may hint at potential inhibitory effects towards TMPRSS2.
99 Ambroxol may also exhibit weak but broad anti-viral activity as it was shown to reduce the
100 occurrence of respiratory infects (17) and to inhibit proteolytic activation of influenza virus
101 by triggering release of antiviral factors (18), and it is used to treat acute respiratory distress
102 syndrome (ARDS) in adults and antenatally in infants (19, 20). Further, two recent preprints,
103 one describing modulation of the ACE2-SARS2-S interaction by both Bromhexine and
104 Ambroxol (21) and the other reporting weak inhibitory activity of Ambroxol against SARS-
105 CoV-2 replication (22) in Vero E6 cells, point at a potential utility of these molecules in the
106 therapy of COVID-19.

107

108 **RESULTS**

109

110 SARS-CoV-2 spike protein mediates robust fusion of 293T cells transfected with ACE2 with 111 and without coexpression of TMPRSS2.

112 In order to investigate the fusion mechanism of the SARS-CoV-2 spike protein we generated
113 several SARS2-S mutants (Fig. 1 A, schematic drawn after (23)). It was reported that a
114 mutation of the S1/S2 cleavage site of SARS2-S plays a role in infection of airway cells like
115 Calu-3 but is dispensable in other cell types (11, 12). Thus, we generated a mutant called
116 S1/S2 where the furin-recognition motif and the cleavage site were replaced by alanines (Fig
117 1 A). In contrast to already published S1/S2 mutants (24, 25) we did not delete the site as we
118 suspected that this may influence protein conformation and flexibility but we mutated the
119 proposed furin cleavage site (26) to fully abrogate processing at this site. Clearly detectable
120 bands of lower molecular weight, indicative of proteolytic processing, were only observed
121 with wt SARS2-S (Fig. 1 B). As expected, the S1/S2-mutant exhibited no processing at the
122 S1/S2 site indicated by a missing S2 fragment in Western Blot from transfected 293T lysate
123 (Fig. 1 B). This is similar to the SARS-1-spike (SARS1-S) which has no furin cleavage site at this
124 position. We furthermore generated an S2' site mutant by exchanging the K814 and the
125 R815 to alanine. The S2' site was shown to be important for proteolytic priming in SARS1-S
126 and is highly conserved among coronavirus spikes (27). We therefore suspected that this site
127 is also important for proteolytic processing of SARS2-S. As not all mutants might be
128 efficiently expressed at the cell surface, we performed cell surface staining with a COVID-19
129 convalescent serum followed by flow cytometry (Fig. 1 C, middle column group) which
130 revealed detectable but strongly reduced cell surface expression of the S2' mutant, as well
131 as reduced ACE2 binding when the same assay was performed with an ACE2-Fc fusion
132 protein (Fig. 1 C, right column group). SARS1-S is only weakly recognized by the COVID-19
133 convalescent serum. RRV gHΔ21-27-Fc, an Fc fusion protein of RRV gH that lacks any
134 detectable receptor interactions (28), served as control.

135 In order to study spike mediated cell-cell fusion, we established a quantitative reporter gene
136 assay. We chose 293T cells as effector cells, i.e. the cell expressing the viral glycoproteins,
137 because i) 293T exhibit high transfection efficiency and protein expression and ii) 293T can
138 be lifted without trypsinization. We resorted to a system that is also used for two-hybrid
139 screenings, using a VP16-Gal4 transcription factor in one cell and a Gal4-response element-
140 driven reporter construct in the other cell, which results in strong transactivation and
141 reporter gene expression after cell-cell fusion. We transfected 293T target cells with ACE2
142 and TMPRSS2 expression plasmids and a Gal4 response element driven TurboGFP-luciferase
143 reporter plasmid (Gal4-TurboGFP-Luc) and effector cells with spike expression constructs, as
144 well as with a plasmid encoding the Gal4 DNA binding domain fused to the VP16

145 transactivator. Apparent expression levels of SARS1-S as assayed by Western blot were
146 seemingly lower than those of SARS2-S (Fig. 1 B), but this may be owed to different
147 glycosylation, proteolytic cleavage and transfer or detection and was not reflected in its
148 surface expression as measured by ACE2-binding (Fig. 1 C) and its fusion activity (Fig. 1 D).
149 We found that when only ACE2 was overexpressed (Fig. 1 D, left), all SARS2-S constructs
150 exhibited fusion activity that was statistically different from background. SARS1-S had visible
151 activity but that did not remain significant after correction for multiple comparisons. On
152 293T cells that were co-transfected with ACE2/TMPRSS2 expression constructs, all spike
153 variants exhibited fusion activity significantly over background (Fig. 1 D), except for the
154 SARS2-S2'-AA mutant, which exhibited visible but not statistically significant activity. We
155 chose a logarithmic scale in Fig. 1 D for an initial overview of the considerably different
156 fusion activities and how they relate to background activity. Testing activity in a time-lapse
157 experiment, we observed that luciferase activity was increasing up to 18h for SARS1-S and
158 SARS2-S1/S2-mut and possibly 24h for SARS2-S (Fig. 1 E). Also, activity between SARS1-S,
159 SARS2-S, and SARS2-S1/S2-mut was not meaningfully different at any timepoint. Activity is
160 shown on a linear scale here, which allows for discrimination of smaller differences and
161 which we use from here on.

162

163 The S1/S2 site is critical for syncytium size.

164 Our results demonstrated mostly normal fusion activity of the S1/S2 mutant in our system
165 when TMPRSS2 was present. Therefore, we wanted to address how mutation of the S1/S2
166 site translates into syncytium formation in our system, as several reports clearly
167 demonstrated that the S1/S2 site is important for this process (24, 26). It should be noted
168 that initial cell-cell fusion and syncytium formation may not necessarily be the exact same
169 thing. After the initial fusion event, all factors that were originally present in separate cells,
170 i.e. viral glycoprotein, receptor, and activating proteases are then together in a single
171 syncytial cell and can interact directly upon coexpression. As our reporter also encodes a
172 TurboGFP that is fused to firefly luciferase, syncytium formation can be conveniently
173 visualized. Under the microscope, we indeed observed, that in the presence of ACE2 and
174 TMPRSS2 the S1/S2 mutant formed small but numerous syncytia, while wt SARS2-S formed
175 larger syncytia (Fig. 1 F). Reporter activity was comparable. Formation of extended syncytia
176 is obviously a quality that our luciferase reporter does not capture, and interestingly, this is
177 not a matter of the timing of the measurement (Fig 1 E), as even at earlier time points, the
178 luciferase activity between wt and the S1/S2 mutant as well as SARS1-S were similar. We
179 conclude that our luciferase assay measures primarily the initial fusion between effector and
180 target cell and not the formation of extended syncytia.

181

182 SARS2-S-mediated cell-cell fusion is dependent on ACE2 receptor expression and is less
183 restricted by TMPRSS2-mediated activation in trans than SARS1-S-mediated fusion.

184 As we found SARS2-S capable of fusing 293T cells efficiently when ACE2 was expressed
185 without TMPRSS2, while SARS1-S was only fully fusogenic in the presence of TMPRSS2, we
186 decided to analyze SARS2-S, SARS1-S and SARS2-S1/S2-mut as well as the SARS2-S2'-AA
187 mutant in the context of different ACE2 and TMPRSS2 expression levels (Fig 2). In this
188 setting, we again observed robust fusion activity of SARS2-S that was essentially unaltered
189 by different levels of TMPRSS2 but required the presence of ACE2 (Fig. 2 A). SARS1-S on the
190 other hand exhibited high activity under all conditions with TMPRSS2 present, whether ACE2
191 was expressed or not. Activity by the SARS2-S2'-AA mutant was low under all conditions but
192 was highest under the condition with maximal ACE2 expression and not responsive to

193 changes in TMPRSS2 levels. The SARS2-S1/S2-mut on the other hand exhibited an interesting
194 behavior in that it exhibited reduced fusion activity when either ACE2 or TMPRSS2 were
195 absent but was fully fusion competent in all conditions in between, with probably a slight
196 trend towards highest activity with comparatively low TMPRSS2 levels, similar in that respect
197 to SARS1-S. The respective protein levels as present at the end of the co-culture are shown
198 in Fig. 2 B. We labelled the fully processed S2 fragment with an asterisk, as the exact nature
199 of this fragment can't be deduced with full confidence from its apparent molecular size,
200 even if it could be the so-called S2' fragment after cleavage at this site. Interestingly, the S0
201 and S2 fragments are visibly processed to a large degree into smaller fragments under
202 conditions that allow for high fusion activity. We decided to continue with transfecting equal
203 amounts of ACE2 and TMPRSS2 expression plasmids.

204

205 Differential effect of the TMPRSS2 inhibitor Camostat, Bromhexine, and the Bromhexine 206 metabolite Ambroxol on SARS1-S and SARS2-S-mediated fusion.

207 For a comprehensive analysis, we measured fusion with target cells that were co-transfected
208 with ACE2 and TMPRSS2 expression plasmids, in addition to cells transfected with either
209 ACE2 or TMPRSS2 expression plasmid alone. As fusion effectors, SARS1-S, SARS2-S as well as
210 SARS2-S1/S2-mut and SARS2-S2'-AA were included. To test the effects of TMPRSS2 inhibition
211 by small molecules on the activation of wt SARS2-S and the two mutants as well as SARS1-S,
212 we incubated the different target cells with Bromhexine, reportedly a specific inhibitor of
213 TMPRSS2 (15), the chemically related compound Ambroxol, or Camostat, an irreversible
214 inhibitor of TMPRSS2 and many serine proteases in general (29, 30), at 50 μ M (Fig. 3 A). We
215 chose this high concentration, which is most likely outside of any therapeutic range except
216 for Ambroxol, as overexpression of TMPRSS2 may shift the EC50 considerably upwards.

217 As observed before (Fig. 1 D), in the presence of ACE2 and TMPRSS2, both SARS1-S and
218 SARS2-S exhibited strong fusion activity, as did the SARS2-S1/S2-mut protein. SARS2-S2'-AA
219 on the other hand was strongly impaired under these conditions.

220 ACE2 expression alone was sufficient for induction of high fusion activity of SARS2-S but
221 induced only moderate activity of SARS1-S. Levels of ACE2 expression were higher in single-
222 transfected cells (Fig. 3 B). This observation is compatible with data from the literature
223 stating that ACE2 is cleaved by TMPRSS2 (10), which conceivably reduces detection by
224 Western blot, in addition to potential competition effects between expression plasmids.

225 Nevertheless, SARS2-S-driven fusion was clearly not limited by TMPRSS2 expression, and
226 reached highest activity when only ACE2 was expressed. The S1/S2 cleavage site mutant of
227 SARS-CoV-2 on the other hand exhibited reduced activation in the presence of ACE2 without
228 additional TMPRSS2 activity, whereas the SARS-S2'-AA mutant exhibited again low but
229 detectable fusion activity when ACE2 was overexpressed. Overexpression of TMPRSS2 did
230 not increase fusion activity of SARS-S2'-AA. Conversely, SARS1-S-driven fusion was clearly
231 more enhanced by overexpression of TMPRSS2 than by overexpression of ACE2, reaching
232 highest activity under conditions where only TMPRSS2 was recombinantly expressed, and
233 was only weakly activated by ACE2 expression in the absence of recombinant TMPRSS2
234 expression (Fig. 2 A and 3 A).

235 We observed that cell-cell fusion by SARS1-S and SARS2-S was not inhibited by Bromhexine,
236 and only SARS1-S activity was slightly inhibited by Ambroxol in the presence of TMPRSS2.
237 Surprisingly, we observed an induction of SARS2-S fusion activity in the presence of
238 Bromhexine, significantly so when ACE2 and TMPRSS2 were coexpressed. Similarly,
239 Camostat did not reduce SARS2-S-mediated fusion in this setting unless TMPRSS2 was
240 overexpressed without ACE2. However, both SARS2-S1/S2-mut and even more

241 pronouncedly SARS1-S exhibited a significantly reduced fusion activity in the presence of
242 Camostat. The strong induction of SARS1-S-mediated fusion by TMPRSS2 was clearly
243 reversed by Camostat but not by Bromhexine. Notably, Camostat did not exert any inhibitory
244 effect on the remaining fusion activity of the SARS2-S2'-AA mutant, nor did TMPRSS2
245 expression induce activity of this mutant, compatible with the S2' site being the primary
246 target of TMPRSS2 *in trans*.

247 The results were also mirrored by Western blot analysis (Fig. 3 B) of SARS2-S under the same
248 conditions, if generation of the fully processed S2 fragment is analyzed, which we labelled
249 with an asterisk. Generation of this fragment was clearly visible under all conditions that
250 allowed for high fusion activity, e.g. when ACE2 was present, less so with TMPRSS2 alone.
251 Interestingly, addition of Camostat increased the detectable amount of ACE2, probably
252 explaining the slight trend towards higher activity in its presence. Further, Ambroxol
253 reproducibly induced the generation of an atypical TMPRSS2 autoproteolytic fragment,
254 which may hint at some sort of modulating activity of Ambroxol towards TMPRSS2 (Fig. 3 B,
255 fourth lane).

256 Taken together, we observed robust SARS-CoV-2 spike protein-mediated cell-cell fusion that
257 was not dependent on exogenous TMPRSS2 expression and that was not inhibited by
258 Bromhexine. Instead, fusion was enhanced by Bromhexine. Cell-cell fusion mediated by
259 SARS2-S was clearly not at all or to a much lesser degree restricted by serine protease
260 activity on target cells than fusion by SARS1-S. Interestingly, Ambroxol exhibited some
261 activity against TMPRSS2-mediated activation of SARS1-S.

262

263 Bromhexine enhances SARS2-S-mediated fusion in the presence of TMPRSS2.

264 To further explore the paradoxical effect of the putative TMPRSS2 inhibitor Bromhexine on
265 fusion activity, we performed fusion reactions in the presence of Bromhexine and Ambroxol
266 at different concentrations (Fig. 3 C). In order to eliminate potential systematic errors, we
267 deviated from our previous protocol, cocultured for 48h instead of 24h, and co-transfected
268 the reporter plasmid into the effector instead of the target cells, this time using a different
269 luciferase reporter without TurboGFP. We again did not observe inhibition by Bromhexine,
270 but a dose-dependent enhancement. Ambroxol treatment on the other hand did not lead to
271 a similar enhancement, but to a slight decrease in activity at 50 μ M. As a control fusion
272 protein that works with practically any cell type, we included VSV-G. While VSV-G is
273 physiologically pH-activated for full fusion activity (31), it reportedly exhibits considerable
274 activity without pH priming (32, 33). VSV-G-mediated fusion activity was not increased by
275 Bromhexine.

276

277 SARS2-S-mediated cell-cell fusion is sensitive to inhibition of matrix metalloproteases.

278 The robust cell-cell-fusion that we observed with SARS2-S in the absence of TMPRSS2
279 activity should most likely be triggered by proteolytic processing, if the mechanism is
280 analogous to what was observed for SARS-CoV (8, 34). Therefore, we tested the effects of
281 different protease inhibitors on SARS2-S-mediated fusion of ACE2 expressing 293T cells
282 without exogenous TMPRSS2 activity. As we wanted to exclude the possibility that pre-
283 activation on the producer cells could play a role, we tested the inhibitors both in the
284 coculture (Fig. 4 A), and with pre-incubation of both effector and target cells (Fig. 4 B).
285 Values were normalized to the respective solvent control for better comparison. We
286 observed some inhibitory effect on SARS2-S and SARS1-S fusion activity by the broadband
287 serine protease inhibitor AEBSF, and by a protease inhibitor cocktail whose main ingredients
288 are the serine protease inhibitors AEBSF and Aprotinin, and the cysteine protease inhibitors

289 E64 and Leupeptin. The S1/S2 cleavage site mutant was not sensitive to this inhibitor
290 cocktail, suggestive of action at this site in the SARS2 wildtype spike. These effects were
291 more pronounced and significant with pre-incubation of the effector cells (Fig. 4B), in
292 particular for the S2' mutant. Interestingly, the inhibitor cocktail almost completely
293 abrogated the remaining fusion activity of SARS1-S. The furin inhibitor CMK did not
294 significantly inhibit any of the spikes except the S2' mutant (Fig. 4 A and B). This was
295 somewhat surprising for us, but it may reflect the fact that proteases other than furin can
296 cleave at the S1/S2 site (35), which may in turn partially obviate furin cleavage in our system.
297 We also tested EDTA/EGTA, Bromhexine, Ambroxol and included Camostat, which as
298 expected had no effect in this TMPRSS2-free system. EDTA/EGTA had a mild impact on
299 SARS1-S fusion activity with and without preincubation (Figs. 4 A, B). Bromhexine and
300 Ambroxol exhibited an interesting behavior in this assay: We observed inhibitory activity of
301 Bromhexine and Ambroxol towards SARS1-S and the SARS2-S1/S2-mut and SARS2-S2'-AA
302 mutants in this TMPRSS2-free cell system, suggesting that these substances somehow
303 interact with the spike proteins or ACE2. Luciferase activity of control cells, which were
304 transfected with both the Gal4 reporter and transactivator constructs, was only mildly
305 affected by AEBSF, the inhibitor cocktail, EDTA/EGTA and Bromhexine, not by the other
306 substances (Fig. 4 C). In particular, any reductions observed with Ambroxol can't be
307 explained by non-specific effects on the luciferase reporter system and most likely represent
308 real inhibitory activity against SARS1-S-mediated fusion activity and fusion mediated by the
309 two SARS2-S cleavage site mutants.

310 Western blot analysis suggested that the protease inhibitor cocktail may have had a
311 somewhat stabilizing effect on the S2 intermediate form of S (Fig. 4 D), which resulted in less
312 processing into the putative S2' form. CMK both reduced "smear" at higher molecular
313 weight, which likely represents glycosylation variants, and reduced abundance of the S2
314 proteolytic product, that should be generated through cleavage at the polybasic cleavage
315 site, compatible with furin inhibition. As none of the tested inhibitors resulted in meaningful
316 reduction of fusion activity with wt SARS2-S, we decided to test a more potent inhibitor of
317 metalloproteases than EDTA/EGTA, whose maximum concentration is limited by its effects
318 on cell adhesion and viability. The EDTA/EGTA concentration that was used by us was most
319 likely too low to meaningfully impact protease activity, in particular as the cell culture
320 medium contains calcium and magnesium. We therefore tested Batimastat, which inhibits
321 matrix metalloproteases (36, 37).

322 Batimastat indeed inhibited SARS2-S-dependent fusion in the absence of TMPRSS2 in a dose
323 dependent manner (Fig. 5 A). Interestingly, no inhibition was observed in the presence of
324 both ACE2 and TMPRSS2, and in the presence of TMPRSS2 alone unless TMPRSS2 was
325 inhibited by Camostat (Fig. 5 A). Therefore, Batimastat-sensitive metalloproteases cleave
326 SARS2-S to activate cell-cell fusion. This notion is supported by the finding, that TMPRSS2
327 expression can overcome the Batimastat-induced block. Western blot analysis of the fusion
328 reactions indicated that Batimastat probably induced a subtle change in the migration
329 pattern of SARS2-S in the presence of ACE2 but without TMPRSS2 (Fig. 5 B). We next decided
330 to test the effect of Batimastat on the fusion activity of the S1/S2 mutant and the S2' mutant
331 under conditions of ACE2 overexpression without TMPRSS2 (Fig. 5 C). Both mutants were
332 inhibited by Batimastat, indicating that matrix metalloproteases can cleave irrespective of an
333 intact S1/S2 or S2' cleavage site, although this does not necessarily rule out an modulating
334 effect in particular by S1/S2 cleavage, as mutation of S1/S2 leads to impaired activity
335 without TMPRSS2. Under conditions of ACE2 and TMPRSS2 coexpression, which leads to
336 lower ACE2 levels (compare Fig 2 B and 3 B), SARS2-S1/S2-mut was not impacted by

337 Batimastat unless TMPRSS2 was again inhibited by addition of Camostat (Fig 5 C), whereas
338 activity of the S2' mutant was inhibited in the presence of Batimastat alone, strongly
339 suggesting that TMPRSS2 activates via the S2' site. Under conditions of TMPRSS2
340 overexpression without ACE2 overexpression Batimastat was again without effect. Results
341 with the SARS2-S2'-AA mutant come with the caveat that this mutant was barely active at all
342 under these conditions (e.g. Figs. 2 A and 3 A). In summary, these experiments demonstrate
343 that in the presence of the ACE2 receptor, matrix metalloproteases can efficiently activate
344 SARS2-S for cell-cell fusion.

345

346 The SARS2-S S2' site is the TMPRSS2 target site

347 While our results with the SARS2-S2'-AA mutant were already strongly suggestive of S2'
348 being the target site for TMPRSS2, this conclusion remained slightly ambiguous in light of the
349 relatively low surface expression and inefficient proteolytic processing of this mutant (Fig. 1
350 B and C). We therefore set out to generate an S2' mutant that is still efficiently processed
351 and expressed at the cell surface. We permuted several amino acids to replace the original
352 "KR" (Fig. 1 A) sequence motif and tested fusion activity in the presence of ACE2, TMPRSS2
353 and ACE2/TMPRSS2. We found that SARS2-S2'-GH and HH mutants were active in our fusion
354 assay, whereas EE and ES resulted in abrogation of fusion activity, below the levels achieved
355 with the AA mutant (Fig. 6 A). The GH mutant was also processed (Fig. 6B), efficiently
356 expressed at the cell surface, and exhibited high ACE2 binding capacity (Fig. 6 C). For further
357 experiments, we continued with the SARS2-S2'-GH mutant. Interestingly, when we tested
358 the furin inhibitor CMK for its effects in absence of TMPRSS2, all spike variants were slightly
359 less active, but only the S2'-GH variant was significantly inhibited, suggesting increasing
360 dependence on pre-priming by furin in absence of the S2' site (Fig. 6 D).

361 Confirming the results of our prior fusion assays with the AA mutant, also the SARS2-S2'-GH
362 and SARS2-S2'-HH fusion activity on 293T cells in the presence of only ACE2 were sensitive to
363 Batimastat (Fig. 6 E, left), and on 293T cells expressing ACE2/TMPRSS2 both SARS2-S2'-GH
364 and SARS2-S2'-HH were insensitive to Camostat, but again highly sensitive to Batimastat (Fig.
365 6 E, right). Fusion activity of the S2 mutants was even increased in the presence of Camostat,
366 likely because inhibition of TMPRSS2 increases ACE2 levels, as demonstrated in Fig. 3 B. This
367 unequivocally identifies the S2' site as the TMPRSS2 target site, and interestingly as the only
368 TMPRSS2 target site, at least for activation of fusion.

369

370 Entry of SARS2-S-pseudotyped lentiviruses is enhanced by TMPRSS2 and is not inhibited by 371 Bromhexine

372 To compare our findings on cell-cell fusion to spike protein-driven entry, we used lentiviral
373 particles expressing GFP as reporter gene, pseudotyped with SARS2-S. We found that
374 TMPRSS2 expression was clearly required for efficient infection of 293T cells by SARS2-S-
375 pseudotyped particles (Fig. 7 A). ACE2 overexpression alone also enhanced infection, but
376 considerably less efficiently and barely above the detection limit, which may be owed to our
377 lentiviral GFP system. The TMPRSS2-mediated enhancement was reversed by addition of
378 Camostat, but not by addition of Bromhexine or Ambroxol, both of which may even slightly
379 enhance infection in this setting. These observations were corroborated by fluorescence
380 microscopy (Fig. 7 B). As luciferase is more sensitive than GFP as a reporter gene, we
381 switched to luciferase detection (Fig. 7 C). We also included the SARS2-S D614G variant now.
382 As previously reported, D614G-driven infection was more efficient (38). It was also strongly
383 enhanced by TMPRSS2, as evidenced by potent Camostat-mediated inhibition. Ambroxol
384 and Bromhexine had no activity in this system, as opposed to Camostat. Batimastat did not

385 alter SARS2-S driven entry. A VSV-G pseudotyped lentivirus was not significantly affected by
386 either substance.

387

388 Mutation of the S2' site uncouples infection from TMPRSS2

389 Next, we aimed to corroborate our findings regarding the S2' site as TMPRSS2 target site for
390 cell-cell-fusion in particle infection. Our SARS2-S2'-GH mutant was efficiently incorporated
391 into lentiviral particles, as was SARS2-S1/S2-mut (Fig. 7 D). Both spike mutants could drive
392 entry into 293T cells expressing ACE2/TMPRSS2, but SARS2-S2'-GH with reduced efficiency
393 and SARS2-S1/S2-mut probably with increased efficiency, although we did not test for the
394 latter. None of the spike variants was inhibited by Batimastat. The wt and S1/S2 mutants
395 were inhibited by Camostat, but not by Batimastat or E64D, indicating proteolytic activation
396 by TMPRSS2. The S2' mutant on the other hand was exclusively inhibited by E64D, indicating
397 that it was refractory to activation by TMPRSS2 and dependent on activation by Cathepsins.

398

399 SARS-CoV-2 is weakly inhibited by Ambroxol on Calu-3 lung cells

400 As transfected 293T cells express TMPRSS2 at high and possibly variable levels between cells
401 and allow for at least some entry via endocytosis, weak modulatory effects on ACE2 or
402 TMPRSS2 might be missed in that system. Calu-3 express TMPRSS2 to much higher levels
403 than 293T (Fig 8 A), which are practically negative, but still at endogenous levels. We
404 therefore infected the Calu-3 lung cell line with our lentiviral pseudoparticles (Fig. 8 B).
405 These cells allow for infection by our lentiviral pseudoparticles only at very low levels (not
406 shown). In order to achieve infection at faithfully detectable levels, we used the D614G
407 variant, which exhibited the same sensitivity profile to inhibitors but was about one log
408 more efficient at driving entry (Fig. 7 C). D614G by now has become the dominant variant
409 globally and is therefore probably also more relevant. As expected, SARS2-S-driven entry
410 was practically abrogated by 50 μ M Camostat. Bromhexine again had no detectable impact
411 on SARS2-S-driven entry. Ambroxol on the other hand exhibited a weakly inhibitory effect on
412 SARS2-S-driven infection in this system, even if that needs a linear scale for proper
413 visualization. Interestingly, both substances, but Bromhexine more so, affected entry of VSV-
414 G-pseudotyped particles negatively. This is likely owed to the targeting of lysosomal
415 processes by these two substances (39, 40). Finally, we wanted to test whether this small
416 but detectable effect would translate into inhibition of authentic virus. We therefore
417 infected Calu-3 with a clinical isolate of SARS-CoV-2 at low MOI and quantified the viral RNA
418 after 20-24h by qRT-PCR (Fig. 8 C). We chose 5 μ M and 50 μ M as concentrations. For
419 Ambroxol, which is heavily enriched in lung tissue, 50 μ M might be a clinically attainable
420 concentration. Interestingly, for both substances, viral RNA copy number trended lower
421 upon treatment, and in a dose-dependent manner as can be observed in the raw Ct values
422 (Fig. 8 C) and after relative quantification (Fig. 8 D). Both, reduction by Ambroxol and by
423 Bromhexine at 50 μ M was significant, even if inhibition by Ambroxol remained only
424 significant without correction for multiple comparisons, which is appropriate in light of a
425 dose response (ANOVA with post test for linear trend in Ct values at 0 μ M, 5 μ M, and 50 μ M,
426 significant for Ambroxol and Bromhexine). 10 μ M Batimastat (compared to the DMSO
427 solvent) had no significant effect on SARS-CoV-2 mRNA level, even if DMSO alone had quite
428 some impact compared to water, most likely due to the high concentration needed, which
429 was 1%. In a cell viability assay with Calu-3 using dilutions of commercial over-the-counter
430 cough thinners, neither substance exhibited significant effects up to 10 μ M (Fig. 8E). We used
431 the cough thinners as an alternative source of Ambroxol and Bromhexine for some control
432 experiments, which were not included in this manuscript, to control for specificity of the

433 observed effects and their independence from the source of the two substances.
434 Bromhexine but not Ambroxol clearly impacted cell viability at 100 μ M, which is compatible
435 with our observations on 293T (Fig. 4C), although it should be noted that toxicity of
436 Bromhexine may have been overestimated here due to non-active ingredients of the cough
437 thinner.

438
439

440 **DISCUSSION**

441 We have established a two-hybrid-based protocol for measuring spike-mediated cell-cell
442 fusion that allows for the quantitation of cell-cell fusion by luciferase activity and
443 visualization of syncytia by GFP fluorescence. Our findings that SARS-CoV and SARS-CoV-2
444 spike protein-mediated fusion activity is activated by the ACE2 receptor is in accordance
445 with published data (11), whereas our finding that SARS2-S-mediated fusion depends
446 relatively more on receptor expression and less on proteolytic activation than SARS1-S-
447 mediated fusion is novel. Further, we have faithfully established the S2' site of SARS2-S as
448 the target for TMPRSS2-mediated activation through generation of a mutant that is
449 defective for TMPRSS2 activation but otherwise fully functional.

450 In our system, TMPRSS2 co-expression on ACE2 expressing target cells was not required for
451 SARS2-S-mediated fusion of 293T cells comparable with results of Ou et al. where ACE2
452 expression alone was also sufficient to induce cell-cell fusion without addition of exogenous
453 protease (11), and also corroborated by a very recent report (41). Furthermore, we did not
454 observe any effect on SARS2-S-mediated fusion activity upon inhibition of TMPRSS2 on
455 target cells by the serine protease inhibitor Camostat. Together, these results imply that
456 proteolytic activation by TMPRSS2 may not be a limiting factor for cell-cell fusion in 293T
457 cells. A recent report demonstrated that upon co-transfection of spike, ACE2, and TMPRSS2,
458 TMPRSS2 accelerates fusion. The size of the resulting syncytia only showed a TMPRSS2
459 dependency within the first 12h but was independent after 24h in that report (42), which is
460 compatible with our observations of efficient cell-cell-fusion without TMPRSS2 in 293T.

461 While SARS1-S-mediated cell-cell fusion was also weakly activated when ACE2 was
462 expressed alone, activation was much higher in the presence of TMPRSS2, indicating
463 stronger dependence of SARS1-S on TMPRSS2, compatible with the monobasic S1/S2
464 cleavage site in the SARS-CoV spike protein. Surprisingly, we even observed maximal
465 activation with overexpression of only TMPRSS2, indicating that SARS1-S-mediated cell-cell
466 fusion is mostly protease- and not ACE2-driven.

467 In line with this observation, SARS1-S-mediated cell-cell fusion was clearly sensitive to
468 Camostat, which reversed the TMPRSS2-mediated activation (Fig. 3 A).

469 Interestingly, mutational ablation of the S1/S2 cleavage site of SARS2-S rendered the
470 mutated spike protein sensitive to inhibition by Camostat in the presence of TMPRSS2 (Fig,
471 3A), suggesting that TMPRSS2 or a related protease is required for processing at the S2' site
472 to reach full activation when the S1/S2 site is not cleaved. In addition, in the absence of
473 recombinantly expressed TMPRSS2, SARS2-S1/S2-mut was clearly impaired with regard to
474 fusion activity (Fig. 2 A). Conversely, mutation of the S2' priming site abrogated any effects
475 of TMPRSS2 on SARS-CoV-2-mediated fusion, e.g. when TMPRSS2 alone was provided by
476 means of recombinant expression (Fig 3 A and 6), or when TMPRSS2 was inhibited by
477 Camostat (Fig. 6 E). It should be noted that the S2' mutants were still fusogenic in the
478 presence of high levels of ACE2 receptor (Figs. 2 A, 3 A and 6 A, E), in the case of the GH and
479 HH S2' mutants even at moderate levels, and in the absence of TMPRSS2 with similar activity
480 as wildtype (Fig. 6 A). The S2' GH mutant was also efficiently incorporated (Fig. 7 D) and able

481 to drive infection of pseudotyped lentiviral particles (Fig. 7 E). With wt SARS2-S or SARS2-
482 S1/S2-mut, but not with the SARS2-S S2' mutants, directed expression of TMPRSS2 led to
483 low but detectable fusion activity (Figs. 2 A, 6 A). Collectively, these findings identify the S2'
484 site as the primary target of TMPRSS2 for fusion activation.

485 Another observation was that the S2'-AA mutant, as observed in Figs. 1 C and 6 C, exhibited
486 drastically reduced surface expression. In fact, a similar incorporation defect has been
487 described in the literature for SARS-CoV (43). Whatever the reason for this defect, we were
488 able to overcome it completely by replacing the S2' motif KR with the amino acids GH, which
489 restored surface expression (Fig. 6 C) and processing into S1 and S2 (Fig. 6 B), and particle
490 incorporation (Fig. 7 D). The reasons for this phenomenon are unclear. Charge reversal of S2'
491 from KR to EE was definitely detrimental to activity, indicating that solubility may not be the
492 critical point. As histidine may carry a positive charge depending on the local environment,
493 our findings might hint at a requirement for at least one positive charge at this position.

494
495 Our results clearly demonstrate that cleavage at the S1/S2 site alone is not sufficient for
496 fusion activity in the presence of ACE2 and likely requires additional processing at S2' or
497 another site (26). This has been established for particle entry (24), but it was not entirely
498 clear for cell-cell fusion, as the pre-cleaved spike was clearly fusogenic also in conditions
499 without exogenous protease activity in several reports (11, 12, 26, 42), which could have
500 been interpreted as a cell-cell fusion-ready state after S1/S2 cleavage. While our initial
501 attempts to block the fusion activity of wt SARS2-S and the S1/S2 mutant in the presence of
502 ACE2 receptor but without TMPRSS2 were relatively unsuccessful, treatment with the
503 metalloprotease inhibitor Batimastat reduced fusion by both wt (Fig. 5 A) and the S1/S2
504 mutant (Fig 5 C), as well as fusion by the S2' mutant (Fig. 6 E). These findings indicate that
505 metalloproteases can activate SARS2-S, and that this activation occurs at least in part
506 independently of the S1/S2 site and of the S2' site, as both mutants were still Batimastat-
507 sensitive. On the other hand, the S1/S2 mutant was clearly less active in the absence of
508 TMPRSS2, indicating that matrix metalloproteases activate more efficiently when the S1/S2
509 site is present. These findings are in line with a very recent report describing similar
510 observations using different inhibitors (41).

511 As SARS2-S did not require TMPRSS2 on target cells for robust cell-cell fusion, our attempts
512 to test the impact of Bromhexine as a specific inhibitor of TMPRSS2 on SARS2-S-mediated
513 fusion activity were somewhat artificial. Nevertheless, SARS1-S-mediated fusion was clearly
514 enhanced by TMPRSS2, as was fusion by the SARS2-S1/S2-mut mutant, and both were
515 inhibited by Camostat but not by Bromhexine. Therefore, our finding that Bromhexine
516 specifically enhanced fusion of 293T cells in the presence of SARS2-S, ACE2, and TMPRSS2 is
517 something that we cannot explain easily. According to our results the Bromhexine-mediated
518 enhancement was specific for SARS-CoV-2 spike protein and was not seen with VSV-G as
519 fusion effector (Fig. 3 C), nor did we observe significant effects with the SARS2-S mutants
520 and SARS1-S (Fig. 3 A). We observed some inhibition of SARS1-S-mediated fusion in the
521 presence of 50 μ M Ambroxol (Fig. 3 A) and also with SARS2-S with longer incubation times
522 (Fig. 3 C), which may hint at some activity of this substance against TMPRSS2, which would
523 fit with the observation of an atypical autoproteolytic fragment of TMPRSS2 in the presence
524 of Ambroxol. The observation of the paradoxical effect of Bromhexine in the presence of
525 TMPRSS2 suggests that Bromhexine somehow modulates proteolytic processing. It is at the
526 moment not clear by what mechanism of action Bromhexine modulates TMPRSS2 activity, if
527 it does so, and we therefore cannot exclude that processing of some substrates is actually
528 enhanced or altered instead of inhibited, as reported for several substrates (15, 44).

529 Recently, another study also reported lack of inhibitory activity of Bromhexine against
530 TMPRSS2 (30). Activity of Bromhexine against TMPRSS2-mediated receptor shedding, which
531 could also explain our observations, was not observed, in contrast to Camostat that
532 increased ACE2 expression levels in the presence of TMPRSS2 (Fig. 3 B, 5 B). This may explain
533 the slight increase, even if not always statistically significant, in fusion activity that we
534 observed in some experiments with SARS2-S in the presence of Camostat when ACE2 and
535 TMPRSS2 were coexpressed (Figs. 3 A, 5 A).

536 Compared to another study (45), our fusion assay yields slightly different results, with
537 SARS2-S-mediated fusion appearing less dependent on activation by TMPRSS2. This could be
538 due to differences in the protocol. The study by Yamamoto et al. allowed only for very short
539 contact times of 4h and used non-adherent 293T FT cells, whereas we cocultured the cells
540 for a longer time, which allows for extended contact between cells and may enable the
541 action of matrix metalloproteases. Our finding that TMPRSS2 is not required for fusion is in
542 line with several reports making the same observation (11, 24, 26, 41, 42, 46). In general, we
543 observed a higher fusion activity with our SARS-CoV-2 S1/S2 spike mutant than was
544 observed with furin cleavage site mutants in previous studies (12, 26), but we observed this
545 only when TMPRSS2 was recombinantly overexpressed together with ACE2. When only ACE2
546 or only TMPRSS2 were recombinantly expressed, SARS2-S1/S2-mut fusion activity was
547 strongly impaired (Fig. 2 A). It should be noted, that we left the loop intact and only replaced
548 the basic residues with alanine in our mutant whereas other groups deleted the loop
549 structure, which may result in a less flexible conformation. Nevertheless, our mutational
550 approach for ablating the furin cleavage site clearly rendered the spike protein more
551 dependent on additional serine protease activity by recombinantly expressed TMPRSS2. This
552 proteolytic activity was directed towards the S2' site, as SARS2-S1/S2-mut fusion activity was
553 dependent on TMPRSS2 and was significantly inhibited by Camostat (Fig. 3 A) in the
554 presence of TMPRSS2.

555 Taken together, our results actually reconcile several seemingly conflicting observations by
556 other groups. The strong reduction in fusion activity by mutation of the S1/S2 site observed
557 in one study in Vero (12) is reflected in our experimental conditions with only TMPRSS2 and
558 endogenous levels of ACE2 expression, whereas our findings of more or less normal fusion
559 activity under conditions of high-level ACE2 and TMPRSS2 expression are similar to the
560 findings of another group with ACE2 overexpressing cells and addition of Trypsin or HAT
561 (26).

562 Overall, we propose that the dependence on S1/S2 cleavage, activity of TMPRSS2 or a
563 related protease and receptor expression are to a certain degree interdependent, where one
564 factor can at least partially compensate for another, e.g. more extensive proteolytic
565 activation at S2' can render the spike more fusogenic even with lower receptor levels, which
566 was particularly observed for SARS1-S, and to a lesser degree for SARS2-S (Fig. 2). Similarly,
567 Batimastat-sensitive metalloproteases can activate SARS2-S for cell-cell fusion (Fig. 5 A). This
568 is partially dependent on the S1/S2 site, as SARS2-S1/S2-mut was still impaired in the
569 absence of TMPRSS2 but completely independent of the S2' site, as demonstrated by full
570 fusion activity of the SARS2-S2'-GH spike mutant on ACE2 expressing 293T cells (Fig. 6 A).

571 According to our results the requirements for cell-cell fusion and virus-cell fusion differ:
572 Additional TMPRSS2 activity drastically enhanced pseudotype entry into transfected 293T
573 (Fig. 7 A) but was not needed for cell-cell fusion with identically transfected 293T cells (Figs.
574 2 A, 3 A). In addition, the matrix metalloprotease inhibitor Batimastat did not affect particle
575 entry in the presence of the TMPRSS2 inhibitor Camostat, indicating that matrix
576 metalloproteases can activate cell-cell fusion but not particle-cell fusion (Fig. 7 C&E), at least

577 not in our experimental system. Similar observations were previously made for SARS-CoV
578 (34). The interpretation of these results is complicated by the ability of virus particles to
579 enter cells through both direct membrane fusion or through an endocytotic pathway, and by
580 different pre-priming states of viral spike proteins depending on proteolytic activity in the
581 producer cell (47). As activation of the spike protein is expected to differ between organ
582 systems depending on the presence of different proteolytic activities, these processes
583 ultimately need to be studied in appropriate tissue systems or animal models. It is tempting
584 to speculate that the relative to SARS-CoV more relaxed requirements for cell-cell fusion
585 with regard to proteolytic activation contribute to the broad organ tropism and
586 neuroinvasion by SARS-CoV-2, as well as the clinically observed formation of extended
587 syncytia (13). Irrespective of the role of cell-cell fusion in COVID-19, in light of the observed
588 paradoxical activation of cell-cell fusion by Bromhexine and its lack of inhibitory activity
589 against entry of SARS-CoV-2 spike-pseudotyped lentiviruses on TMPRSS2 expressing cells, we
590 would at the moment caution against clinical use of Bromhexine for treatment or
591 prophylaxis of COVID-19, at least at higher concentrations that aim at the inhibition of
592 TMPRSS2. A recent small randomized trial showed promising results for Bromhexine at 3x
593 8 mg per day combined with hydroxychloroquine (48), which should result in Bromhexine
594 plasma concentrations in the range of 0.1 μM (49). We are fairly confident to postulate that
595 these favorable results are unlikely due to inhibition of TMPRSS2, although we can't fully
596 exclude extremely weak activity. This view is supported by a recent study that found no
597 effect of Bromhexine on TMPRSS2 activity (30). More likely, favorable patient outcomes are
598 attributable to the beneficial effects of Bromhexine or its main metabolite Ambroxol on lung
599 function, general defense mechanisms against airway infections, and inflammatory response
600 (16–19, 50). Another recent study by Olaleye et al (51) specifically analyzed the effects of
601 Bromhexine and Ambroxol on the interaction of ACE2 with the SARS-CoV-2 spike receptor
602 binding domain (RBD), and reported a very peculiar behavior of these substances which in
603 part may explain the paradoxical results of our fusion assays and would support a beneficial
604 effect of low-dose Bromhexine, which is converted to Ambroxol in vivo: While Ambroxol
605 weakly inhibited the ACE2-RBD interaction up to 100 μM concentration, Bromhexine
606 exhibited a biphasic behavior and was weakly inhibitory below 10 μM but increased ACE2-
607 RBD binding at higher concentrations in that study. Both substances were reported to
608 weakly inhibit SARS-CoV-2-mediated cytopathic effect in culture (22), and Ambroxol was also
609 shown to moderately impact replication of SARS-CoV-2 in that report (22), albeit on Vero
610 cells and not lung cells. Our results suggest that Ambroxol can weakly inhibit spike-driven
611 entry of lentiviral pseudotypes into Calu-3 cells at high but potentially attainable
612 concentrations (Fig. 8 B), and our experiments with authentic virus on Calu-3 (Fig. 8 D)
613 demonstrated a trend towards inhibition of replication by both Ambroxol and Bromhexine,
614 with Bromhexine possibly being slightly more potent, but also more toxic (Figs. 4 C, 8 E).
615 Thus, the specificity of Bromhexine-mediated inhibition is questionable. In sum, it seems
616 likely that Ambroxol acts weakly on TMPRSS2, which would explain its modest but significant
617 effect on TMPRSS2-mediated activation of SARS1-S-mediated fusion (Fig 3 A). It should be
618 noted that replication of the authentic virus can be influenced at numerous points, not
619 necessarily only during entry, and that effects can be amplified over several replication
620 cycles. Of course, compared to the potency of Camostat, the effect of both substances is
621 marginal. Nevertheless, Ambroxol can be administered in high doses of 1g and more
622 intravenously (19) or orally (52) and reportedly accumulates strongly in lung tissue (53).
623 Thus, Ambroxol, which exhibited a trend towards inhibition of SARS2-Spike-mediated entry
624 and fusion in several assays without enhancing effects as were observed with Bromhexine at

625 high concentrations may represent an interesting option for supportive therapy at higher
626 dosage, in particular as it is a proven therapeutic for antenatal respiratory distress syndrome
627 (54) and has shown efficacy in the treatment of radiation-induced lung injury (50).

628

629 MATERIAL AND METHODS

630

631 Cell Culture

632 All cell lines in this study were incubated at 37°C and 5% CO₂. 293T (a kind gift from Vladan
633 Rankovic and originally purchased from the ATCC, Göttingen) and Calu-3 cells (a kind gift
634 from Stefan Ludwig) were cultured in Dulbecco's Modified Eagle Medium (DMEM), high
635 glucose, GlutaMAX, 25mM HEPES (Thermo Fisher Scientific) supplemented with 10% fetal
636 calf serum (FCS) (Thermo Fisher Scientific), and 50µg/ml gentamycin (PAN Biotech). For Calu-
637 3 cells 1mM Sodium-Pyruvate (Thermo Fisher Scientific) was added. For seeding and sub-
638 culturing of cells the medium was removed, the cells were washed with PBS (PAN-Biotech)
639 and detached with Trypsin (PAN-Biotech). All transfections were performed using PEI
640 (Polysciences) in a 1:3 Ratio (µg DNA/µg PEI) mixed in OptiMEM. The cell viability assay with
641 Calu-3 (Fig. 8 E) was performed as described previously (7); unlike for the other assays in this
642 series of experiments Bromhexine and Ambroxol were used in the form of commercial
643 cough suppressants (Bromo 12mg/ml, Krewel Meuselbach, and Mucosolvan 30mg/5ml,
644 Sanofi-Aventis).

645

646

647 Plasmids

648 Expression plasmids for pQCXIPBL-hTMPRSS2 (55), pCG1-SARS-2-S_humanized (56), pCG1-
649 ACE2 (7) and pCG1-SARS S (57) are described elsewhere. For generation of pVAX1-SARS2-S
650 the codon-optimized sequence encoding the spike protein of SARS-CoV-2 was amplified by
651 PCR and cloned into the pVAX1 backbone. psPAX2 and pMD2.G were a gift from Didier
652 Trono (Addgene plasmid # 12260, Addgene plasmid # 12259) and pLenti CMV GFP Neo (657-
653 2) was a gift from Eric Campeau & Paul Kaufman (Addgene plasmid # 17447). Expression
654 plasmids SARS2-S2'-AA and SARS2-S1/S2-mut were generated from pCG1_SL-Cov_Wuhan-
655 S_humanized SARS2-S by PCR based mutation of the SARS2-S S1/S2 and the S2' cleavage site
656 using around-the-horn PCR mutagenesis using Phusion PCR, T4 PNK and Quick ligase (all
657 from New England Biolabs) and using the following primers: primers S1-S2 AAAA mut for V2
658 (CTGCCTCTGTGGCCAGCCAGAGCATC), S1-S2 AAAA mut rev V2
659 (CAGCGGCGGGGCTGTTTGTCTGTGTCTG), S2 to AA mut_Forward
660 (GCCAGTTCATCGAGGACCTGCTG) and S2 to AA mut_Reverse
661 (AGCGCTGGGCTTGCTAGGATCGG), SARS2S R815 H for (CACAGCTTCATCGAGGACCTGCTG),
662 SARS2S K814H rev (GTGGCTGGGCTTGCTAGGATCGG), SARS2S R815E for
663 (GAGAGCTTCATCGAGGACCTGCTG), SARS2S K814E rev (CTCGCTGGGCTTGCTAGGATCGG),
664 SARS2S R815E for (GAGAGCTTCATCGAGGACCTGCTG), SARS2S R815S for
665 (AGCAGTTCATCGAGGACCTGCTG), SARS2S K814G rev (TCCGCTGGGCTTGCTAGGATCGG).
666 Sequence integrity was verified by sequencing of the coding region. Plasmid pCG1-SARS2-
667 S_S2'mut contains a silent G to T mutation in the codon for leucine 441.
668 Expression plasmids pVAX1-SARS2-S_S2'-GH, pVAX1-SARS2-S1/S2-mut and pVAX1-SARS2-
669 S_D614G were generated from pVAX1-SARS2-S by PCR-based mutation in a similar manner.
670 The Gal4-Luc reporter plasmid encoding firefly luciferase under the control of an activator
671 sequence that binds the Gal4 transcription factor has been described elsewhere (34). The
672 Gal4 DNA binding domain VP16 fusion plasmid corresponds to Genbank identifier X85976.

673 The TurboGFP-Luciferase fusion reporter gene was constructed using Gibson Assembly
674 Master Mix (New England Biolabs) to insert the TurboGFP open reading frame with a Ser-
675 Gly-Ser-Gly Linker in front of the Met codon of the luciferase open reading frame. Before
676 assembly, the two fragments were generated using Phusion PCR (New England Biolabs) by
677 amplifying the TurboGFP open reading frame from the vector pGIPZ (Thermo Scientific Open
678 Biosystems) using the primers TurboGFP for Gal4Luc before ATG ov
679 (GGTACTGTTGGTAAAATGGAGAGCGACGAGAGC) and TurboGFP rev
680 (TTCTTACCGGCATCTGCATC), and the Gal4-Luc backbone by amplification with primer
681 Gal4Luc before ATG rev (TTTACCAACAGTACCGGAATGC) and primer Luc for SGS TurboGFP
682 overhang
683 (GATGCAGATGCCGGTGAAGAAAGCGGTAGCGGTATGGAAGACGCCAAAAACATAAAG).

684 The pLentiCMV-TurboGFP::Luciferase fusion reporter gene was constructed using Gibson
685 Assembly Master Mix (New England Biolabs) to exchange the insert in pLenti-CMV-Blast-
686 EphA7-Strep (described elsewhere (58)) with the TurboGFP::Luc open reading frame without
687 the Strep-Tag, the two fragments were generated using CloneAmp™ HiFi PCR Premix
688 (Takara Bio) by amplifying the TurboGFP::Luc open reading frame from the Vector Gal4-
689 TurboGFP-Luc using the primers GA_TurboGFP::Luc_pLentiBlast-StrepOneOv_For
690 (acaaaaaagcaggctccaccATGGAGAGCGACGAGAGC) and GA_TurboGFP::Luc_pLentiBlast-
691 StrepOneOv_Rev (tgtggatggctccaagcctTTACAATTTGGACTTCCGCC), and the pLenti-CMV-
692 Blast-EphA7-Strep backbone by amplification with primer pLenti attB1 rev at ATG
693 (CATGGTGGAGCCTGCTTTTTGTAC) and OneStrep for (AGCGCTTGAGCCATCCAC).

694
695

696 **Western Blot**

697 Protein expression was analyzed by polyacrylamide gel electrophoresis on 8%-16% gradient
698 precast gradient gels (Thermo) and Western blotting using antibodies to ACE2 (AF933, R&D
699 Systems), c-Myc-epitope (clone 9E10, Santa Cruz Biotechnology), SARS-Spike (NB100-56578,
700 Novus Biologicals), HIV-1 Gag p24 Antibody (clone 749140, R&D), and GAPDH (GenScript) in
701 NETT-G (150 mM NaCl, 5mM EDTA, 50 mM Tris, 0.05% Triton X-100, 0.25% gelatin, pH 7.5)
702 and donkey anti-mouse horseradish peroxidase (HRP)-coupled (Dianova), goat anti-rabbit
703 HRP-coupled (Life Technologies) or rabbit anti-goat HRP-coupled (Proteintech) secondary
704 antibody in 5% dry milk powder in PBS with 0.05% Tween 20. Imaging was performed using
705 Immobilon Forte substrate (Merck) on an INTAS ECL ChemoCam system.

706

707 **Flow cytometry**

708 293T cells were transfected with the respective spike expression constructs. On day two post
709 transfection, the cells were harvested by gentle pipetting in PBS and were fixed in 2%
710 methanol-free formaldehyde in PBS for 15 min. The cells were then washed once in PBS and
711 then incubated in 10% FCS in PBS for 30 min to block non-specific binding. The cells were
712 then incubated in either convalescent serum at 1:1000 dilution or soluble ACE2-Fc fusion
713 protein, both described elsewhere (59), at 2 ng/μl for 1h in 10% FCS in PBS, followed by one
714 wash in a large volume of PBS and then incubation with Alexa647-coupled anti-human
715 secondary antibody (Thermo) at 1:200 in 10% FCS in PBS. The RRV gHΔ21-27-Fc fusion
716 protein, which was used as a control protein, was generated from RRV 26-95 gH-Fc (60) by
717 deletion of the codons for amino acid 21-27, which are important for receptor binding (28),
718 and was produced analogous to the gH-Fc protein in Hahn et al. 2013 (60). The cells were
719 then washed once in a large volume of PBS and post-fixated in 2% PFA in PBS before analysis
720 on an LSRII flow cytometer (BD Biosciences). Data was analyzed using Flowing software

721 (version 2.5) and GraphPad Prism, version 6, for Windows (GraphPad Software). COVID-19
722 convalescent serum was collected previously (59) in accordance with ethical requirements
723 (ethics committee UK Erlangen, license number AZ. 174_20 B).

724

725 **Fusion-Assay**

726 293T target-cells were seeded in a 48-well plate at 50.000 cells/well and transfected with
727 Vp16-Gal4 (Fig. 3 C) or Gal4-TurboGFP-Luciferase expression plasmid (Gal4-TurboGFP-Luc, all
728 other experiments) as well as expression plasmids for ACE2 and TMPRSS2 as indicated. In
729 case only ACE2 or TMPRSS2 were transfected the missing amount of DNA was replaced by
730 empty vector. 293T effector-cells were seeded in a 10 cm dish at 70-80% confluency and
731 transfected with either the Vp16-Gal4 (all experiments except Fig. 3 C) or Gal4-Luciferase
732 (Fig. 3 C) expression plasmid as well as expression plasmids for SARS2-S, SARS2-S2'-AA,
733 SARS2-S2'-GH, SARS2-S2'-HH, SARS2-S2'-EE, SARS2-S2'-ES, SARS1-S, VSV-G glycoproteins or
734 pcDNA6/V5-HisA (Thermo). For effector -cell pre-incubation experiments, the medium of
735 effector-cells was changed to Bromhexine hydrochloride (Merck), Ambroxol hydrochloride
736 (Merck), Camostat mesylate (Tocris), Batimastat (Merck), AEBSF (Merck), EDTA (Merck),
737 EGTA (Merck), 100x Cocktail Set V, Animal-Free - Calbiochem (Merck) or Decanoyl-RVKR-
738 CMK (Merck) containing medium at final concentration 6h after transfection. 24h after
739 transfection, target-cells were pre-incubated with Bromhexine hydrochloride (Merck),
740 Ambroxol hydrochloride (Merck), Camostat mesylate (Tocris), Batimastat (Merck), AEBSF
741 (Merck), EDTA (Merck), EGTA (Merck) or Decanoyl-RVKR-CMK (Merck) for 30 min at twice
742 the indicated concentration. Effector-cells were then added to the target-cells in a 1:1 ratio
743 reaching the final inhibitor concentration. After 24-48h GFP-fluorescence was detected using
744 a Vert.A1 Fluorescence Microscope and ZEN-Software (Zeiss), luciferase activity was
745 analyzed using the PromoKine Firefly luciferase Kit or Beetle-Juice Luciferase Assay according
746 to manufacturer's instructions and a Biotek Synergy 2 plate reader. Statistical analysis was
747 performed using GraphPad Prism, version 6, for Windows (GraphPad Software).

748

749 **Production of lentiviral and pseudoparticles and pseudoparticle infection experiments**

750 Lentiviral pseudoparticles were produced by transfecting 293T cells with expression
751 plasmids for psPAX2, pLenti-CMV-GFP or pLentiCMV-TurboGFP::Luciferase and either SARS2-
752 Spike variants (pVAX1-SARS2-S_S2'-GH, pVAX1-SARS2-S1/S2-mut and pVAX1-SARS2-
753 S_D614G) or VSV-G (pMD2.G Addgene #12259) which was a gift from Didier Trono. The cell
754 culture supernatants were harvested 24-72 h post transfection followed by addition of fresh
755 media and again after 48-72h. The supernatants were passed through a 0.45µm CE-Filter,
756 and the SARS-CoV-2-S pseudoparticles were concentrated via low speed centrifugation at
757 4°C for 16h at 4200xg. For detection of particle incorporation virus supernatant was further
758 concentrated by centrifugation at 4°C for 2h at 21000xg on 5% Optiprep (Merck), the
759 supernatant was removed and pellet was resuspended and addressed to Western Blot
760 analysis. The SARS-CoV-2 spike and VSV-G lentiviral pseudoparticles were used to transduce
761 293T transfected with TMPRSS2 and ACE2 expression plasmids or Calu-3 cells. 48 h after
762 transfection with control or ACE2 and TMPRSS2 expression plasmids, the pseudoparticles
763 were added to the cells pre-incubated with inhibitors Bromhexine hydrochloride (Merck),
764 Ambroxol hydrochloride (Merck), Camostat mesylate (Tocris), Batimastat (Merck), AEBSF
765 (Merck) and E64-d (Biomol) for 30 min at twice the indicated concentration. Cells transduced
766 with pLenti-CMV-GFP pseudoparticles were harvested 48 h after transduction using trypsin.
767 Bald particles from 293T cells that were transfected with empty vector instead of
768 glycoprotein expression plasmids and the lentiviral packaging system were used as

769 background control for normalization. Trypsin activity was inhibited by adding 5% FCS in PBS,
770 and after washing with PBS the cells were fixed with 4% formaldehyde (Roth) in PBS. The
771 percentages of GFP-positive cells were determined using a LSRII flow cytometer, and at least
772 10000 cells were analyzed. Cells transduced with pLentiCMV-TurboGFP::Luciferase
773 pseudoparticles were lysed after 48h with Luciferase-Lysis Buffer (Promega) and detected
774 using Beetle-Juice Luciferase Assay according to manufacturer's instructions and a Biotek
775 Synergy 2 platereader. Statistical analysis was performed using GraphPad Prism 6.

776

777 **SARS-CoV-2 infections**

778 Primary SARS-CoV-2 isolate ER-PR2 was a kind gift from Klaus Überla, Erlangen, and was
779 originally isolated on Vero cells. The virus stock was then grown on Calu-3 cells in EMEM +
780 2% FCS + Penicillin/Streptomycin. Virus containing supernatant was harvested after CPE was
781 clearly visible, and the supernatant was cleared by low-speed centrifugation at 1200 rpm for
782 10min before passage through a 0.2µm syringe filter (Mini-Sart, Sartorius). Virus stocks were
783 aliquoted in 200µl aliquots and stored at -150°C. Infectivity was determined by the method
784 of Reed and Muench (61) at $10^{6.1}$ TCID₅₀/ml. Calu-3 cells were seeded one day (first
785 experiment) or two day (other two experiments) before infection and approx. 100000 Calu-3
786 cells were infected at a multiplicity of infection (MOI) of approximately 0.002 in a 96-well
787 plate in triplicates. The cells were preincubated with the respective inhibitors in 50µl at
788 twice the concentration for ~1.5h, the virus was then added in 50µl medium. Total RNA from
789 the cells and the culture supernatant was harvested 20h (experiment 3) and 24h
790 (experiments 1&2) post infection.

791

792 **RNA isolation, cDNA synthesis, and qRT-PCR**

793 RNA was isolated using the Direct-zol RNA Miniprep Plus Kit (Zymo) according to the
794 manufacturer's instructions. For quantification of viral RNA in infected cultures, the cells and
795 cellular supernatant in a volume of 100µl were lysed and inactivated by addition of 300µl TRI
796 reagent (Zymo). qPCR on viral genomes was performed using the N1 CDC primer set (2019-
797 nCoV_N1-F GACCCCAAATCAGCGAAAT and 2019-nCoV_N1-R
798 TCTGGTACTGCCAGTTGAATCTG, both 500nM, 2019-nCoV_N1-P FAM-ACC CCG CAT TAC GTT
799 TGG TGG ACC-BHQ1, 125nM) and SensiFAST Probe Hi-ROX One-Step Kit (Bioline) according
800 to the manufacturer's instructions in a 20µl reaction with 5µl sample. All PCR reactions were
801 performed in technical duplicates on a StepOne Plus (Thermo) realtime cyler. PCR
802 conditions were 45°C for 10min, 95°C for 2min, and then 45 cycles 95°C for 5sec followed by
803 55°C for 20sec. To determine the PCR efficiency across the whole dynamic range, a 7-step
804 10-fold dilution series with the H₂O-treated SARS-CoV-2-infected Calu-3 sample was
805 performed. These datapoints with the undiluted sample set to 1 was approximated by an
806 exponential function using Microsoft Excel 2020. The measured PCR efficiency was
807 additionally fitted by multiplication with a constant factor to match our RNA standard
808 (Charite, Berlin), which was only available at 50, 500, and 5000 copies, which confirmed our
809 approach, but was not used for relative quantification. Fit was performed by minimizing the
810 sum of the squared relative deviations from the standard concentrations with an exactness
811 of two digits.

812 For quantification of cellular TMPRSS2 and GAPDH expression cDNA synthesis and qPCR
813 were performed according to the manufacturer's instructions using the SensiFast cDNA kit
814 and SensiFAST SYBR qPCR kit (both from Bioline). The qPCR was run on a StepOnePlus
815 realtime PCR cyler (Thermo) and analyzed using the StepOne Software, which was also used
816 to calculate $\Delta\Delta C_t$ values and error estimates for TMPRSS2 expression. TMPRSS2 mRNA was

817 detected using primer set Hs.PT.58.39408998 (IDT) (forward primer
818 GTCAAGGACGAAGACCATGT, reverse primer TGCCAAAGCTTACAGACCAG). GAPDH mRNA was
819 detected using primers GAPDH_Hs-Mm_s (CTTTGGTATCGTGAAGGACTC) and GAPDH_Hs-
820 Mm_as (GTAGAGGCAGGGATGATGTTTC). Amplifications with Ct above 35 and non-matching
821 melting curve were scored as not detected.

822

823

824

825

826 **ACKNOWLEDGEMENTS**

827 We thank Stefan Pöhlmann and Markus Hofmann for sharing reagents and for critical
828 reading of the manuscript and helpful discussions. We thank Klaus Überla for sharing SARS-
829 CoV-2 ER-PR2. We also thank Armin Ensser and Florian Full for helpful discussions.

830

831 **FUNDING**

832 This work was supported by grant HA 6013/4-1 to A.S.H. from the Deutsche
833 Forschungsgemeinschaft and by grant 2019.027.1 to A.S.H. from the Wilhelm-Sander-
834 Stiftung.

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838 **REFERENCES**

839

- 840 1. Zhou P, Yang X-L, Wang X-G, Hu B, Zhang L, Zhang W, Si H-R, Zhu Y, Li B,
841 Huang C-L, Chen H-D, Chen J, Luo Y, Guo H, Jiang R-D, Liu M-Q, Chen Y, Shen X-R,
842 Wang X, Zheng X-S, Zhao K, Chen Q-J, Deng F, Liu L-L, Yan B, Zhan F-X, Wang Y-Y,
843 Xiao G-F, Shi Z-L. 2020. A pneumonia outbreak associated with a new coronavirus of
844 probable bat origin. *Nature* 579:270–273.
- 845 2. Zang R, Gomez Castro MF, McCune BT, Zeng Q, Rothlauf PW, Sonnek NM, Liu Z,
846 Brulois KF, Wang X, Greenberg HB, Diamond MS, Ciorba MA, Whelan SPJ, Ding S. 2020.
847 TMPRSS2 and TMPRSS4 promote SARS-CoV-2 infection of human small intestinal
848 enterocytes. *Sci Immunol* 5.
- 849 3. Bojkova D, McGreig JE, McLaughlin K-M, Masterson SG, Widera M, Krähling V,
850 Ciesek S, Wass MN, Michaelis M, Cinatl J. 2020. SARS-CoV-2 and SARS-CoV differ in
851 their cell tropism and drug sensitivity profiles. *bioRxiv* 2020.04.03.024257.
- 852 4. Hui KPY, Cheung M-C, Perera RAPM, Ng K-C, Bui CHT, Ho JCW, Ng MMT, Kuok
853 DIT, Shih KC, Tsao S-W, Poon LLM, Peiris M, Nicholls JM, Chan MCW. 2020. Tropism,
854 replication competence, and innate immune responses of the coronavirus SARS-CoV-2 in
855 human respiratory tract and conjunctiva: an analysis in ex-vivo and in-vitro cultures. *Lancet*
856 *Respir Med* [https://doi.org/10.1016/S2213-2600\(20\)30193-4](https://doi.org/10.1016/S2213-2600(20)30193-4).
- 857 5. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M,
858 Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. 2003. Angiotensin-converting
859 enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 426:450–454.
- 860 6. Letko M, Marzi A, Munster V. 2020. Functional assessment of cell entry and receptor
861 usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat Microbiol* 5:562–569.
- 862 7. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S,
863 Schiergens TS, Herrler G, Wu N-H, Nitsche A, Müller MA, Drosten C, Pöhlmann S. 2020.
864 SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically
865 Proven Protease Inhibitor. *Cell* <https://doi.org/10.1016/j.cell.2020.02.052>.
- 866 8. Heald-Sargent T, Gallagher T. 2012. Ready, set, fuse! The coronavirus spike protein

- 867 and acquisition of fusion competence. *Viruses* 4:557–580.
- 868 9. Matsuyama S, Nao N, Shirato K, Kawase M, Saito S, Takayama I, Nagata N,
869 Sekizuka T, Katoh H, Kato F, Sakata M, Tahara M, Kutsuna S, Ohmagari N, Kuroda M,
870 Suzuki T, Kageyama T, Takeda M. 2020. Enhanced isolation of SARS-CoV-2 by TMPRSS2-
871 expressing cells. *Proc Natl Acad Sci U S A* 117:7001–7003.
- 872 10. Shulla A, Heald-Sargent T, Subramanya G, Zhao J, Perlman S, Gallagher T. 2011. A
873 transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus
874 receptor and activates virus entry. *J Virol* 85:873–882.
- 875 11. Ou X, Liu Y, Lei X, Li P, Mi D, Ren L, Guo L, Guo R, Chen T, Hu J, Xiang Z, Mu Z,
876 Chen X, Chen J, Hu K, Jin Q, Wang J, Qian Z. 2020. Characterization of spike glycoprotein
877 of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. 1. *Nat*
878 *Commun* 11:1–12.
- 879 12. Hoffmann M, Kleine-Weber H, Pöhlmann S. 2020. A Multibasic Cleavage Site in the
880 Spike Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. *Mol Cell*
881 78:779–784.e5.
- 882 13. Giacca M, Bussani R, Schneider E, Zentilin L, Collesi C, Ali H, Braga L, Secco I,
883 Volpe MC, Colliva A, Zanconati F, Berlot G, Silvestri F, Zacchigna S. 2020. Persistence of
884 viral RNA, widespread thrombosis and abnormal cellular syncytia are hallmarks of COVID-
885 19 lung pathology. *medRxiv* 2020.06.22.20136358.
- 886 14. Ziegler CGK, Allon SJ, Nyquist SK, Mbanjo IM, Miao VN, Tzouanas CN, Cao Y,
887 Yousif AS, Bals J, Hauser BM, Feldman J, Muus C, Wadsworth MH, Kazer SW, Hughes TK,
888 Doran B, Gatter GJ, Vukovic M, Taliaferro F, Mead BE, Guo Z, Wang JP, Gras D, Plaisant
889 M, Ansari M, Angelidis I, Adler H, Sucre JMS, Taylor CJ, Lin B, Waghray A, Mitsialis V,
890 Dwyer DF, Buchheit KM, Boyce JA, Barrett NA, Laidlaw TM, Carroll SL, Colonna L,
891 Tkachev V, Peterson CW, Yu A, Zheng HB, Gideon HP, Winchell CG, Lin PL, Bingle CD,
892 Snapper SB, Kropski JA, Theis FJ, Schiller HB, Zaragosi L-E, Barbry P, Leslie A, Kiem H-P,
893 Flynn JL, Fortune SM, Berger B, Finberg RW, Kean LS, Garber M, Schmidt AG, Lingwood
894 D, Shalek AK, Ordovas-Montanes J, HCA Lung Biological Network. Electronic address:
895 lung-network@humancellatlas.org, HCA Lung Biological Network. 2020. SARS-CoV-2
896 Receptor ACE2 Is an Interferon-Stimulated Gene in Human Airway Epithelial Cells and Is
897 Detected in Specific Cell Subsets across Tissues. *Cell* 181:1016–1035.e19.
- 898 15. Lucas JM, Heinlein C, Kim T, Hernandez SA, Malik MS, True LD, Morrissey C,
899 Corey E, Montgomery B, Mostaghel E, Clegg N, Coleman I, Brown CM, Schneider EL,
900 Craik C, Simon J, Bedalov T, Nelson PS. 2014. The Androgen-Regulated Protease TMPRSS2
901 Activates a Proteolytic Cascade Involving Components of the Tumor Microenvironment and
902 Promotes Prostate Cancer Metastasis. *Cancer Discov* 4:1310–1325.
- 903 16. Renovanz VK. 1975. [Results of some clinical-pharmacological studies on ambroxol
904 (NA 872)]. *Arzneimittelforschung* 25:646–652.
- 905 17. Nobata K, Fujimura M, Ishiura Y, Myou S, Nakao S. 2006. Ambroxol for the
906 prevention of acute upper respiratory disease. *Clin Exp Med* 6:79–83.
- 907 18. Yang B, Yao DF, Ohuchi M, Ide M, Yano M, Okumura Y, Kido H. 2002. Ambroxol
908 suppresses influenza-virus proliferation in the mouse airway by increasing antiviral factor
909 levels. *Eur Respir J* 19:952–958.
- 910 19. Wu X, Li S, Zhang J, Zhang Y, Han L, Deng Q, Wan X. 2014. Meta-analysis of high
911 doses of ambroxol treatment for acute lung injury/acute respiratory distress syndrome based
912 on randomized controlled trials. *J Clin Pharmacol* 54:1199–1206.
- 913 20. Wauer RR, Schmalisch G, Menzel K, Schröder M, Müller K, Tiller R, Methfessel G,
914 Sitka U, Koepke E, Plath C, Schlegel C, Böttcher M, Köppe I, Fricke U, Severin K, Jacobi R,
915 Schmidt W, Hinkel GK, Nitz I, Kunze D, Reichmann G, Lachmann B, Lampe K, Grauel EL.
916 1982. The antenatal use of ambroxol (bromhexine metabolite VIII) to prevent hyaline
917 membrane disease: a controlled double-blind study. *Int J Biol Res Pregnancy* 3:84–91.

- 918 21. Ambroxol Hydrochloride Inhibits the Interaction between Severe Acute Respiratory
919 Syndrome Coronavirus 2 Spike Protein's Receptor Binding Domain and Recombinant Human
920 ACE2 | bioRxiv.
- 921 22. Bradfute SB, Ye C, Clarke EC, Kumar S, Timmins GS, Deretic V. 2020. Ambroxol
922 and Ciprofloxacin Show Activity Against SARS-CoV2 in Vero E6 Cells at Clinically-
923 Relevant Concentrations. bioRxiv 2020.08.11.245100.
- 924 23. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh C-L, Abiona O, Graham BS,
925 McLellan JS. 2020. Cryo-EM structure of the 2019-nCoV spike in the prefusion
926 conformation. *Science* 367:1260–1263.
- 927 24. Hoffmann M, Kleine-Weber H, Pöhlmann S. 2020. A Multibasic Cleavage Site in the
928 Spike Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. *Mol Cell*
929 S1097276520302641.
- 930 25. Zhu Y, Feng F, Hu G, Wang Y, Yu Y, Zhu Y, Xu W, Cai X, Sun Z, Han W, Ye R,
931 Chen H, Ding Q, Cai Q, Qu D, Xie Y, Yuan Z, Zhang R. 2020. The S1/S2 boundary of
932 SARS-CoV-2 spike protein modulates cell entry pathways and transmission. preprint,
933 *Microbiology*.
- 934 26. Xia S, Lan Q, Su S, Wang X, Xu W, Liu Z, Zhu Y, Wang Q, Lu L, Jiang S. 2020. The
935 role of furin cleavage site in SARS-CoV-2 spike protein-mediated membrane fusion in the
936 presence or absence of trypsin. *Signal Transduct Target Ther* 5.
- 937 27. Belouzard S, Chu VC, Whittaker GR. 2009. Activation of the SARS coronavirus spike
938 protein via sequential proteolytic cleavage at two distinct sites. *Proc Natl Acad Sci* 106:5871–
939 5876.
- 940 28. Großkopf AK, Schlagowski S, Ensser A, Desrosiers RC, Hahn AS. 2020. Plxdc family
941 members are novel receptors for the rhesus monkey rhadinovirus (RRV). bioRxiv
942 2020.01.20.912246.
- 943 29. Nimishakavi S, Raymond WW, Gruenert DC, Caughey GH. 2015. Divergent Inhibitor
944 Susceptibility among Airway Lumen-Accessible Tryptic Proteases. *PLOS ONE* 10:e0141169.
- 945 30. Shrimp JH, Kales SC, Sanderson PE, Simeonov A, Shen M, Hall MD. 2020. An
946 Enzymatic TMPRSS2 Assay for Assessment of Clinical Candidates and Discovery of
947 Inhibitors as Potential Treatment of COVID-19. *ACS Pharmacol Transl Sci* 3:997–1007.
- 948 31. Kim IS, Jenni S, Stanifer ML, Roth E, Whelan SPJ, van Oijen AM, Harrison SC.
949 2017. Mechanism of membrane fusion induced by vesicular stomatitis virus G protein. *Proc*
950 *Natl Acad Sci U S A* 114:E28–E36.
- 951 32. Hoffmann M, Wu Y-J, Gerber M, Berger-Rentsch M, Heimrich B, Schwemmler M,
952 Zimmer G. 2010. Fusion-active glycoprotein G mediates the cytotoxicity of vesicular
953 stomatitis virus M mutants lacking host shut-off activity. *J Gen Virol* 91:2782–2793.
- 954 33. Hoffmann D, Bayer W, Wildner O. 2007. Therapeutic immune response induced by
955 intratumoral expression of the fusogenic membrane protein of vesicular stomatitis virus and
956 cytokines encoded by adenoviral vectors. *Int J Mol Med* 20:673–681.
- 957 34. Simmons G, Bertram S, Glowacka I, Steffen I, Chaipan C, Agudelo J, Lu K,
958 Rennekamp AJ, Hofmann H, Bates P, Pöhlmann S. 2011. Different host cell proteases
959 activate the SARS-coronavirus spike-protein for cell-cell and virus-cell fusion. *Virology*
960 413:265–274.
- 961 35. Jaimes JA, Millet JK, Whittaker GR. 2020. Proteolytic Cleavage of the SARS-CoV-2
962 Spike Protein and the Role of the Novel S1/S2 Site. *iScience* 23:101212.
- 963 36. Davies B, Brown PD, East N, Crimmin MJ, Balkwill FR. 1993. A synthetic matrix
964 metalloproteinase inhibitor decreases tumor burden and prolongs survival of mice bearing
965 human ovarian carcinoma xenografts. *Cancer Res* 53:2087–2091.
- 966 37. Wojtowicz-Praga SM, Dickson RB, Hawkins MJ. 1997. Matrix metalloproteinase
967 inhibitors. *Invest New Drugs* 15:61–75.
- 968 38. Hou YJ, Chiba S, Halfmann P, Ehre C, Kuroda M, Dinnon KH, Leist SR, Schäfer A,

- 969 Nakajima N, Takahashi K, Lee RE, Mascenik TM, Graham R, Edwards CE, Tse LV, Okuda
970 K, Markmann AJ, Bartelt L, de Silva A, Margolis DM, Boucher RC, Randell SH, Suzuki T,
971 Gralinski LE, Kawaoka Y, Baric RS. 2020. SARS-CoV-2 D614G variant exhibits efficient
972 replication ex vivo and transmission in vivo. *Science* <https://doi.org/10.1126/science.abe8499>.
973 39. Fois G, Hobi N, Felder E, Ziegler A, Miklavc P, Walther P, Radermacher P, Haller T,
974 Dietl P. 2015. A new role for an old drug: Ambroxol triggers lysosomal exocytosis via pH-
975 dependent Ca^{2+} release from acidic Ca^{2+} stores. *Cell Calcium* 58:628–637.
976 40. Takeda H, Misawa M, Yanaura S. 1983. A role of lysosomal enzymes in the
977 mechanism of mucolytic action of bromhexine. *Jpn J Pharmacol* 33:455–461.
978 41. Nguyen HT, Zhang S, Wang Q, Anang S, Wang J, Ding H, Kappes JC, Sodroski J.
979 2020. Spike glycoprotein and host cell determinants of SARS-CoV-2 entry and cytopathic
980 effects. *J Virol* <https://doi.org/10.1128/JVI.02304-20>.
981 42. Buchrieser J, Dufloo J, Hubert M, Monel B, Planas D, Rajah MM, Planchais C, Porrot
982 F, Guivel-Benhassine F, Van der Werf S, Casartelli N, Mouquet H, Bruel T, Schwartz O.
983 2020. Syncytia formation by SARS-CoV-2-infected cells. *EMBO J* 39:e106267.
984 43. Belouzard S, Chu VC, Whittaker GR. 2009. Activation of the SARS coronavirus spike
985 protein via sequential proteolytic cleavage at two distinct sites. *Proc Natl Acad Sci* 106:5871–
986 5876.
987 44. Azouz NP, Klingler AM, Rothenberg ME. 2020. Alpha 1 Antitrypsin is an Inhibitor of
988 the SARS-CoV2–Priming Protease TMPRSS2. *bioRxiv* 2020.05.04.077826.
989 45. Yamamoto M, Kiso M, Sakai-Tagawa Y, Iwatsuki-Horimoto K, Imai M, Takeda M,
990 Kinoshita N, Ohmagari N, Gohda J, Semba K, Matsuda Z, Kawaguchi Y, Kawaoka Y, Inoue
991 J. 2020. The anticoagulant nafamostat potently inhibits SARS-CoV-2 infection in vitro: an
992 existing drug with multiple possible therapeutic effects. *bioRxiv* 2020.04.22.054981.
993 46. Liu S, Selvaraj P, Lien CZ, Wu WW, Chou C-K, Wang TT. 2020. The PRRA insert at
994 the S1/S2 site modulates cellular tropism of SARS-CoV-2 and ACE2 usage by the closely
995 related Bat raTG13. *bioRxiv* 2020.07.20.213280.
996 47. Shang J, Wan Y, Luo C, Ye G, Geng Q, Auerbach A, Li F. 2020. Cell entry
997 mechanisms of SARS-CoV-2. *Proc Natl Acad Sci U S A*
998 <https://doi.org/10.1073/pnas.2003138117>.
999 48. Ansarin K, Tolouian R, Ardalan M, Taghizadieh A, Varshochi M, Teimouri S, Vaezi
1000 T, Valizadeh H, Saleh P, Safiri S, Chapman KR. 2020. Effect of bromhexine on clinical
1001 outcomes and mortality in COVID-19 patients: A randomized clinical trial. *BioImpacts BI*
1002 10:209–215.
1003 49. Bechgaard E, Nielsen A. 1982. Bioavailability of bromhexine tablets and preliminary
1004 pharmacokinetics in humans. *Biopharm Drug Dispos* 3:337–344.
1005 50. Xia D-H, Xi L, Xu C, Mao W-D, Shen W-S, Shu Z-Q, Yang H-Z, Dai M. 2010. The
1006 protective effects of ambroxol on radiation lung injury and influence on production of
1007 transforming growth factor beta1 and tumor necrosis factor alpha. *Med Oncol Northwood*
1008 *Lond Engl* 27:697–701.
1009 51. Olaleye OA, Kaur M, Onyenaka CC. 2020. Ambroxol Hydrochloride Inhibits the
1010 Interaction between Severe Acute Respiratory Syndrome Coronavirus 2 Spike Protein’s
1011 Receptor Binding Domain and Recombinant Human ACE2. *bioRxiv* 2020.09.13.295691.
1012 52. Mullin S, Smith L, Lee K, D’Souza G, Woodgate P, Elflein J, Hällqvist J, Toffoli M,
1013 Streeter A, Hosking J, Heywood WE, Khengar R, Campbell P, Hehir J, Cable S, Mills K,
1014 Zetterberg H, Limousin P, Libri V, Foltynie T, Schapira AHV. 2020. Ambroxol for the
1015 Treatment of Patients With Parkinson Disease With and Without Glucocerebrosidase Gene
1016 Mutations: A Nonrandomized, Noncontrolled Trial. *JAMA Neurol* 77:427–434.
1017 53. Li Q, Yao G, Zhu X. 2012. High-dose Ambroxol Reduces Pulmonary Complications
1018 in Patients with Acute Cervical Spinal Cord Injury After Surgery. *Neurocrit Care* 16:267–272.
1019 54. Zhang H, Liu J, Liu T, Wang Y, Dai W. 2018. Antenatal maternal medication

- 1020 administration in preventing respiratory distress syndrome of premature infants: A network
1021 meta-analysis. *Clin Respir J* 12:2480–2490.
- 1022 55. Kleine-Weber H, Elzayat MT, Hoffmann M, Pöhlmann S. 2018. Functional analysis
1023 of potential cleavage sites in the MERS-coronavirus spike protein. *Sci Rep* 8:16597.
- 1024 56. Hoffmann M. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is
1025 Blocked by a Clinically Proven Protease Inhibitor 19.
- 1026 57. Hoffmann M, Müller MA, Drexler JF, Glende J, Erdt M, Gützkow T, Losemann C,
1027 Binger T, Deng H, Schwegmann-Weßels C, Esser K-H, Drosten C, Herrler G. 2013.
1028 Differential Sensitivity of Bat Cells to Infection by Enveloped RNA Viruses: Coronaviruses,
1029 Paramyxoviruses, Filoviruses, and Influenza Viruses. *PLoS ONE* 8:e72942.
- 1030 58. Großkopf AK, Schlagowski S, Hörnich BF, Fricke T, Desrosiers RC, Hahn AS. 2019.
1031 EphA7 functions as receptor on BJAB cells for cell-to-cell transmission of the Kaposi's
1032 sarcoma-associated herpesvirus (KSHV) and for cell-free infection by the related rhesus
1033 monkey rhadinovirus (RRV). *J Virol* <https://doi.org/10.1128/JVI.00064-19>.
- 1034 59. Lapuente D, Maier C, Irrgang P, Hübner J, Peter AS, Hoffmann M, Ensser A, Ziegler
1035 K, Winkler TH, Birkholz T, Kremer AE, Steininger P, Korn K, Neipel F, Überla K, Tenbusch
1036 M. 2020. Rapid response flow cytometric assay for the detection of antibody responses to
1037 SARS-CoV-2. *Eur J Clin Microbiol Infect Dis Off Publ Eur Soc Clin Microbiol*
1038 <https://doi.org/10.1007/s10096-020-04072-7>.
- 1039 60. Hahn AS, Desrosiers RC. 2013. Rhesus Monkey Rhadinovirus Uses Eph Family
1040 Receptors for Entry into B Cells and Endothelial Cells but Not Fibroblasts. *PLoS Pathog*
1041 9:e1003360.
- 1042 61. REED LJ, MUENCH H. 1938. A SIMPLE METHOD OF ESTIMATING FIFTY PER
1043 CENT ENDPOINTS12. *Am J Epidemiol* 27:493–497.

1044
1045
1046

1047 FIGURE LEGENDS

1048

1049 **Figure 1: SARS2-S mediates robust fusion activity in the presence of ACE2 or ACE2 and**
1050 **TMPRSS2 on target cells, and ablation of the S1/S2 or S2' proteolytic cleavage site affects**
1051 **fusion activity differently.**

1052

1053 **A** Schematic illustration of the coronavirus spike protein showing the signal peptide (SP), the
1054 receptor binding domain (RBD), the fusion peptide (FP), the transmembrane domain (TM),
1055 the S1/S2 cleavage site (S1/S2) and the S2 cleavage site (S2'), together with amino acid
1056 sequence alignments of the spike proteins of SARS-CoV-2, SARS-CoV, and the SARS-2
1057 cleavage site mutants analyzed in this study (not exactly drawn to scale).

1058

1059 **B** Expression of analyzed spike variants in 293T cells. The unprocessed spike (S0) and the
1060 S1/S2-site processed (S2) spike are indicated by arrows. The expression of GAPDH served as
1061 loading control.

1062

1063 **C** Cell surface expression and ACE2 binding. Cell surface expression as measured by antibody
1064 binding from a COVID-19 convalescent serum and binding of soluble ACE2-Fc to 293T cells
1065 expressing the indicated spike proteins was determined via flow cytometry analysis and
1066 detection with an Alexa647-coupled secondary antibody to human IgG. The percentages of
1067 Alexa647-positive cells are shown.

1068

1069 **D** Cell-cell fusion-assay: Effector cells (293T transfected with either empty vector or
1070 expression plasmids for the indicated spike variants and Vp16-Gal4 transactivator) were
1071 cocultured together with target cells (293T transfected with empty vector or ACE2/TMPRSS2
1072 expression plasmids and Gal4-TurboGFP-Luc reporter plasmid). After 24h luciferase activity

1069 was measured. The data shows averaged relative luminescence units, error bars represent
1070 the standard deviations of one representative experiment performed in triplicates.
1071 **E** Experiment as shown in **D**, except that only ACE2/TMPRSS2 target cells were analyzed.
1072 After 12h, 18h and 24h luciferase activity was measured. The data shows averaged fusion
1073 activity normalized to empty vector transfected effector cells, error bars represent the
1074 standard deviations of one representative experiment performed in triplicates.
1075 **F** Representative GFP fluorescence microscopy images of ACE2 and ACE2/TMPRSS2
1076 expressing cells from a cell-cell fusion assay (200 μ m scale bar).
1077 Statistical significance in **C**, **D** and **E** was determined by Two-Way ANOVA, p-values were
1078 corrected for multiple comparisons by Sidak's method ($p > 0.05$, ns; $p \leq 0.05$, *; $p \leq 0.01$, **;
1079 $p \leq 0.001$, ***; $p \leq 0.0001$, ****).

1080

1081 **Figure 2: SARS2-S-mediated cell-cell fusion depends on ACE2 receptor expression whereas**
1082 **SARS1-S-mediated fusion depends on TMPRSS2 activity in 293T cells.**

1083 **A** Cell-Cell Fusion-Assay: Effector cells (293T transfected with either empty vector or
1084 expression plasmids for the indicated spike variants and Vp16-Gal4 transactivator) were
1085 cocultured together with target cells (293T transfected with ACE2 or TMPRSS2 expression
1086 plasmids at the indicated ratios and Gal4-TurboGFP-Luc reporter plasmid). After 24h
1087 luciferase activity was measured. The data shows averaged relative luminescence units,
1088 error bars represent the standard deviations of one representative experiment performed in
1089 triplicates. Comparisons were made against the condition with maximum activation using
1090 Two-Way ANOVA, p-values were corrected for multiple comparisons by Sidak's method
1091 ($p > 0.05$, ns; $p \leq 0.05$, *; $p \leq 0.01$, **; $p \leq 0.001$, ***; $p \leq 0.0001$, ****).

1092 **B** The expression of proteins in target cells and effector cells after co-cultivation was
1093 analyzed by Western blot from lysates harvested for determination of luciferase activity in **A**.
1094 The unprocessed Spike (S0) and the S1/S2-site processed (S2) Spike are indicated by arrows.
1095 An additional cleavage product marked with * was observed. The predominant, processed
1096 low molecular weight TMPRSS2 fragment is shown. The expression of GAPDH served as
1097 loading control. One representative Western blot is shown.

1098

1099

1100 **Figure 3: SARS2-S mediated cell-cell Fusion of 293T cells is enhanced by Bromhexine in the**
1101 **presence of TMPRSS2.**

1102 **A** Cell-cell fusion-Assay: Effector cells (293T transfected with either empty vector or
1103 expression plasmids for the indicated spike variants together with Vp16-Gal4 expression
1104 plasmid) were added to target cells (293T transfected with empty vector, expression
1105 plasmids for ACE2, TMPRSS2 alone or in combination and Gal4-TurboGFP-Luc reporter
1106 plasmid), which had been pre-incubated for 30 min with Bromhexine, Ambroxol or
1107 Camostat. After addition of effector cells, effector and target cells were cocultured in the
1108 presence of the respective inhibitors at 50 μ M. After 24h luciferase activity was measured.
1109 The data shows averaged relative luminescence units and the error bars represent the
1110 standard error of the mean of four independent experiments, each performed in triplicates.
1111 Statistical significance was determined by Two-Way ANOVA, p-values were corrected for
1112 multiple comparisons by Sidak's method ($p > 0.05$, ns; $p \leq 0.05$, *; $p \leq 0.01$, **; $p \leq 0.001$, ***;
1113 $p \leq 0.0001$, ****). For the comparison between inhibitor treatments, the three comparisons
1114 within each family were corrected for. The p-values for comparisons between different H2O
1115 (control) treated target cell populations were corrected for multiple comparison of each

1116 target cell and effector cell combination in the inhibitor group (in total 190 possible
1117 comparisons).

1118 **B** The expression of proteins in treated target cells and effector cells after co-cultivation was
1119 analyzed by Western blot from lysates harvested for determination of luciferase activity
1120 shown in **A**. The unprocessed Spike (S0) and the S1/S2-site processed (S2) Spike are
1121 indicated by arrows. An additional cleavage product marked with * was observed. The
1122 predominant, processed low molecular weight TMPRSS2 fragment is shown. The expression
1123 of GAPDH served as loading control. One representative Western blot is shown. ev = empty
1124 vector.

1125 **C** Cell-Cell Fusion-Assay: Effector cells (293T cells transfected with either empty vector or the
1126 indicated glycoprotein expression plasmids and Gal4-Luc reporter plasmid) were cocultured
1127 with target cells (293T transfected with empty vector or ACE2 and TMPRSS2 expression
1128 plasmids and Vp16-Gal4 expression plasmid) that were pre-incubated for 30 min with
1129 Bromhexine or Ambroxol. After addition of effector cells, effector and target cells were
1130 cocultured with inhibitors at indicated concentrations, and luciferase activity of cell lysates
1131 was measured after 48h. Data shows averaged relative luminescence units of one
1132 experiment performed in triplicates, error bars represent the standard deviation.

1133

1134 **Figure 4: Sensitivity of SARS2-S-mediated cell-cell fusion 293T to different inhibitors.**

1135 **A** Cell-cell fusion-assay: Effector cells (293T transfected with expression plasmids for the
1136 indicated spike variants together with Vp16-Gal4 expression plasmid) were added to target
1137 cells (293T transfected with expression plasmids for ACE2 and Gal4-TurboGFP-Luc reporter
1138 plasmid), which had been pre-incubated for 30 min with twice the final concentration of
1139 AEBSF (200 μ M), furin-Inhibitor CMK (10 μ M), proteinase inhibitor cocktail, EDTA/EGTA
1140 (2.5mM each), Bromhexine (50 μ M), Ambroxol (50 μ M) and Camostat (50 μ M). After addition
1141 of effector cells, effector and target cells were cocultured in the presence of the respective
1142 inhibitors. After 24h luciferase activity was measured. The data shows values normalized to
1143 solvent treatment which was set to 100 and the error bars represent the standard deviation
1144 of three independent experiments, each performed in triplicates.

1145 **B** Cell-cell-fusion assay as shown in **A**, except that effector cells were pre-incubated with
1146 indicated inhibitors for 16h before co-culture with target cells. The target cells were pre-
1147 incubated with indicated inhibitors for 30min before addition of effector cells. After 24h
1148 luciferase activity was measured. The data shows values normalized to solvent treatment
1149 which was set to 100 and the error bars represent the standard deviation of three
1150 independent experiments, each performed in triplicates.

1151 **C** 293T cells transfected with Vp16-Gal4 and Gal4-TurboGFP-Luc reporter expression
1152 plasmids and were incubated with inhibitors similar as described in **A**. After 24h luciferase
1153 activity was measured. The data shows averaged relative luminescence units, error bars
1154 represent the standard deviations of one representative experiment performed in
1155 triplicates.

1156 **D** The expression of proteins in treated target cells and effector cells after co-cultivation was
1157 analyzed by Western blot from lysates harvested for determination of luciferase activity
1158 shown in **A**. The unprocessed spike (S0) and the S1/S2-site processed (S2) spike are indicated
1159 by arrows. An additional cleavage product marked with * was observed. The predominant,
1160 processed low molecular weight TMPRSS2 fragment is shown. The expression of GAPDH
1161 served as loading control. One representative Western blot is shown.

1162

1163 **Figure 5 The matrix metalloproteinase inhibitor Batimastat inhibits SARS2-S-mediated cell-**
1164 **cell fusion.**

1165 **A** Cell-cell fusion assay: Effector cells (293T transfected with expression plasmids for the
1166 indicated spike variants together with Vp16-Gal4 expression plasmid) were added to target
1167 cells (293T transfected with expression plasmids for ACE2, ACE2/TMPRSS2, TMPRSS2 and
1168 Gal4-TurboGFP-Luc reporter plasmid), which had been pre-incubated with Batimastat or
1169 Camostat for 30 min at twice the indicated final concentration. After 24h luciferase activity
1170 was measured. The data shows averaged relative luminescence units, error bars represent
1171 the standard deviations of one representative experiment performed in triplicates.
1172 Statistical significance in **A**, **C** and **F** was determined by Two-Way ANOVA, p-values were
1173 corrected for multiple comparisons by Sidak's method ($p > 0.05$, ns; $p \leq 0.05$, *; $p \leq 0.01$, **;
1174 $p \leq 0.001$, ***; $p \leq 0.0001$, ****).

1175 **B** The expression of proteins in treated target cells and effector cells after co-cultivation was
1176 analyzed by Western blot from lysates harvested for determination of luciferase activity
1177 shown in **A**. The unprocessed Spike (S0) and the S1/S2-site processed (S2) Spike are
1178 indicated by arrows. An additional cleavage product marked with * was observed. The
1179 predominant, processed low molecular weight TMPRSS2 fragment is shown. The expression
1180 of GAPDH served as loading control. One representative Western blot is shown. ev = empty
1181 vector.

1182 **C** Cell-Cell Fusion-Assay: Effector cells (293T transfected with expression plasmids for the
1183 indicated spike variants together with Vp16-Gal4 expression plasmid) were added to target
1184 cells (293T transfected with expression plasmids for ACE2, ACE2/TMPRSS2, TMPRSS2 and
1185 Gal4-TurboGFP-Luc reporter plasmid), which had been pre-incubated for 30 min with
1186 Batimastat (10 μ M) and/or Camostat (50 μ M) at twice the final concentration. After 24h
1187 luciferase activity was measured. The data shows values normalized to solvent treatment
1188 which was set to 100 and the error bars represent the standard deviation of three
1189 independent experiments, each performed in triplicates. Statistical significance was
1190 determined by Two-Way ANOVA, p-values were corrected for multiple comparisons by
1191 Sidak's method ($p > 0.05$, ns; $p \leq 0.05$, *; $p \leq 0.01$, **; $p \leq 0.001$, ***; $p \leq 0.0001$, ****).

1192

1193 **Figure 6: The conserved S2'-site is the site of TMPRSS2-mediated activation of SARS2-S for**
1194 **cell-cell fusion.**

1195 **A** Cell-cell fusion-assay: Effector cells (293T transfected with expression plasmids for the
1196 indicated spike variants together with Vp16-Gal4 expression plasmid) were added to target
1197 cells (293T transfected with expression plasmids for ACE2, ACE2/TMPRSS2, TMPRSS2 and
1198 Gal4-TurboGFP-Luc reporter plasmid). After 24h luciferase activity was measured. The data
1199 shows values fold empty vector control and the error bars represent the standard deviation
1200 of three independent experiments, each performed in triplicates.

1201 **B** Expression of analyzed spike variants in 293T cells. The unprocessed spike (S0) and the
1202 S1/S2-site processed (S2) spike are indicated by arrows. The expression of GAPDH served as
1203 loading control.

1204 **C** Cell surface expression and ACE binding. Cell surface expression and binding of soluble
1205 ACE2-Fc by the indicated spike variants was determined by flow cytometry. Analysis was
1206 performed as in Fig. 1 C.

1207 **D** Cell-cell fusion assay: Effector cells (293T transfected with expression plasmids for the
1208 indicated spike variants together with Vp16-Gal4 expression plasmid) were pre-incubated
1209 with furin-Inhibitor CMK (10 μ M) and after 16h added to target cells (293T transfected with
1210 expression plasmids for ACE2 and Gal4-TurboGFP-Luc reporter plasmid), which had been

1211 pre-incubated for 30 min with the same inhibitor concentration. After addition of effector
1212 cells, effector and target cells were cocultured in the presence of CMK. After 24h luciferase
1213 activity was measured. The data shows values normalized to solvent treatment which was
1214 set to 100 and the error bars represent the standard deviation of two independent
1215 experiments, each performed in triplicates.

1216 **E** Cell-cell fusion assay: Effector cells (293T transfected with expression plasmids for the
1217 indicated spike variants together with Vp16-Gal4 expression plasmid) were added to target
1218 cells (293T transfected with expression plasmids for ACE2, ACE2/TMPRSS2, TMPRSS2 and
1219 Gal4-TurboGFP-Luc reporter plasmid), which had been pre-incubated with Batimastat
1220 and/or Camostat for 30 min at twice the final concentration; final concentrations were
1221 Batimastat 10 μ M and/or Camostat 50 μ M. After 24h luciferase activity was measured. The
1222 data shows values normalized to solvent treatment which was set to 100 and the error bars
1223 represent the standard deviation of two independent experiments, each performed in
1224 triplicates.

1225 Statistical significance in **A**, **C**, **D** and **E** was determined by Two-Way ANOVA, p-values were
1226 corrected for multiple comparisons by Sidak's method ($p > 0.05$, ns; $p \leq 0.05$, *; $p \leq 0.01$, **;
1227 $p \leq 0.001$, ***; $p \leq 0.0001$, ****).

1228

1229 **Figure 7: Requirements for the entry of SARS2-S pseudotyped lentiviral particles differ**
1230 **from SARS2-S-mediated cell-cell fusion.**

1231 **A** 293T cells transfected with empty vector, ACE2/TMPRSS2, ACE2 or TMPRSS2 were pre-
1232 incubated with Bromhexine, Ambroxol or Camostat at indicated concentration before
1233 addition of lentiviral particles pseudotyped with SARS2-Spike. 48h after transduction the
1234 cells were analysed via flow-cytometry. Data shows averaged percent of GFP-positive cells,
1235 error bars represent the standard deviations of one representative experiment performed in
1236 triplicates. **B** Images of ACE2 and TMPRSS2 transfected cells that were infected with the
1237 respective lentiviral pseudotype particles.

1238 **C** 293T cells transfected with ACE2/TMPRSS2 were pre-incubated with Batimastat (10 μ M),
1239 Bromhexine (50 μ M), Ambroxol (50 μ M), AEBSF (200 μ M), Camostat (50 μ M) or Batimastat
1240 (10 μ M) in combination with Camostat (50 μ M) before addition of lentiviral particles
1241 pseudotyped with respective glycoprotein. 48h after transduction the cells were lysed and
1242 luciferase activity was determined. Data shows log-fold change over background, error bars
1243 represent the standard deviations of three independent experiments, each performed in
1244 triplicates, raw values were log₁₀-transformed before analysis.

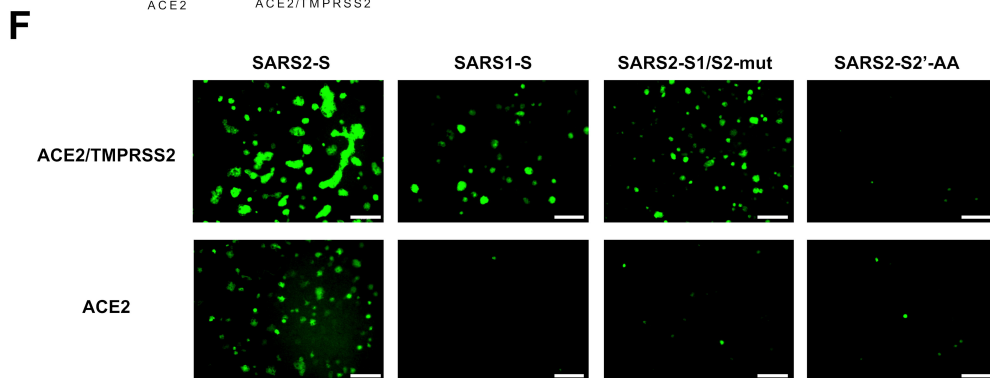
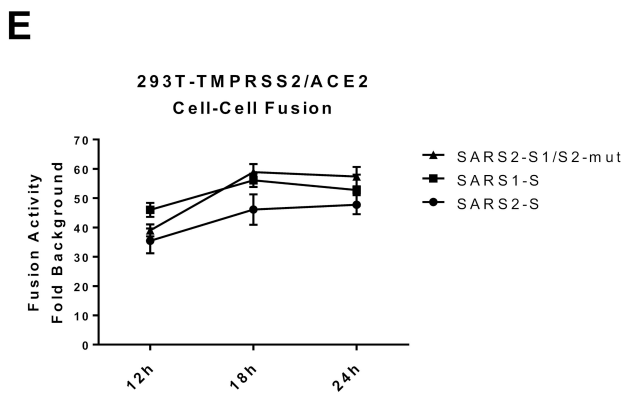
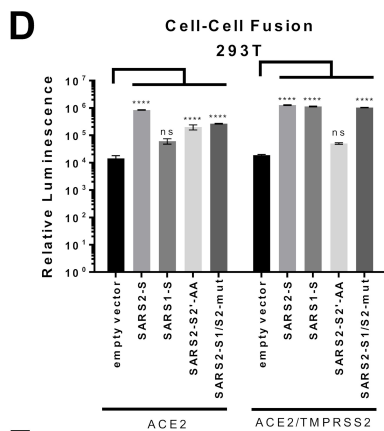
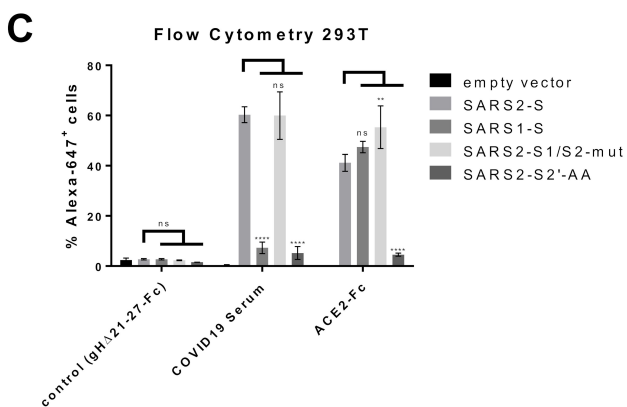
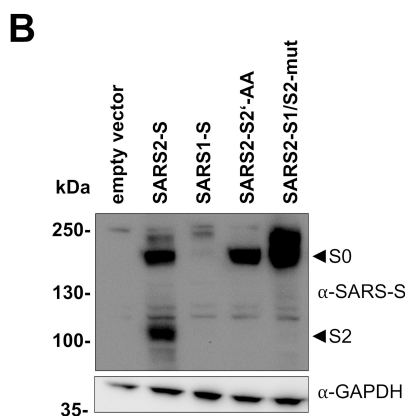
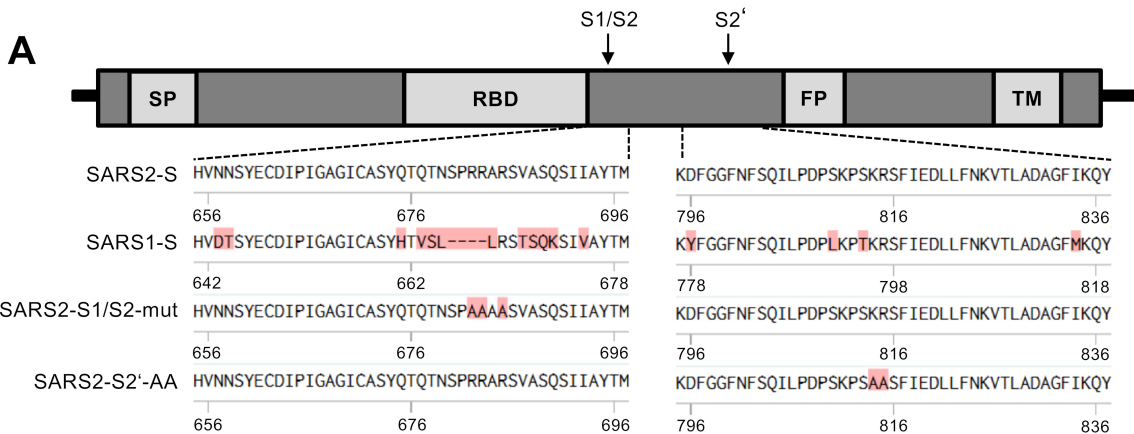
1245 **D** Western Blot analysis of incorporation of the respective spike variants into lentiviral
1246 particles used in **E** and lysate control of transfected 293T cells used for production of
1247 lentiviral particles. p24 and GAPDH served as loading control.

1248 **E** 293T cells transfected with ACE2/TMPRSS2 were pre-incubated with Batimastat (10 μ M),
1249 E64-d (25 μ M), Camostat (50 μ M) or Batimastat (10 μ M)/ E64-d (10 μ M) in combination with
1250 Camostat (50 μ M) before addition of lentiviral particles pseudotyped with the respective
1251 glycoproteins. 48h after transduction the cells were lysed and luciferase activity was
1252 determined. Data shows log-fold change over background, error bars represent the standard
1253 deviations of two independent experiments, each performed in triplicates, raw values were
1254 log₁₀-transformed before analysis. Statistical significance in **A**, **C** and **E** was determined by
1255 Two-Way ANOVA, p-values were corrected for multiple comparisons by Sidak's method
1256 method ($p > 0.05$, ns; $p \leq 0.05$, *; $p \leq 0.01$, **; $p \leq 0.001$, ***; $p \leq 0.0001$, ****).

1257

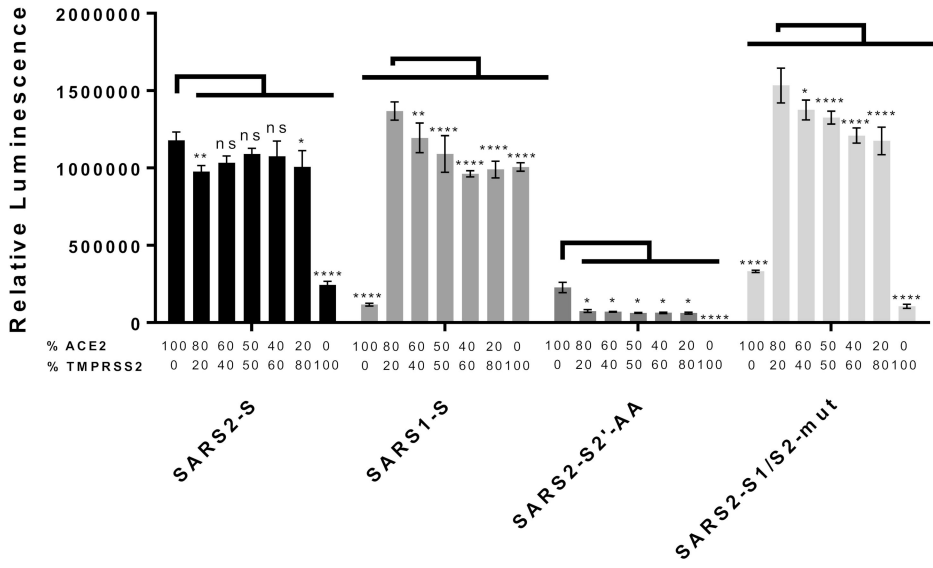
1258 **Figure 8: SARS-CoV-2 is weakly inhibited by Bromhexine and Ambroxol on Calu-3 cells.**

1259 **A** RT-qPCR analysis of TMPRSS2 expression. Fold TMPRSS2 mRNA expression in Calu-3 cells,
1260 293T cells, and A549 cells was measured by RT-qPCR using the $\Delta\Delta C_t$ method. The -RT control
1261 with C_t 30.33 for the GAPDH mRNA in Calu-3 was considered irrelevant as it was more than
1262 19 cycles over the value in the sample, representing a contamination of less than 0.01%.
1263 **B** Calu-3 cells were infected with lentiviral particles encoding a TurboGFP-luciferase
1264 reporter gene pseudotyped with SARS2-Spike in the presence of 50 μ M Bromhexine,
1265 Ambroxol or Camostat. 48h after transduction the cells were lysed and luciferase activity
1266 was determined. The data shows values normalized to solvent treatment, which was set to
1267 100, and the error bars represent the standard deviation of three independent experiments,
1268 each performed in triplicates.
1269 **C** Viral RNA load. Calu-3 cells were infected with SARS-CoV-2 in the presence of Bromhexine,
1270 Ambroxol, Camostat, or Batimastat at the indicated concentrations. Viral RNA was
1271 quantified by RT-qPCR 24h (experiment 1 and 2) and 20h (experiment 3) post infection. The
1272 median C_t values of three experiments (each experiment was performed in biological
1273 triplicates) are plotted (experiment 1: dots, experiment 2: triangles, experiment 3: squares,
1274 and the mean was determined. Significant differences to solvent controls are indicated by
1275 asterisks. Significance was determined using repeated measures one way ANOVA and
1276 Fisher's LSD without correcting for multiple comparisons. Differences were also significant
1277 using two way ANOVA without correction for multiple comparisons and all available data,
1278 but use of the median from each experiment significantly reduced variance. All samples
1279 were compared to water except for Batimastat, which was compared to DMSO.
1280 **D** Relative viral RNA expression. Using the median C_t values from each experiment series as
1281 above and the experimentally determined PCR efficiency, the amount of viral RNA was
1282 calculated as percent of solvent control for each inhibitor.
1283 **E** Cell viability. Cell viability of Calu-3 was determined after culture in the presence of the
1284 indicated compounds in two independent assays, each performed in biological triplicates.
1285 Statistical significance was determined using Two-Way ANOVA, only significant reductions in
1286 viability are shown.
1287

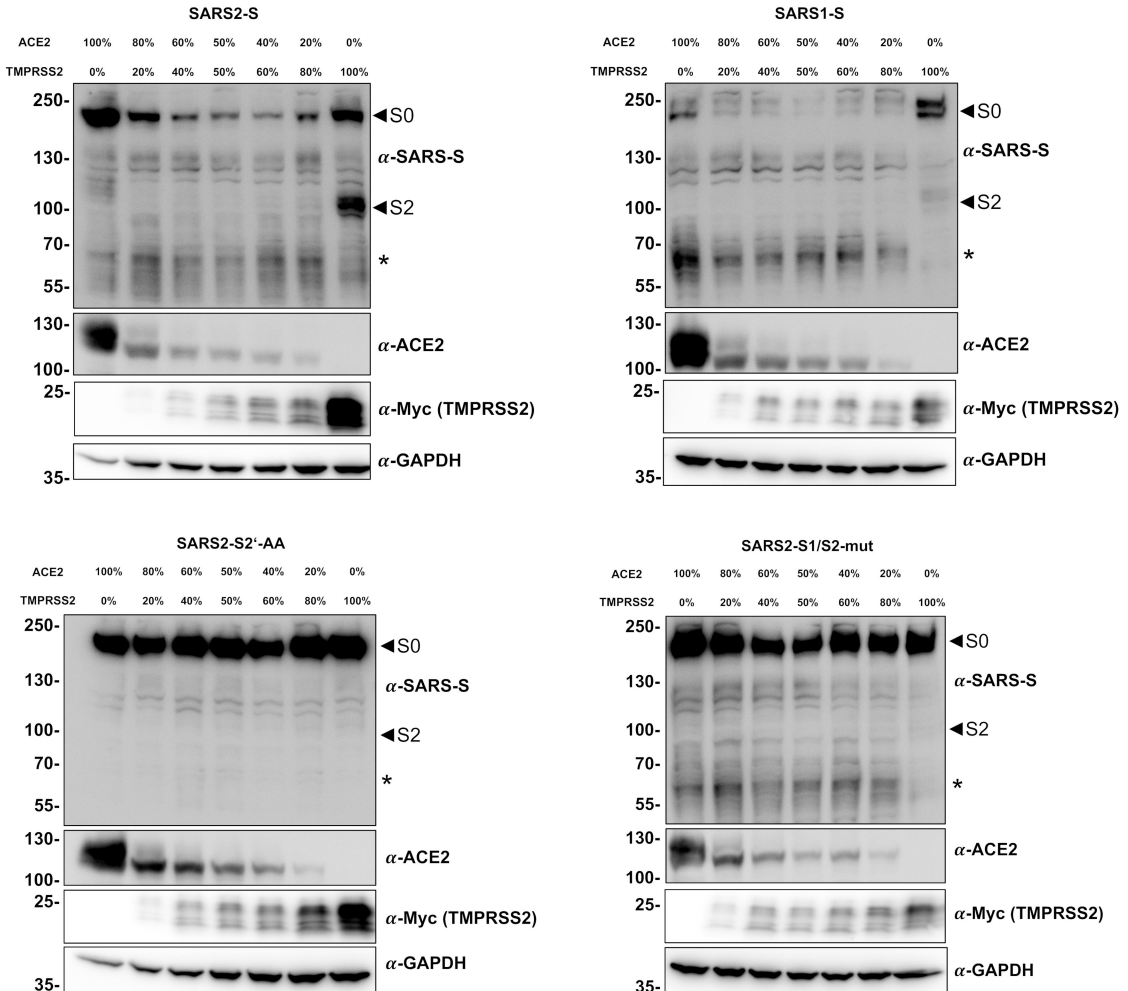


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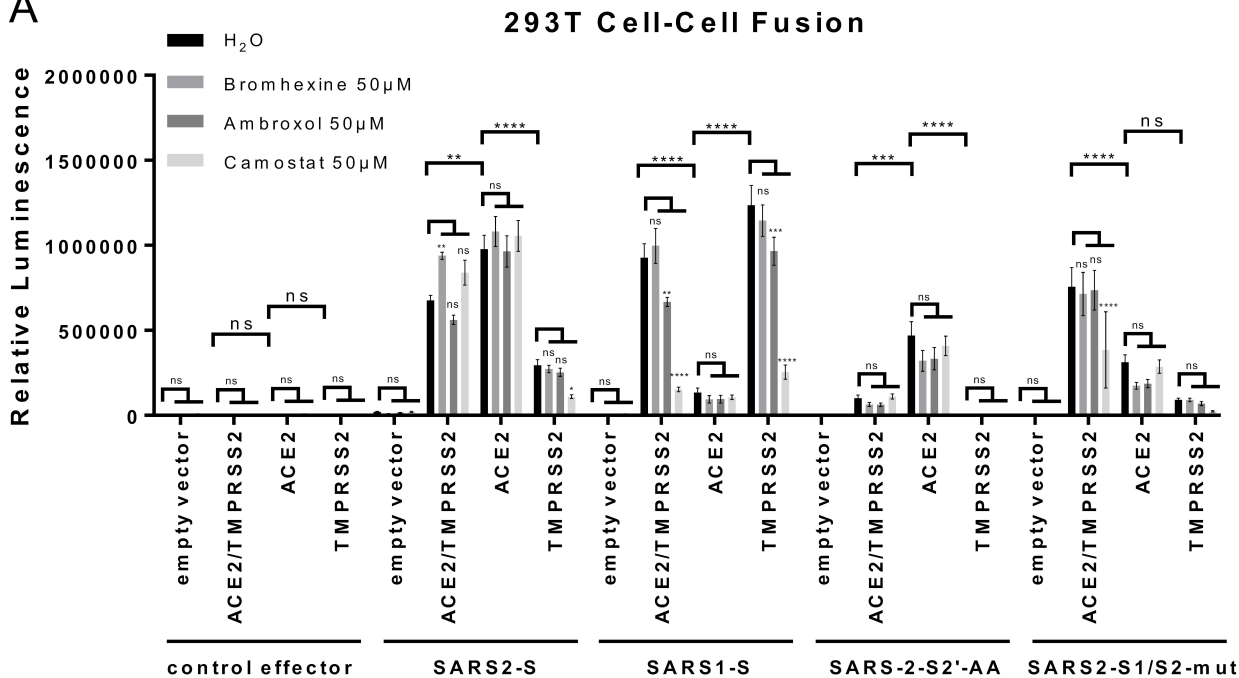
293T Cell-Cell Fusion



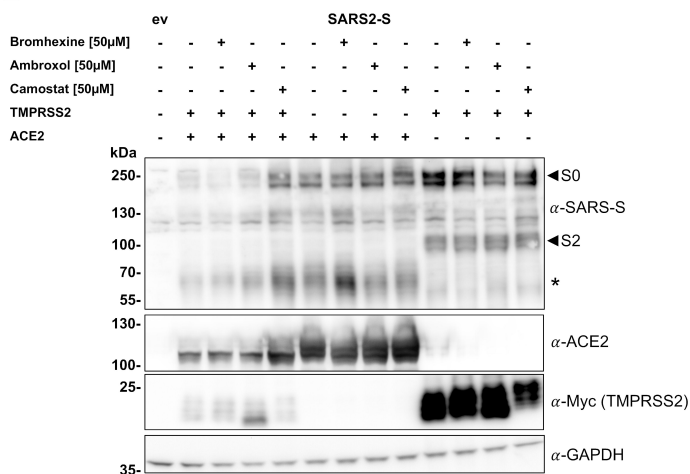
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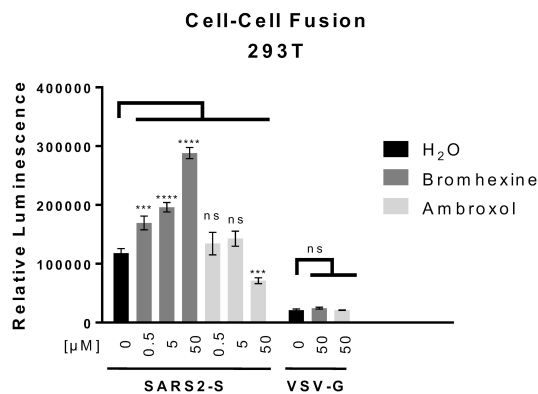
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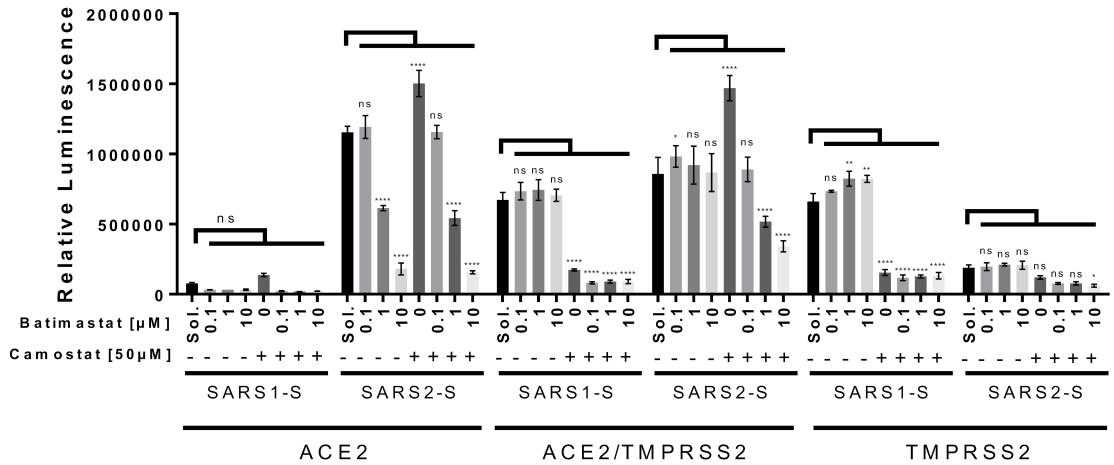


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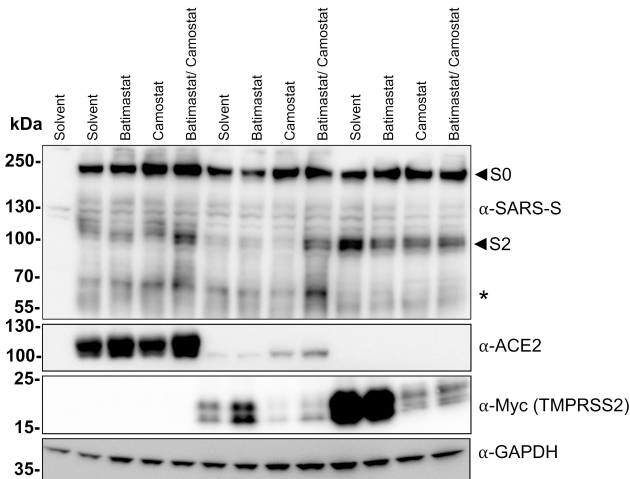
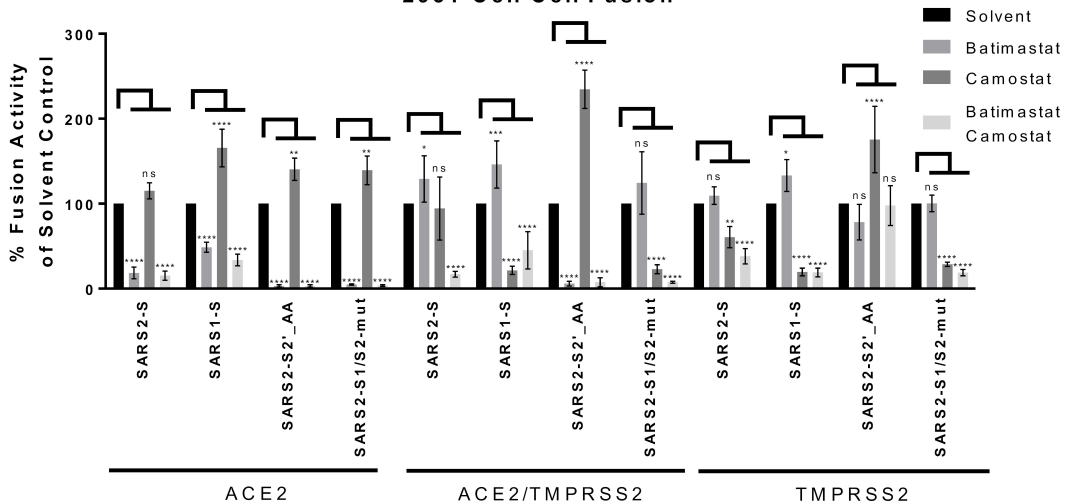
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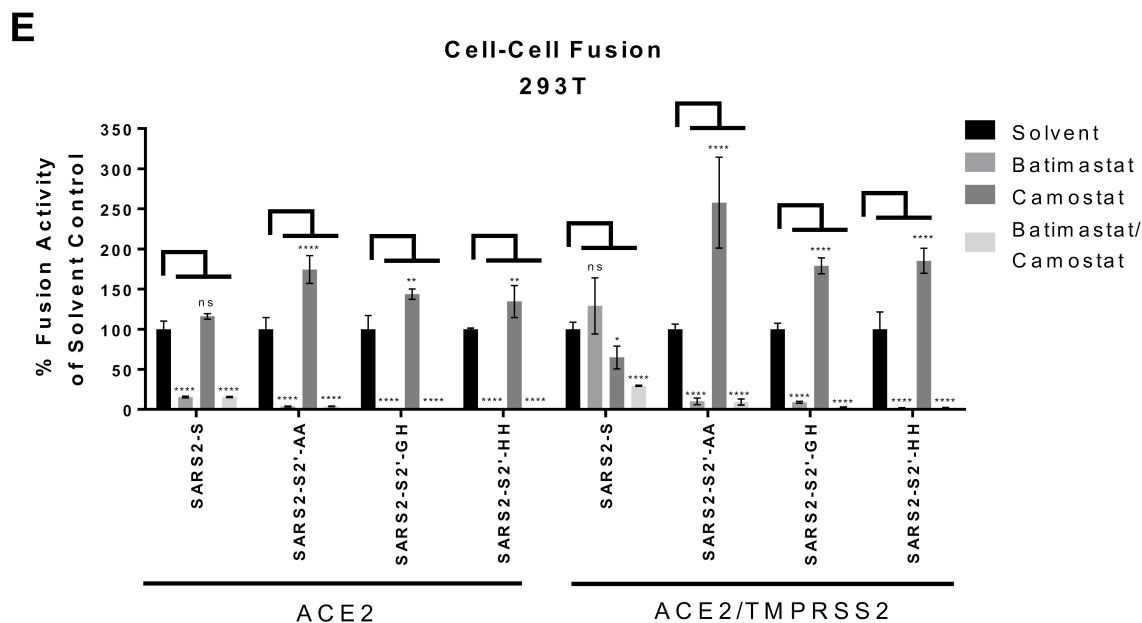
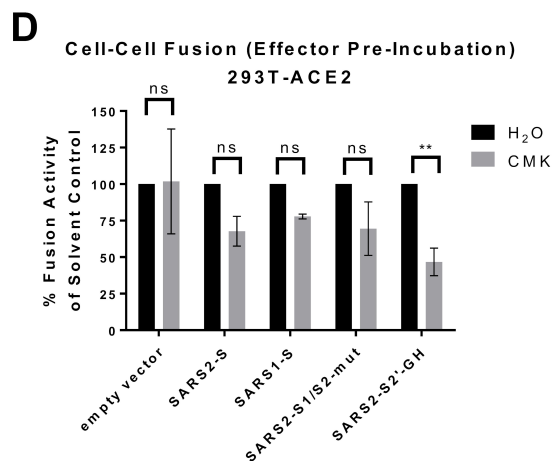
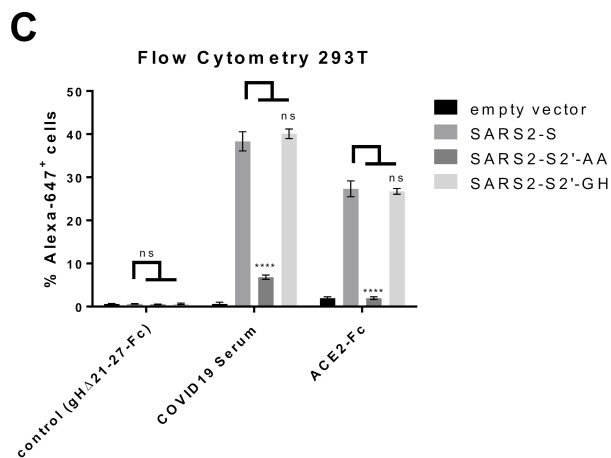
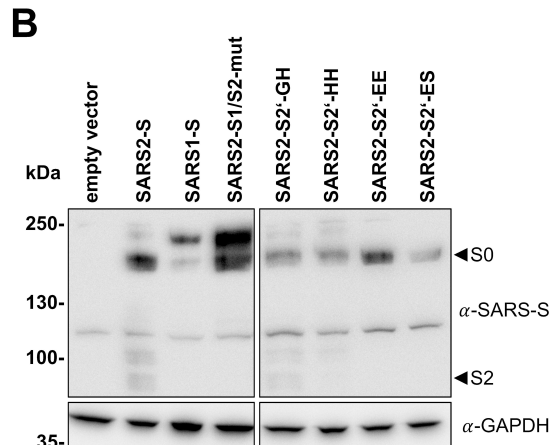
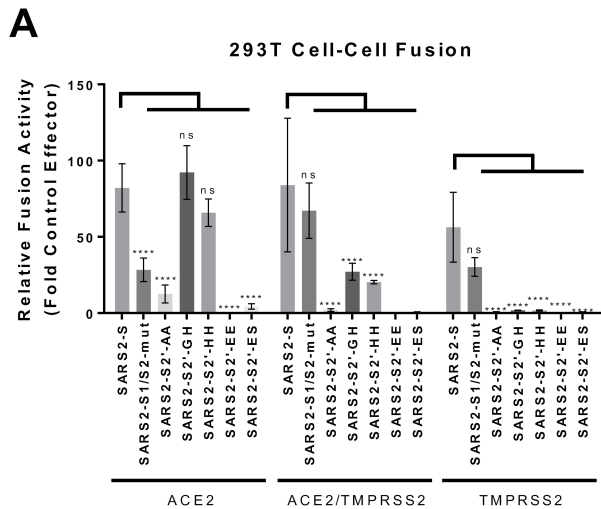


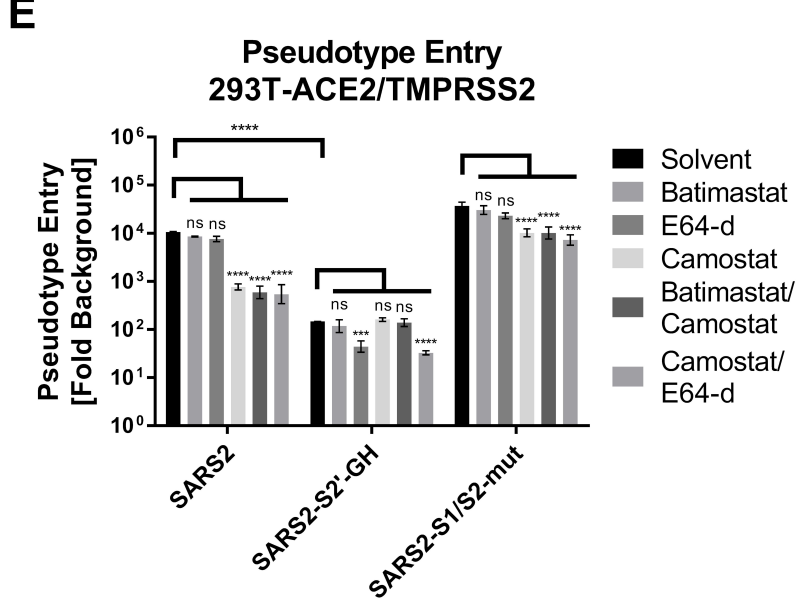
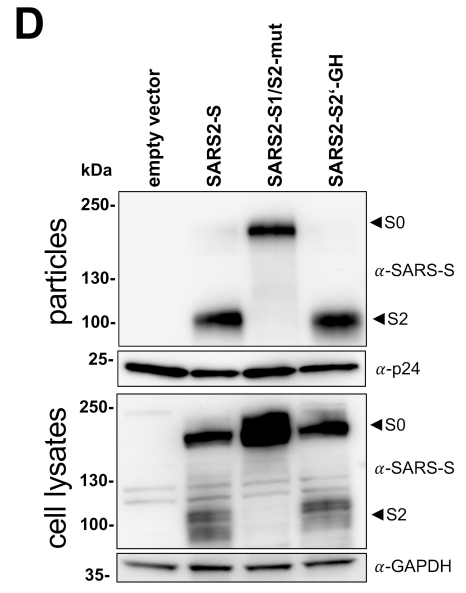
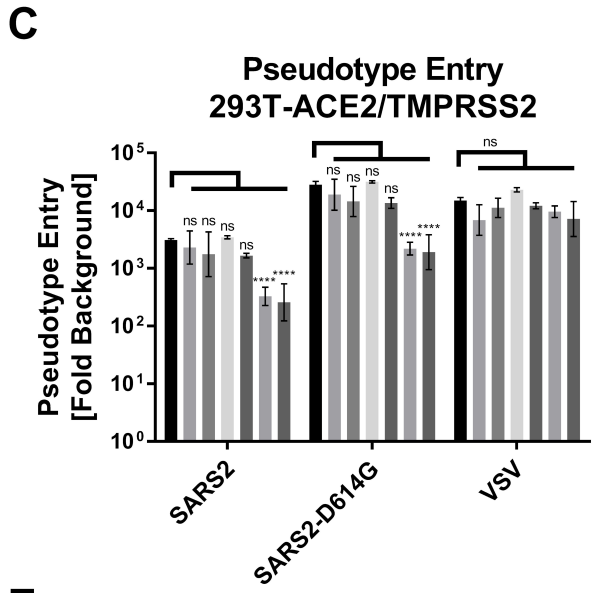
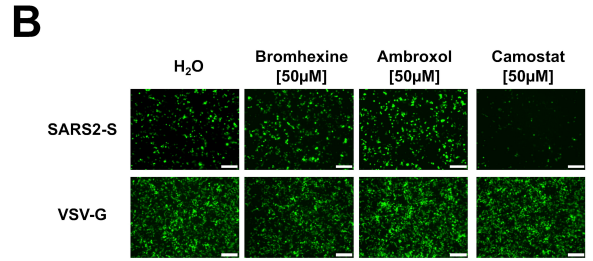
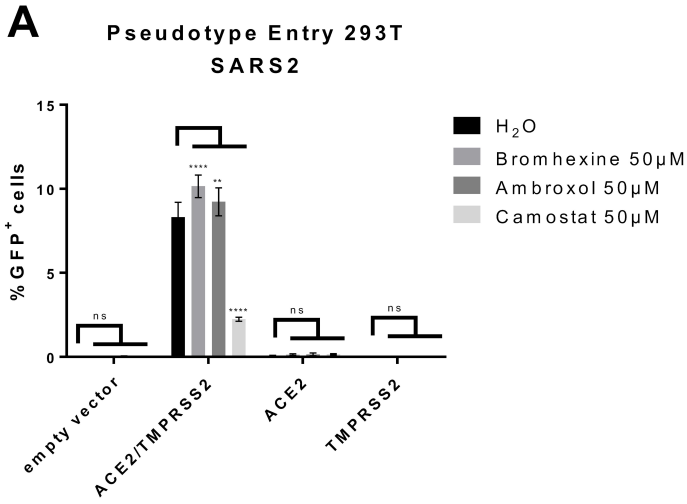
A**293T Cell-Cell Fusion****B**

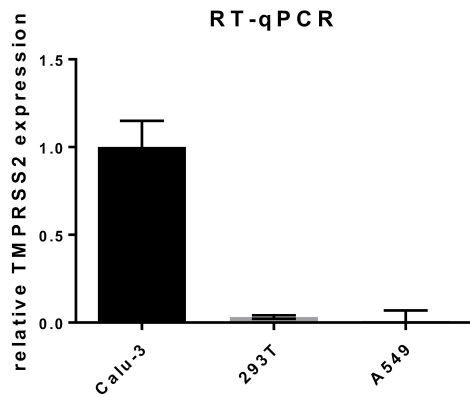
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Target: ev | ACE2 | ACE2/TMPRSS2 | TMPRSS2

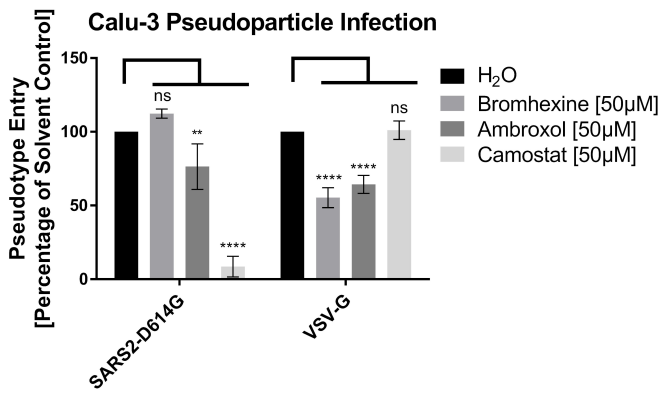
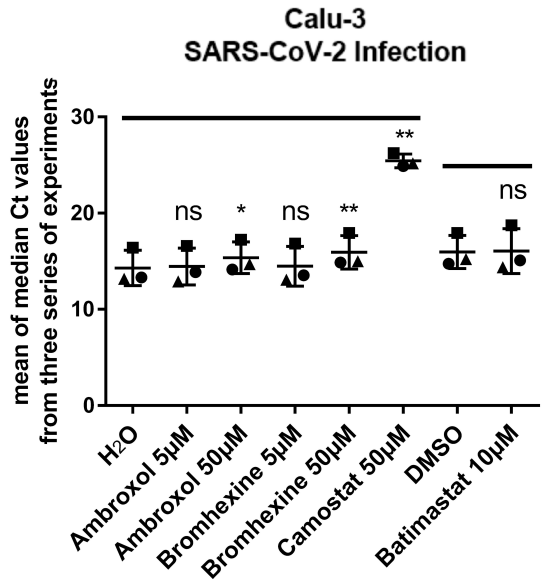
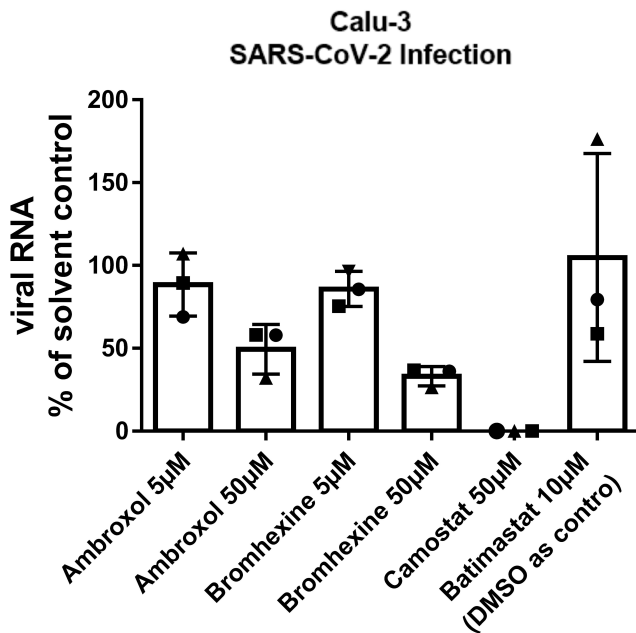
**C****293T Cell-Cell Fusion**





A

	Calu-3		293T		A549	
	Ct	SD	Ct	SD	Ct	SD
Ct TMPRSS2	23.17	0.11	28.67	0.18	29.80	1.76
Ct GAPDH	10.81	0.04	11.33	0.06	10.72	0.26
Δ Ct	12.36	0.07	17.33	0.11	19.08	1.03
$\Delta\Delta$ Ct	0.00	0.10	4.97	0.15	6.72	1.46
$2^{\text{exp}}(-\Delta\Delta\text{Ct})$	1.00	0.15	0.03	0.01	0.01	0.06
Ct TMPRSS2 -RT	n.d.		n.d.		n.d.	
Ct GAPDH -RT	30.33		n.d.		n.d.	

B**C****D****E**