

1 **Camostat mesylate inhibits SARS-CoV-2 activation by TMPRSS2-related**  
2 **proteases and its metabolite GBPA exerts antiviral activity**

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47 **Antiviral therapy is urgently needed to combat the coronavirus disease 2019 (COVID-19)**  
48 **pandemic, which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-**

49 **CoV-2). The protease inhibitor camostat mesylate inhibits SARS-CoV-2 infection of lung**  
50 **cells by blocking the virus-activating host cell protease TMPRSS2. Camostat mesylate has**  
51 **been approved for treatment of pancreatitis in Japan and is currently being repurposed for**  
52 **COVID-19 treatment. However, potential mechanisms of viral resistance as well as**  
53 **camostat mesylate metabolism and antiviral activity of metabolites are unclear. Here, we**  
54 **show that SARS-CoV-2 can employ TMPRSS2-related host cell proteases for activation and**  
55 **that several of them are expressed in viral target cells. However, entry mediated by these**  
56 **proteases was blocked by camostat mesylate. The camostat metabolite GBPA inhibited the**  
57 **activity of recombinant TMPRSS2 with reduced efficiency as compared to camostat**  
58 **mesylate and was rapidly generated in the presence of serum. Importantly, the infection**  
59 **experiments in which camostat mesylate was identified as a SARS-CoV-2 inhibitor involved**  
60 **preincubation of target cells with camostat mesylate in the presence of serum for 2 h and**  
61 **thus allowed conversion of camostat mesylate into GBPA. Indeed, when the antiviral**  
62 **activities of GBPA and camostat mesylate were compared in this setting, no major**  
63 **differences were identified. Our results indicate that use of TMPRSS2-related proteases for**  
64 **entry into target cells will not render SARS-CoV-2 camostat mesylate resistant. Moreover,**  
65 **the present and previous findings suggest that the peak concentrations of GBPA established**  
66 **after the clinically approved camostat mesylate dose (600 mg/day) will result in antiviral**  
67 **activity.**

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71 **INTRODUCTION**

72 The outbreak of the novel coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-  
73 CoV-2) in the city of Wuhan, China, in the winter of 2019 and its subsequent pandemic spread  
74 has resulted in more than 14 million cases of coronavirus disease 2019 and more than 600.00  
75 deaths (1). Antivirals designed to combat SARS-CoV-2 are not available and repurposing of  
76 existing drugs developed against other diseases is considered the fastest option to close this gap  
77 (2). Remdesivir, a drug generated to inhibit Ebola virus infection, has recently been shown to  
78 reduce the duration of hospitalization for COVID-19 (3). However, the drug failed to reduce  
79 fatality significantly (3) and beneficial effects were not observed in a previous clinical trial (4),  
80 indicating that additional therapeutic options are needed.

81 We previously showed that the SARS-CoV-2 spike protein (S) uses the host cell factors  
82 angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) for  
83 entry into target cells (5). TMPRSS2 is a cellular type II transmembrane serine protease (TTSP)  
84 expressed in human respiratory epithelium that cleaves and thereby activates the viral S protein.  
85 Activation is essential for viral infectivity and we found that the protease inhibitor camostat  
86 mesylate, which is known to block TMPRSS2 activity (6), inhibits SARS-CoV-2 infection of  
87 lung cells (5). Camostat mesylate has been approved for treatment of pancreatitis in Japan (7-9)  
88 and it is currently being investigated as a treatment of COVID-19 in several clinical trials in  
89 Denmark, Israel and USA (NCT04321096, NCT04353284, NCT04355052, NCT04374019).

90 The activity of TMPRSS2 is essential for SARS-CoV and MERS-CoV lung infection and  
91 disease development (10, 11). Whether TMPRSS2-independent pathways for S protein activation  
92 exist and contribute to viral spread outside the lung is not fully understood. The S proteins of  
93 SARS-CoV-2 and several other coronaviruses can be activated by the pH-dependent endosomal  
94 cysteine protease cathepsin L in certain cell lines (5, 12-15). However, this auxiliary S protein  
95 activation pathway is not operative in the lung, likely due to low cathepsin L expression (16).

96 Whether this pathway contributes to the recently reported extrapulmonary spread of SARS-CoV-  
97 2 is unknown (17). Similarly, it is unclear whether TTSPs other than TMPRSS2 can promote  
98 extrapulmonary SARS-CoV-2 spread. Finally, camostat mesylate is rapidly hydrolyzed into the  
99 active metabolite 4-(4-guanidinobenzoyloxy)phenylacetic acid (GBPA) in patients (18-20) but it  
100 is unknown to what extend GBPA inhibits TMPRSS2 activity.

101 Here, we identify TTSPs other than TMPRSS2 that can be used by SARS-CoV-2 for S  
102 protein activation and demonstrate that they are inhibited by camostat mesylate. Moreover, we  
103 provide evidence that camostat mesylate is rapidly converted into GBPA in cell culture and that  
104 GBPA inhibits SARS-CoV-2 entry with almost identical efficiency as compared to camostat  
105 mesylate when cells are preincubated with these compounds.

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119 **RESULTS**

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121 **Identification of novel SARS-CoV-2 S protein activating proteases**

122 The TTSP family comprises several enzymes which have previously been shown to activate  
123 surface glycoproteins of coronaviruses and other viruses, at least upon directed expression (21-  
124 23). Therefore, we asked whether the S protein of SARS-CoV-2 (SARS-2-S) can employ TTSPs  
125 other than TMPRSS2 for its activation. For this, we expressed different TTSPs along with the  
126 SARS-CoV-2 receptor, ACE2, in the otherwise poorly susceptible BHK-21 cells, treated the cells  
127 with ammonium chloride, which blocks the cathepsin L-dependent, auxiliary activation pathway,  
128 and transduced the cells with previously described vesicular stomatitis virus (VSV)-based  
129 pseudotypes bearing SARS-2-S (5). Ammonium chloride treatment strongly reduced SARS-2-S-  
130 driven transduction and this effect was rescued upon expression of TMPRSS2 (Fig. 1), as  
131 expected. Notably, this effect was also efficiently rescued by expression of TMPRSS13 and, to a  
132 lesser degree, TMPRSS11D, TMPRSS11E and TMPRSS11F (Fig. 1). Thus, SARS-2-S can use  
133 diverse TTSPs for S protein activation upon overexpression, with S protein activation by  
134 TMPRSS13 being particularly robust.

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136 **Several novel SARS-2-S activators are expressed in the airways and throat**

137 In order to obtain insights into whether SARS-2-S activating TTSPs could contribute to viral  
138 spread in the infected host, we asked whether these enzymes are expressed in viral target cells.  
139 For this, we analyzed single-cell RNA-Seq datasets collected from human lungs (24) and airways  
140 (25). As previously reported (26-31), ACE2 was expressed in the lung epithelial compartment,  
141 particularly including alveolar type 2 cells, secretory (goblet/club) cells, and ciliated cells (Fig.  
142 2A and Fig. S1). TMPRSS2 and TMPRSS13 were similarly expressed across epithelial cells,  
143 although TMPRSS13 expression was generally less robust. In contrast, expression of

144 TMPRSS11-family members was only rarely detected (Fig. 2A). We found that 53% of ACE2<sup>+</sup>  
145 cells in the lung co-express TMPRSS2, while 21% of ACE2<sup>+</sup> cells do not express TMPRSS2 but  
146 do express another TTSP capable of activating SARS-CoV-2 (Fig. S1). Within the airways, we  
147 observed ACE2 expression in secretory cells, ciliated cells, and suprabasal cells in both the nasal  
148 turbinate and the trachea (Fig. 2B). Interestingly, the expression pattern of the TTSPs in the  
149 airways was largely distinct: TMPRSS2 was primarily expressed in ciliated and secretory cells,  
150 TMPRSS11D was primarily expressed in basal cells, TMPRSS11E was primarily expressed in  
151 ionocytes, and TMPRSS13 was primarily expressed in nasal secretory cells (Fig. 2B). Within this  
152 dataset, 21% of ACE2<sup>+</sup> cells co-expressed TMPRSS2, while 24% of ACE2<sup>+</sup> cells co-expressed a  
153 different TTSP (Fig. S1). In total, these results suggest that TMPRSS2 is the dominant SARS-  
154 CoV-2-activating protease in the lung, in keeping with findings made for SARS-CoV and MERS-  
155 CoV, while the virus may use other activating proteases for spread in the airways.

156 A recent study provided evidence for extrapulmonary replication of SARS-CoV-2 in liver,  
157 colon, heart, kidney and blood in some patients (17). Therefore, we asked whether ACE2,  
158 TMPRSS2 and related SARS-2-S-activating proteases are expressed in these organs, using  
159 published resources (32, 33). Liver, colon, heart and kidney expressed robust levels of ACE2  
160 (Fig. 2C). Similarly, TMPRSS2 expression in colon, liver and kidney was readily detectable,  
161 although expression levels were lower than those measured for lung (Fig. 2C). In contrast, little  
162 to no expression of TMPRSS11D, TMPRSS11E, TMPRSS11F, TMPRSS13 was detected in  
163 liver, colon, heart and kidney. Finally, TMPRSS13 was expressed in lung and blood cells and  
164 expression of TMPRSS11-family members was readily detectable in esophagus and salivary  
165 gland (Fig. 2C). Collectively, the TTSPs able to activate SARS-2-S were not expressed in  
166 appreciable levels in potential extrapulmonary targets of SARS-CoV-2. The only exceptions were

167 TMPRS13 and TMPRSS11-family members that might contribute to SARS-CoV-2 infection of  
168 blood cells and to viral spread in the throat, respectively.

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170 **Newly identified SARS-2-S activators are camostat mesylate sensitive**

171 We next asked whether S protein activation by TTSP other than TMPRSS2 can be inhibited by  
172 camostat mesylate. To address this question, we performed the rescue assay as described above  
173 but investigated whether rescue can be blocked by camostat mesylate. In the absence of TTSP  
174 expression in target cells, ammonium chloride but not camostat mesylate reduced SARS-2-S-  
175 driven entry and the combination of both substances resulted in similar inhibition as observed  
176 upon ammonium chloride treatment alone (Fig. 3). These results are in agreement with only the  
177 cathepsin L-dependent auxiliary pathway being operative in control BHK-21 cells, in agreement  
178 with our published results (5). In TMPRSS2 transfected cells ammonium chloride did not  
179 efficiently block entry (Fig. 3), since under those conditions TMPRSS2 is available for S protein  
180 activation. Similarly, no entry inhibition was observed upon blockade of TMPRSS2 activity by  
181 camostat mesylate (Fig. 3), since the cathepsin L dependent activation pathway remained  
182 operative. Finally, the combination of ammonium chloride and camostat mesylate blocked entry  
183 into these cells (Fig. 3), in keeping with both activation pathways (cathepsin L and TMPRSS2)  
184 not being available under these conditions. Importantly, a comparable inhibition pattern was  
185 observed for all TTSPs able to activate SARS-2-S (Fig. 3), demonstrating that camostat mesylate  
186 will likely suppress SARS-CoV-2 activation by TMPRSS2 and TMPRSS2-related S protein  
187 activating serine proteases.

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189 **The camostat mesylate metabolite GBPA shows reduced inhibition of recombinant**  
190 **TMPRSS2**

191 Multiple studies show that camostat mesylate is rapidly converted into its active metabolite, 4-(4-  
192 guanidinobenzoyloxy)phenylacetic acid (GBPA) in animals and humans, followed by further  
193 conversion of GBPA into the inactive metabolite 4-guanidinobenzoic acid (GBA) (18-20, 34)  
194 (Fig. 6A). However, the capacity of GBPA to inhibit the enzymatic activity of TMPRSS2 has not  
195 been examined. To address this question, we compared inhibition of recombinant TMPRSS2 by  
196 camostat mesylate, GBPA and GBA. For this, we used FOY-251, a methanesulfonate of GBPA.  
197 We found that FOY-251 exerted a 10-fold reduced capacity to inhibit TMPRSS2 as compared to  
198 camostat mesylate, although both compounds completely suppressed TMPRSS2 activity at 1  $\mu$ M  
199 or higher (Fig. 4). In contrast, GBA was less active (Fig. 4). Thus, FOY-251 blocks TMPRSS2  
200 activity but with reduced efficiency as compared to camostat mesylate.

201 In order to obtain insights into the reduced inhibitor activity of FOY-251, we investigated  
202 TMPRSS2 inhibition by GBPA on the molecular level. For this, we used a combination of  
203 extensive all-atom molecular dynamics (MD) simulations and Markov modeling of the  
204 TMPRSS2-GBPA complex (35). Guanidinobenzoate-containing drugs such as camostat mesylate  
205 and GBPA inhibit TMPRSS2 by first forming a noncovalent precomplex which is then catalyzed  
206 to form a long-lived covalent complex that is the main source of inhibition (36). However, the  
207 population of the short-lived precomplex directly relates to the inhibitory activity (35). By  
208 computing the TMPRSS2-GBPA binding kinetics (35), we find that (i) the noncovalent  
209 TMPRSS2-GBPA complex is metastable, rendering it suitable to form a covalent inhibitory  
210 complex, and (ii) its population is 40% lower compared to camostat at equal drug concentrations,  
211 consistent with the finding that FOY-251 is a viable but less potent inhibitor (Fig. 4).  
212 Structurally, we find that GBPA binds in the same manner as camostat (Fig. 5, (35)). The main  
213 stabilizing interaction is its Guanidinium group binding into TMPRSS2's S1 pocket which is  
214 stabilized by a transient salt bridge with Asp 435. The GBPA ester group can interact with the

215 catalytic Ser 441, making it prone for catalysis and formation of the catalytic complex. The  
216 slightly lower stability of the GBPA compared to the camostat mesylate-TMPRSS2 complex is  
217 consistent with GBPA's shorter tail which has less possibilities to interact with the hydrophobic  
218 patch on the TMPRSS2 binding site shown in Fig. 5, left panel.

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220 **Rapid conversion of camostat mesylate to GBPA in cell culture**

221 Although camostat mesylate is rapidly metabolized in animals and humans, it is less clear  
222 whether conversion of camostat mesylate into GBPA and GBA also occurs in cell culture. We  
223 addressed this question by exposing camostat mesylate to culture medium containing fetal calf  
224 serum (FCS), which is standardly used for cell culture, followed by mass spectrometric  
225 quantification of camostat mesylate and GBPA levels. Camostat mesylate levels rapidly declined  
226 with a half-life of approximately 2 h and the compound being barely detectable after 8 h (Fig.  
227 6B). Conversely, the levels of the camostat mesylate metabolite GBPA increased rapidly, with  
228 peak levels attained at 8 h, and then remained relatively stable (Fig. 6B). Finally, the rapid  
229 metabolism of camostat mesylate into GBPA in the presence of serum was further confirmed  
230 by incubation of camostat mesylate in either water or FCS-containing culture medium for 1 min  
231 followed by quantification of camostat mesylate and GBPA levels. While GBPA levels were at  
232 background level when camostat mesylate was incubated in water, ~5.4 % of camostat mesylate  
233 was metabolized into GBPA when incubated in FCS-containing culture medium (Fig. 6C). Thus,  
234 camostat mesylate is rapidly converted into GBPA under standard cell culture conditions, but the  
235 conversion is slower than what is observed in humans (20).

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237 **Camostat mesylate and FOY-251 inhibit SARS-CoV-2 infection with comparable efficiency**

238 We finally compared the antiviral activity of camostat mesylate and FOY-251, the

239 methanesulfonate of GBPA, in cell culture. The reduced ability of FOY-251 to block the  
240 enzymatic activity of recombinant TMPRSS2 as compared to camostat mesylate would suggest  
241 that the compound should also exert reduced antiviral activity. On the other hand, analysis of  
242 antiviral activity encompasses preincubation of target cells with camostat mesylate for 2 h in the  
243 presence of FCS, which allows conversion of camostat mesylate into GBPA, as demonstrated  
244 above. Indeed, titration experiments with VSV pseudotypes and Calu-3 lung cells as targets  
245 revealed that entry inhibition by FOY-251 was only slightly reduced as compared to camostat  
246 mesylate, with EC50 values of 107 nM (camostat mesylate) and 178 nM (FOY-251) (Fig. 7).  
247 Moreover, no marked differences in inhibition of infection of Calu-3 cells with authentic SARS-  
248 CoV-2 were observed (Fig. 8). Thus, under the conditions chosen camostat mesylate and GBPA  
249 exerted comparable antiviral activity, likely due to conversion of camostat mesylate into GBPA.  
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263 **DISCUSSION**

264 With the exception of remdesivir, which reduces disease duration (3), and dexamethasone, which  
265 reduces mortality in ICU patients by targeting inflammation (37), there are currently no drugs  
266 against COVID-19 with efficacy proven in clinical trials. We previously reported that the  
267 protease inhibitor camostat mesylate inhibits SARS-CoV-2 infection of cultured lung cells by  
268 blocking the virus-activating cellular protease TMPRSS2 (5). Camostat mesylate has been  
269 approved for human use in Japan and may thus constitute a COVID-19 treatment option. Here,  
270 we provide evidence that the virus can use TMPRSS2-related proteases for S protein activation  
271 and that these enzymes are also blocked by camostat mesylate. Moreover, we demonstrate that  
272 the camostat mesylate metabolite GBPA exhibits reduced ability to block enzymatic activity of  
273 purified, recombinant TMPRSS2 and is rapidly produced under cell culture conditions. The rapid  
274 conversion of camostat mesylate into GBPA likely accounts for our finding that both compounds  
275 exerted similar antiviral activity.

276 Knock-out of TMPRSS2 in mice markedly reduces SARS-CoV and MERS-CoV infection  
277 (10) and disease development, and similar findings have been reported for influenza A viruses  
278 (IAV) (38-40), which also use TMPRSS2 for glycoprotein activation (41). Thus, TMPRSS2  
279 activity is essential for CoV and IAV infection of the lung. In contrast, several members of the  
280 TTSP family other than TMPRSS2 can activate CoV and IAV glycoproteins and support viral  
281 spread in cell culture, at least upon directed expression (21, 23, 41). Whether these TTSPs play a  
282 role in viral spread in the host is incompletely understood. For IAV, infection by H3N2 viruses  
283 were found not to be fully TMPRSS2 dependent (38, 42) and an auxiliary role of TMPRSS4 in  
284 spread and pathogenesis of H3N2 viruses has been reported (43, 44). Moreover, influenza B  
285 viruses can use a broad range of TTSPs in cell culture (44, 45) and a prominent role of TMPRSS2

286 in viral spread in type II pneumocytes has been reported (46) but viral spread in mice is  
287 TMPRSS2 independent (44, 47).

288 The present study shows that also SARS-CoV-2 can use TTSPs other than TMPRSS2 for  
289 S protein activation. Whether the TTSPs found here to activate SARS-2-S upon directed  
290 expression play a role in viral spread in the host remains to be investigated. Expression analyses  
291 suggest that they may. TMPRSS13 activated SARS-2-S with similar efficiency as TMPRSS2 and  
292 TMPRSS13 mRNA was found to be coexpressed with ACE2 in type II pneumocytes, goblet and  
293 club cells and basal cells. Moreover, TMPRSS13 was expressed in blood cells, which may  
294 constitute a target for SARS-CoV-2 infection in some patients. Finally, and most notably, SARS-  
295 S-2 activating TTSPs showed distinct expression patterns in the upper respiratory tract and  
296 several potential target cells coexpressed ACE2 jointly with a novel S protein activating TTSP  
297 but not TMPRSS2. Although viral spread supported by TMPRSS13 and potentially other SARS-  
298 2-S activating TTSPs could contribute to transmission and pathogenesis, it would still be  
299 sensitive to blockade by camostat mesylate. Thus, usage of auxiliary TTSPs for S protein  
300 activation would not confer camostat mesylate resistance to SARS-CoV-2.

301 In animal and humans camostat mesylate is rapidly hydrolyzed into the active metabolite  
302 4-(4-guanidinobenzoyloxy) phenylacetic acid (GBPA), which is further hydrolyzed to 4-  
303 guanidinobenzoic acid (GBA) (18-20). GBPA was known to retain protease inhibitor activity (34,  
304 48) but it was unclear whether GBPA would block TMPRSS2 activity with the same efficiency  
305 as camostat mesylate. Inhibition studies carried out with recombinant TMPRSS2 demonstrated  
306 that although GBPA robustly blocked TMPRSS2 activity, the compound was about 10-fold less  
307 active than camostat mesylate, which roughly matches results reported for other proteases (49).  
308 This finding raised the question whether camostat mesylate conversion into GBPA also occurs in  
309 cell culture systems used to assess antiviral activity of camostat mesylate. Indeed, camostat

310 mesylate was rapidly converted into GBPA in the presence of serum which may account for  
311 camostat mesylate and FOY-251 exerting roughly comparable antiviral activity when cells were  
312 preincubated with these compounds for 2 h in the presence of serum. This has important  
313 implications for COVID-19 treatment, considering that continuous IV infusion of camostat  
314 mesylate (40 mg) resulted in a maximal plasma GBPA concentration of 0.22  $\mu$ M and peak  
315 plasma concentrations of GBPA in humans upon oral intake of 200 mg camostat mesylate can  
316 reach 0.25  $\mu$ M (<http://www.shijiebiaopin.net/upload/product/201272318373223.PDF>). Provided  
317 that concentrations in plasma and in respiratory epithelium are comparable, this would suggest  
318 that GBPA peak levels attained with the dosage approved for pancreatitis treatment (200 mg  
319 camostat mesylate three times a day) would be sufficient to exert antiviral activity.

320 Collectively, our results indicate that camostat mesylate constitutes a viable treatment  
321 option for COVID-19. Independent of its antiviral activity, camostat mesylate might reduce the  
322 uncontrolled cytokine release observed in severe COVID-19, since TMPRSS2 expression is  
323 required for robust cytokine release upon exposure of mice to polyIC (10).

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334 **MATERIALS AND METHODS**

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336 **Cell culture**

337 BHK-21 (baby hamster kidney; ATCC no. CCL-10) and HEK-293T (human embryonic kidney;  
338 DSMZ no. ACC 635) cells were cultivated in Dulbecco's modified Eagle medium supplemented  
339 with 10 % fetal bovine serum (FCS, Biochrom), 100 U/mL of penicillin and 0.1 mg/mL of  
340 streptomycin (PAN-Biotech). Calu-3 cells (human lung adenocarcinoma) were cultivated in  
341 minimum essential medium (MEM) containing 10 % FCS (Biochrom), 100 U/mL of penicillin  
342 and 0.1 mg/mL of streptomycin (PAN-Biotech), 1x non-essential amino acid solution (from 100x  
343 stock, PAA) and 10mM sodium pyruvate (Thermo Fisher Scientific). Cell lines were incubated at  
344 37 °C in a humidified atmosphere containing 5 % CO<sub>2</sub>. Transfection of 293T cells was performed  
345 by calcium-phosphate precipitation, while Lipofectamine LTX with Plus reagent (Thermo Fisher  
346 Scientific) was used for transfection of BHK-21 cells.

347

348 **Plasmids**

349 We employed pCAGGS-based expression vectors for VSV-G, TMPRSS2, TMPRSS3,  
350 TMPRSS4, TMPRSS10, TMPRSS11A, TMPRSS11B, TMPRSS11D, TMPRSS11E,  
351 TMPRSS11F and TMPRSS13 that have either been previously described elsewhere or  
352 constructed on existing expression vectors (21-23, 50-52). All proteases contained an N-terminal  
353 cMYC-epitope tag. Further, we used pCG1-based expression vectors for human ACE2 (53) and a  
354 SARS-2-S variant with a truncated cytoplasmic tail for improved pseudotype particle production  
355 (deletion of last 18 amino acid residues,(54))

356

357 **Preparation of camostat mesylate and GBPA stocks**

358 Camostat mesylate and GBPA were obtained from Ono pharmaceuticals Co., LTD.  
359 (Osaka/Japan) and reconstituted in DMSO to yield stock solutions of 100 mM. Stocks were  
360 stored at -20 °C, thawed immediately before the experiment and residual compound was  
361 discarded.

362

363 **Mass spectrometric quantification of camostat mesylate metabolism**

364 Camostat mesylate was diluted to a concentration of ~15 µM in either water or MEM containing  
365 10 % FCS and incubated for 1 min (water and medium samples), 15 min, 30 min, 1 h, 2 h, 4 h, 8  
366 h and 24 h (only medium samples) at 37 °C. Next, samples were snap-frozen and stored at -80 °C  
367 until camostat mesylate, GBPA and GBA levels were quantified by mass spectrometry. An ultra-  
368 high-performance liquid chromatography tandem mass spectrometry method using pneumatically  
369 assisted electrospray ionisation (UHPLC-ESI-MS/MS) was used for quantification of camostat  
370 and 4-(4-guanidinobenzoyloxy) phenylacetic acid (GBPA) in liquid samples. Calibrants based  
371 on blank sample were used for the construction of 8-point calibration curves. Calibrants were  
372 prepared with concentrations of 0.1, 1, 25, 50, 75, 100, 500 and 1000 µg/L of camostat and  
373 GBPA. In addition, a blank sample (a processed matrix sample without any added analyte) and a  
374 blank sample spiked with SIL-IS were included to verify the absence of detectable concentrations  
375 of the analytes. The calibration curves were created by weighted (1/x) regression analysis of the  
376 SIL-IS normalised peak areas (analyte area/IS area).

377

378 **Preparation of pseudotype particles**

379 We employed a previously published protocol to generate vesicular stomatitis virus (VSV)  
380 pseudotype particles that is based on a replication-deficient VSV containing eGFP and firefly  
381 luciferase (FLuc) reporter genes, VSV\*ΔG-FLuc (kindly provided by Gert Zimmer, Institute of

382 Virology and Immunology IVI, Mittelhäusern/Switzerland) (5, 55). For this, HEK-293T cells  
383 were first transfected with expression vector for either SARS-2-S or VSV-G (or empty  
384 expression vector, control). At 24 h post transfection, cells were inoculated with VSV-G-  
385 transcomplemented VSV\*ΔG-FLuc at a multiplicity of infection (MOI) of 3 and incubated for 1  
386 h at 37 °C and 5 % CO<sub>2</sub>. Next, the inoculum was removed and cells were washed with PBS  
387 before fresh culture medium was added. In case of cells transfected with SARS-2-S-encoding  
388 vector or empty plasmid, the medium was spiked with anti-VSV-G antibody (supernatant of  
389 CRL-2700 cells, 1:1,000) in order to inactivate residual input virus containing VSV-G. At 16-18  
390 h post inoculation, the culture supernatant was harvested and centrifuged (2,000 x g, 10 min) to  
391 remove cellular debris. Clarified supernatants containing pseudotype particles were aliquoted and  
392 stored at -80 °C until further use.

393

394 **Preparation of TMPRSS2 recombinant protein and substrate**

395 Human TMPRSS2 (Recombinant N-terminus 6xHis, aa106-492) (Cat # LS-G57269-20) protein  
396 was acquired from LifeSpan Biosciences. Peptide Boc-Gln-Ala-Arg-MCA for the enzyme  
397 substrate was acquired from Peptide Institute, Inc.

398

399 **TMPRSS2 enzyme assay**

400 All of different concentrations of test compounds were dissolved in DMSO and diluted with  
401 assay buffer (50 mM Tris-HCl pH 8.0, 154 mM NaCl) to the final DMSO concentration of 1%.  
402 Compound solution and Boc-Gln-Ala-Arg-MCA (10 μM final concentration) were added into the  
403 384-well black plate (Greiner 784076). Then, enzyme reaction was started after adding  
404 TMPRSS2 recombinant protein to a final concentration of 2 μg/mL. Fluorescence intensity was  
405 read using the Envision plate reader with excitation: 380 nm and emission: 460 nm in 2 min

406 intervals over 60 min at room temperature. The IC<sub>50</sub> value was calculated based on the increasing  
407 rate of fluorescence intensity.

408

#### 409 **Molecular dynamics simulations and Markov modeling**

410 We used extensive all-atom molecular dynamics (MD) simulations of TMPRSS2 in complex  
411 with camostat as described in (35) starting from a homology model (56), in which drug binding  
412 and dissociation are sampled multiple times. We have then replaced camostat with GBPA and  
413 simulated a total of 50  $\mu$ s MD with the same simulation setup as in [1] and used Markov  
414 modeling (57) to extract the dominant metastable binding modes of GBPA to the TMPRSS2  
415 target on an atomistic scale. We estimate the binding kinetics of GBPA to the non-covalent  
416 complex by re-estimating the camostat Markov model described in (35) with the TMPRSS2-  
417 GBPA data. At the simulated drug concentration the association constant of GBPA is found to  
418 have a maximum likelihood estimate of 60% compared to that of camostat, resulting in a  
419 correspondingly lower inhibitory activity following the kinetic model of (35). Bootstrapping of  
420 trajectories under the constraint of comparable implied timescales yields a confidence interval of  
421 51-100 % (68 % percentile).

422

#### 423 **Transduction experiments**

424 The day before transduction, BHK-21 cells were transfected with an expression vector for ACE2  
425 and either empty expression plasmid (control) or expression vector encoding TMPRSS2,  
426 TMPRSS3, TMPRSS4, TMPRSS10, TMPRSS11A, TMPRSS11B, TMPRSS11D, TMPRSS11E,  
427 TMPRSS11F or TMPRSS13. For this, the old culture medium was removed and 50  $\mu$ l/well of  
428 fresh culture medium were added. Next, transfection mixtures were prepared. For one well 0.1  $\mu$ g  
429 of ACE2-encoding vector and 0.02  $\mu$ g of protease-encoding vector (or empty plasmid) were

430 mixed with 1  $\mu$ l of Plus reagent, 50  $\mu$ l of Opti-MEM medium (Thermo Fisher Scientific) and 1  $\mu$ l  
431 of Lipofectamine LTX reagent. The transfection mix was vortexed and incubated for 30 min at  
432 room temperature before it was added to the cells. At 6 h post transfection, the transfection  
433 medium was replaced by fresh culture medium and the cells were further incubated for ~18 h.  
434 Then, the cells were either pre-incubated for 2 h with inhibitor (50 mM ammonium chloride  
435 [Sigma-Aldrich], 100  $\mu$ M camostat mesylate or a combination of both; cell treated with DMSO  
436 served as controls) before transduction or directly inoculated with pseudotype particles bearing  
437 SARS-2-S or cells. For transduction of Calu-3 cells, cells were pre-incubated for 2 h at 37 °C and  
438 5 % CO<sub>2</sub> with different concentrations (0.01, 0.1, 1, 10, 100  $\mu$ M) of camostat mesylate, FOY-251  
439 or DMSO (control), before they were inoculated with pseudotype particles bearing SARS-2-S or  
440 VSV-G. At 16 h post inoculation, transduction efficiency was analyzed by measuring the activity  
441 of virus-encoded FLuc in cell lysates. For this, the cell culture medium was removed and cells  
442 were incubated for 30 min with 1x concentrated Cell Culture Lysis Reagent (Promega), before  
443 cell lysates were transferred into white opaque-walled 96-well plates and luminescence was  
444 recorded (1 sec/sample) using a Hidex Sense plate luminometer (Hidex) and a commercial  
445 substrate (Beetle-Juice, PJK).

446

#### 447 **Analysis of cell vitality**

448 For the analysis of cell vitality of Calu-3 cells treated with camostat mesylate or FOY-251 the  
449 CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) was used. For this, Calu-3 cells  
450 were grown in 96-well plates to reach ~50% confluence, before they were incubated in the  
451 presence of different concentrations of camostat mesylate or FOY-251 for 24 h. Cells treated with  
452 DMSO (solvent control) served as controls. Following incubation, 100  $\mu$ l of CellTiter-Glo  
453 substrate were added per well and the samples were incubated for 30 min on a rocking platform.

454 In addition, fresh culture medium (without cells) was also incubated with CellTiter-Glo substrate  
455 in order to define the assay background. Following incubation, the samples were transferred into  
456 white opaque-walled 96-well plates and luminescence was recorded (200 msec/sample) using a  
457 Hidex Sense plate luminometer (Hidex).

458

459 **Infection of Calu-3 cells with authentic SARS-CoV-2**

460 The SARS-CoV-2 isolate hCoV-19/Germany/FI1103201/2020 (GISAID accession EPI-  
461 ISL\_463008) was isolated at the Institute of Virology, Muenster, Germany, from a patient  
462 returning from the Southern Tyrolean ski areas and propagated in Vero-TMPRSS2 cells. Calu-3  
463 cells were pre-incubated for 2 h with 2-fold concentrated camostat mesylate or FOY-251 (2, 20  
464 or 200  $\mu$ M), or DMSO (control), before they were inoculated with SARS-CoV-2 at an MOI of  
465 0.001 or 0.01. For this, the identical volume of virus-containing medium was added to the  
466 inhibitor-containing medium on the cells (resulting in 1-fold concentrated camostat mesylate or  
467 FOY-251; 1, 10 or 100  $\mu$ M). Following 1 h of incubation at 37 °C and 5 % CO<sub>2</sub>, the culture  
468 supernatant was removed and cells were washed two times with excess PBS before culture  
469 medium containing 1-fold concentrated inhibitor was added. Supernatants were harvested at 24 h  
470 post inoculation and subjected to plaque titration. For this, confluent Vero-TMPRSS2 cells were  
471 inoculated with 10-fold serial dilutions of supernatant and incubated for 1 h 37 °C and 5 % CO<sub>2</sub>.  
472 Thereafter, the inoculum was removed and cells were incubated with culture medium containing  
473 1 % (w/v) methyl cellulose. Plaques were counted at 48 h post infection and titers determined as  
474 plaque forming units per ml (pfu/ml).

475

476 **TTSP expression analysis**

477 Bulk tissue expression data were obtained from the GTEx portal (33). Single-cell expression data  
478 from human lungs were obtained from GSE1229603. Only IPF and cryobiopsy lung explants  
479 were used in this analysis. Single-cell expression data from human airways was obtained from  
480 <https://www.genomique.eu/cellbrowser/HCA/4>. The single-cell data was analyzed as described in  
481 Smith et al (29). In short, dimensionality reduction and clustering were performed on normalized  
482 expression data in python using Scanpy and the Multicore-TSNE package (58, 59). Low quality  
483 cells were filtered out by removing cells with fewer than 500 detected genes. Highly variable  
484 genes were computed using the Seurat approach in Scanpy, and then used to calculate the  
485 principle component analysis. T-SNE and Leiden clustering were calculated using nearest  
486 neighbors, with parameters as described in the associated code. Cell clusters were labeled  
487 manually by comparing the expression patterns of established marker genes with the lists of  
488 differentially-expressed genes produced by Scanpy (60-63). The code used for performing these  
489 analyses is available at <https://github.com/joan-smith/covid19-proteases/>.

490

## 491 **Statistical analyses**

492 All statistical analyses were performed using GraphPad Prism (version 8.4.2, GraphPad Software,  
493 Inc.). Statistical significance of differences between two datasets was analyzed by paired, two-  
494 tailed student's t-test, while two-way analysis of variance (ANOVA) with Dunnett's posttest was  
495 used for comparison of multiple datasets (the exact method used is stated in the figure legends).  
496 For the calculation of the turnover time required for metabolism of 50 % of camostat mesylate  
497 ( $T_{1/2}$ ) as well as the effective concentration 50 (EC50) values, which indicate the inhibitor  
498 concentration leading to 50 % reduction of transduction, non-linear fit regression models were  
499 used.

500

501 **SUPPLEMENTARY MATERIALS**

502 Fig. S1, panel A. A track plot displaying the expression of ACE2, S-activating proteases, and  
503 several lineage-enriched genes in different lung cell populations obtained from Leiden clustering.

504 Fig. S1, panel B. A track plot displaying the expression of ACE2, S-activating proteases, and  
505 several lineage-enriched genes in different airway cell populations obtained from Leiden  
506 clustering.

507 Fig. S1, panel C. The percent of cells in the lung that express the indicated single gene or pair of  
508 genes are displayed.

509 Fig. S1, panel D. The percent of cells in the airway that express the indicated single gene or pair  
510 of genes are displayed.

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690 J.C.S., N.K., LK.S., O.S.S., J.B.H., T.H., L.R., S.O., T.Y., K.Y., and J.M.S., performed research.  
691 M.H., J.C.S., H.M., T.H., F.N. J.S.M., M.K. and S.P. analyzed the data. M.W. and S.L. provided  
692 essential reagents. M.H. and S.P. wrote the manuscript. All authors revised the manuscript.  
693 **Competing interests:** J.C.S. is a co-founder of Meliora Therapeutics and is an employee of  
694 Google, Inc. This work was performed outside of her affiliation with Google and used no  
695 proprietary knowledge or materials from Google. J.M.S. has received consulting fees from Ono  
696 Pharmaceuticals, is a member of the Advisory Board of Tyra Biosciences, and is a co-founder of  
697 Meliora Therapeutics. As part of its mission the Deutsches Primatenzentrum (German Primate  
698 Center) performs services for the scientific community including services for pharmaceutical  
699 companies resulting in fees being paid to the German Primate Center. **Data availability**  
700 **statement:** All data associated with this

701 study are shown in the paper or the Supplementary Materials. All of the data used in this  
702 manuscript to determine protease expression are described in Table S1 of (29) and the code used  
703 for performing these analyses is available at [github.com/joan-smith/covid19](https://github.com/joan-smith/covid19).

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725 **FIGURE LEGENDS**

726

727 **Fig. 1. Different TTSPs can activate SARS-2-S in transfected cells.** BHK-21 cells transiently  
728 expressing ACE2 and one of the indicated type-II transmembrane serine protease (or empty  
729 vector) were pre-incubated with either 50 mM ammonium chloride or DMSO (control, indicated  
730 by dashed line) for 2 h, before they were inoculated with pseudotype particles bearing SARS-2-S.  
731 At 16 h post inoculation, SARS-2-S-driven cell entry of viral pseudotypes was analyzed by  
732 measuring the activity of virus-encoded luciferase activity in cell lysates. Data were further  
733 normalized and entry efficiency in the absence of ammonium chloride was set as 100 %. Shown  
734 are the average (mean) data obtained from three biological replicates, each performed in  
735 quadruplicates. Error bars indicate the standard error of the mean (SEM). Statistical significance  
736 of differences in entry efficiency in the presence of ammonium chloride was analyzed by two-  
737 way analysis of variance (ANOVA) with Dunnett's posttest.

738

739 **Fig. 2. SARS-2-S activating proteases are expressed in lung and blood.** (A) T-SNE clustering  
740 of cells from the human lung (24). Cells expressing the coronavirus receptor ACE2 are  
741 highlighted in the right panel. These panels are reproduced with permission from Smith et al.  
742 (29). Cells expressing various S-activating proteases in the human lung are highlighted. (B) T-  
743 SNE clustering of cells from the human airway (25). Cells expressing the coronavirus receptor  
744 ACE2 are highlighted in the right panel. Cells expressing various S-activating proteases in the  
745 human airway are highlighted. (C) Log2-normalized expression data of the indicated gene across  
746 different human tissues from the GTEx consortium (33).

747

748 **Fig. 3. Activation of SARS-2-S by TMPRSS2-related proteases can be suppressed by**  
749 **camostat mesylate.** The experiment was performed as described for figure 1 with the  
750 modifications that only TMPRSS2, TMPRSS11D, TMPRSS11E, TMPRSS11F and TMPRSS13  
751 were investigated and target cells were pre-treated with either 50 mM ammonium chloride (red),  
752 100  $\mu$ M camostat mesylate (blue) or a combination of both (green). DMSO-treated cells served  
753 as controls. At 16 h post inoculation with viral particles bearing SARS-2-S, pseudotype entry was  
754 analyzed by measuring the activity of virus-encoded luciferase activity in cell lysates. Data were  
755 further normalized and entry efficiency into control-treated cells was set as 100 %. Shown are the  
756 average (mean) data obtained from three biological replicates, each performed in quadruplicates.  
757 Error bars indicate the SEM. Statistical significance of differences in entry efficiency in  
758 ammonium chloride-, camostat mesylate- or ammonium chloride + camostat mesylate-treated  
759 cells versus control-treated cells was analyzed by two-way ANOVA with Dunnett's posttest ( $p >$   
760 0.5, not significant [ns],  $p \leq 0.5$ , \*;  $p \leq 0.1$ , \*\*;  $p \leq 0.01$ , \*\*\*).

761  
762 **Fig. 4. Camostat mesylate and FOY-251 inhibit the activity of recombinant TMPRSS2.**  
763 TMPRSS2 cleaved Boc-Gln-Ala-Arg-MCA as substrate and produced the potent fluorophore,  
764 AMC(7-Amino-4-methylcoumarin). TMPRSS2 enzyme activity was evaluated by measuring the  
765 fluorescence intensity using Envision plate reader and all of the data were normalized against the  
766 intensity of the absence of test compounds. The concentration-response data for each test  
767 compound was plotted and modeled by a four-parameter logistic fit to determine the 50%  
768 inhibitory concentration ( $IC_{50}$ ) value. Inhibitory activity of camostat mesylate (blue), FOY-  
769 251(light blue) and GBA (red) against TMPRSS2 recombinant protein were visualized and curve  
770 fitting were performed using GraphPad Prism. The average of two independent experiments, each

771 performed with quadruplicate (camostat mesylate and FOY-251) or duplicate samples (GBA) is  
772 shown. IC<sub>50</sub> values were 4.2 nM (camostat mesylate), 70.3 nM (FOY-251), >10 μM (GBA).

773

774 **Fig. 5. TMPRSS2 protease domain and GBPA interaction.** A TMPRSS2 structure model is  
775 shown in the left panel, the active site is highlighted in cyan and catalytic triad residues are  
776 shown in black. The representative structure of GBPA bound to TMPRSS2 in a reactive complex  
777 is shown in the right panel. The GBPA guanidinium head forms a salt bridge with D435 inside  
778 the S1 pocket. This transient complex, which is similar for Camostat, is prone to be catalyzed at  
779 the ester bond interacting with Ser441, leading to a covalent complex with TMPRSS2 inhibited.

780

781 **Fig. 6. Camostat mesylate is rapidly converted into GBPA in the presence of cell culture**  
782 **medium. (A)** Metabolization of camostat mesylate. **(B)** LC-MS/MS determination of camostat,  
783 GBPA and GBA in culture medium containing FCS. Camostat mesylate was added to FCS-  
784 containing culture medium at a concentration of 15 μM. Samples were taken after incubation for  
785 1, 15, 30, 60, 120, 240, 480, and 1,440 min at 37 °C, snap-frozen and stored at -80 °C. Samples  
786 were analyzed by LC-MS/MS and quantified regarding their content of intact camostat mesylate  
787 and its metabolites GBPA (active) and GBA (inactive). Presented are the mean (average) data  
788 from three biological replicates (single samples). Error bars indicate the SEM. The turnover time  
789 that is required to cause metabolization of 50 % of camostat mesylate (T<sub>1/2</sub>) was further  
790 calculated by a non-linear regression model and was determined to be 141.3 min (95 %  
791 confidence interval = 116.5 to 171.7 min). **(C)** Relative levels of camostat mesylate and GBPA  
792 after incubation of 15 μM camostat mesylate in either water or FCS-containing culture medium.  
793 For normalization, the combined values of camostat mesylate and GBPA were set as 100 % and  
794 the relative fractions of the compounds were calculated. Presented are the mean (average) data

795 from three biological replicates (single samples). Error bars indicate SEM. Statistical significance  
796 of differences in GBPA levels following incubation of camostat mesylate in either water or FCS-  
797 containing culture medium was analyzed by paired, two-tailed student's t-test ( $p \leq 0.01$ , \*\*).  
798 Abbreviations: FOY-51/GBPA = 4-(4-guanidinobenzoyloxy)phenylacetic acid; GBA = 4-  
799 guanidinobenzoic acid.

800

801 **Fig. 7. Camostat mesylate and FOY-251 inhibit SARS-2-S-driven cell entry with**  
802 **comparable efficiency.** Calu-3 cells were pre-incubated with different concentrations of  
803 camostat mesylate (left panel), FOY-251 (right panel) or DMSO (control, indicated by dashed  
804 lines) for 2 h, before they were inoculated with pseudotype particles bearing VSV-G (red) or  
805 SARS-2-S (blue). Alternatively, in order to analyze potential negative effects of camostat  
806 mesylate and FOY-251 on cell vitality (grey bars), cells received medium instead of pseudotype  
807 particles and were further incubated. At 16 h post inoculation, pseudotype entry and cell vitality  
808 were analyzed by measuring the activity of virus-encoded luciferase activity in cell lysates or  
809 intracellular adenosine triphosphate levels (CellTiter-Glo assay), respectively. Data were further  
810 normalized against and entry efficiency/cell vitality in the absence of camostat mesylate and  
811 FOY-251 was set as 100 %. Shown are the average (mean) data obtained from three biological  
812 replicates, each performed in quadruplicates. Error bars indicate SEM. Statistical significance of  
813 differences in entry efficiency/cell vitality in camostat mesylate - or FOY-251-treated cells versus  
814 control-treated cells was analyzed by two-way ANOVA with Dunnett's posttest ( $p > 0.5$ , not  
815 significant [ns],  $p \leq 0.5$ , \*;  $p \leq 0.1$ , \*\*;  $p \leq 0.01$ , \*\*\*).

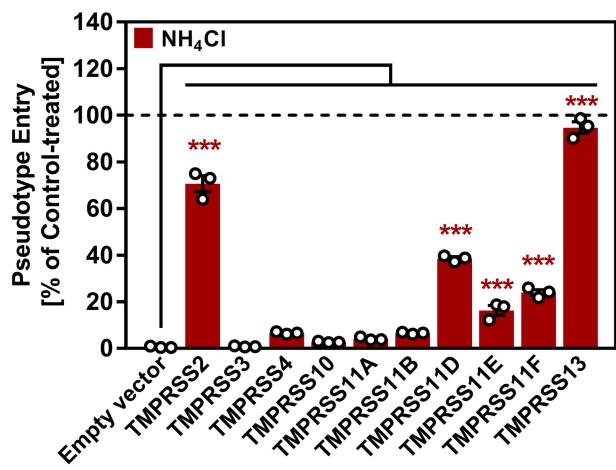
816

817 **Fig. 8. Camostat mesylate and FOY-251 inhibit SARS-CoV-2 infection with comparable**  
818 **efficiency.** Calu-3 cells were pre-incubated for 2h with double concentration of camostat

819 mesylate or FOY-251 as indicated. DMSO-treated cells served as control. Thereafter, cells were  
820 infected with SARS-CoV-2 at an MOI 0.001 or MOI 0.01 by adding the same volume of virus-  
821 containing culture medium to the inhibitor-treated cells. After 1 h of incubation, the inoculum  
822 was removed and cells were washed two times with PBS, before culture medium containing 1-  
823 fold inhibitor concentration was added. Culture supernatants were harvested at 24 h post  
824 infection, stored at -80°C and thereafter subjected to standard plaque formation assays using  
825 Vero-TMPRSS2 target cells and culture medium containing 1 % methyl cellulose. Plaques were  
826 counted at 48 h post infection and titers determined as plaque forming units per ml (pfu/ml).  
827 Presented are the data from a single experiment performed with technical triplicates and the  
828 results were confirmed in a separate experiment with another SARS-CoV-2 isolate. Error bars  
829 indicate the standard deviation.  
830

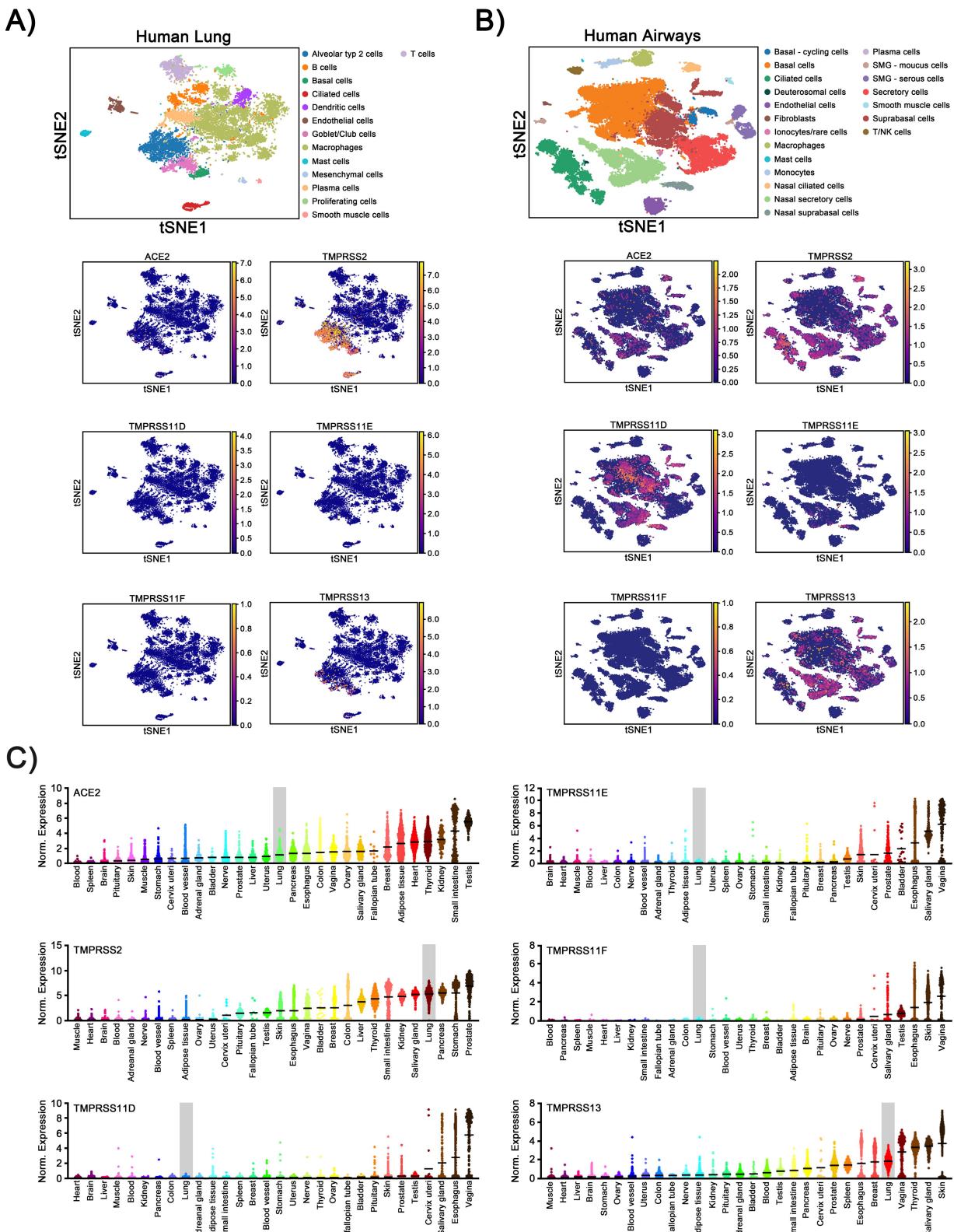
# Figure 1

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## Figure 2

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# Figure 3

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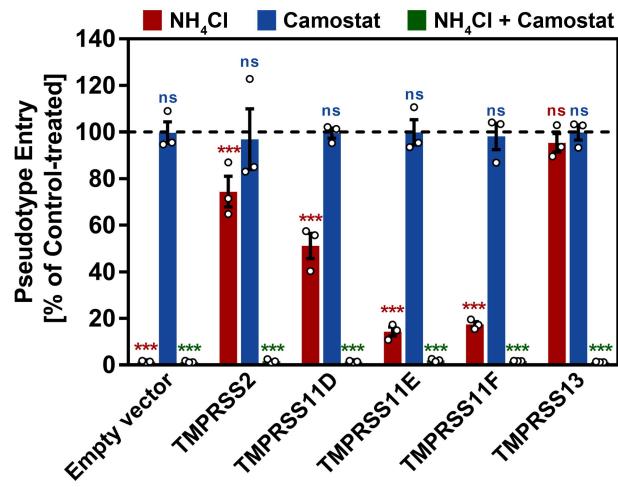


Figure 4

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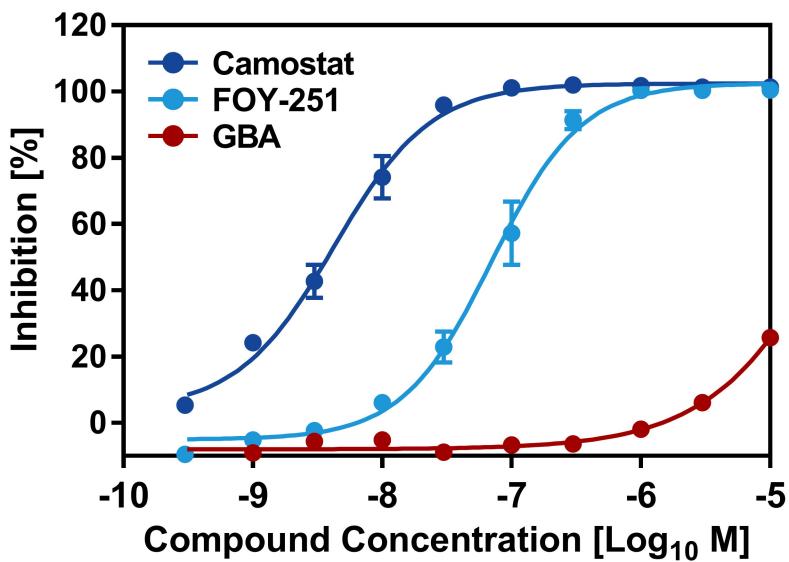
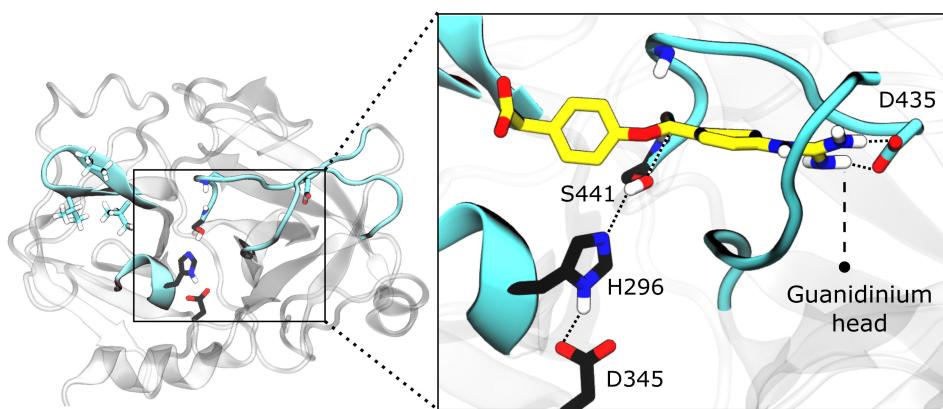


Figure 5

Hoffmann *et al.*, 2020

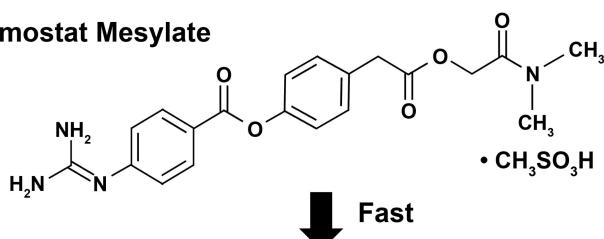


# Figure 6

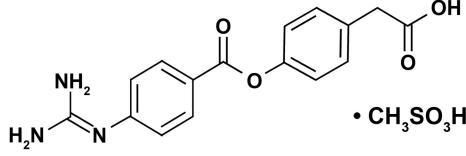
Hoffmann *et al.*, 2020

A)

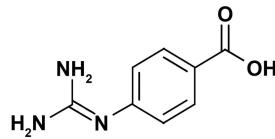
Camostat Mesylate



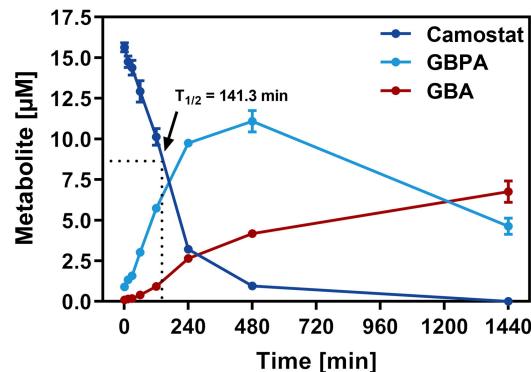
GBPA



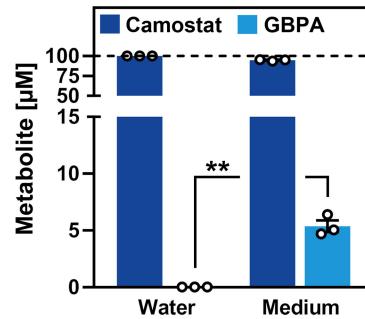
GBA



B)

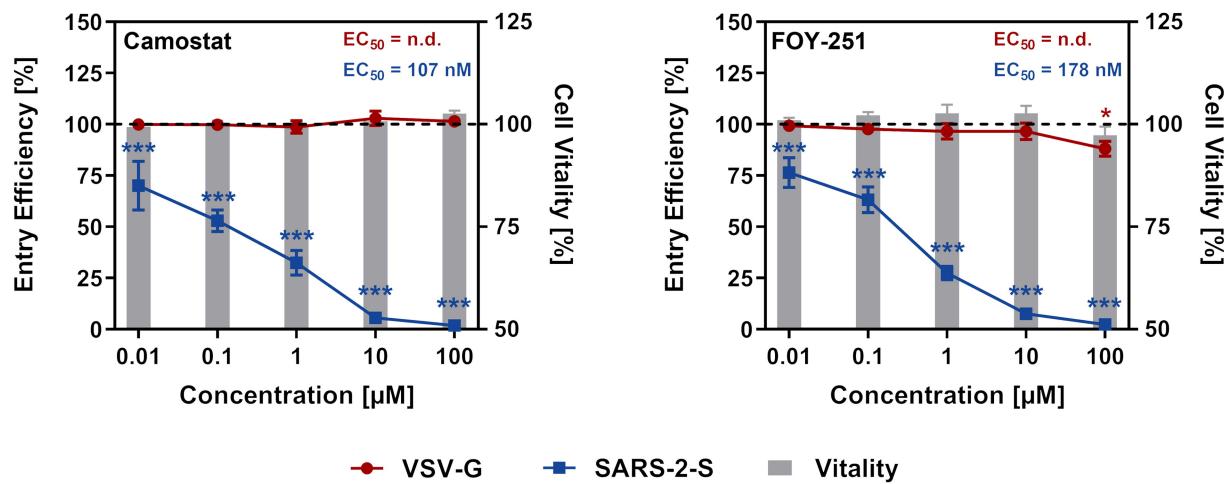


C)



# Figure 7

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# Figure 8

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