

1 **SARS-CoV-2 and SARS-CoV differ in their cell tropism and drug**
2 **sensitivity profiles**

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14 # equal contribution

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17

18 **Abstract**

19 SARS-CoV-2 is a novel coronavirus currently causing a pandemic. We show
20 that the majority of amino acid positions, which differ between SARS-CoV-2 and the
21 closely related SARS-CoV, are differentially conserved suggesting differences in
22 biological behaviour. In agreement, novel cell culture models revealed differences
23 between the tropism of SARS-CoV-2 and SARS-CoV. Moreover, cellular ACE2
24 (SARS-CoV-2 receptor) and TMPRSS2 (enables virus entry via S protein cleavage)
25 levels did not reliably indicate cell susceptibility to SARS-CoV-2. SARS-CoV-2 and
26 SARS-CoV further differed in their drug sensitivity profiles. Thus, only drug testing
27 using SARS-CoV-2 reliably identifies therapy candidates. Therapeutic concentrations
28 of the approved protease inhibitor aprotinin displayed anti-SARS-CoV-2 activity. The
29 efficacy of aprotinin and of remdesivir (currently under clinical investigation against
30 SARS-CoV-2) were further enhanced by therapeutic concentrations of the proton
31 pump inhibitor omeprazole (aprotinin 2.7-fold, remdesivir 10-fold). Hence, our study
32 has also identified anti-SARS-CoV-2 therapy candidates that can be readily tested in
33 patients.

34
35 **Key words:** severe acute respiratory syndrome coronavirus, severe acute respiratory
36 syndrome coronavirus 2, ACE2, 2019-nCoV, COVID-19, antiviral, drug discovery, cell
37 tropism, TMPRSS2, aprotinin, remdesivir, omeprazole

38

39 Introduction

40 In December 2019, SARS-CoV-2, a novel betacoronavirus, was identified that
41 causes a respiratory disease and pneumonia called coronavirus disease 19 (COVID-
42 19) [Chen et al., 2020; Coronaviridae Study Group of the International Committee on
43 Taxonomy of Viruses, 2020; Lu et al., 2020; Wu et al., 2020; Zhou et al., 2020; Zhu et
44 al., 2020]. The first cases seemed to have originated from a wholesale fish market in
45 Wuhan, China [Zhu et al., 2020]. As of 3rd April 2020, this novel virus has resulted in
46 1,041,126 confirmed infections and 55,132 deaths in 181 countries and regions
47 (www.who.int) [Dong et al., 2020].

48 SARS-CoV-2 is the seventh coronavirus known to infect and cause disease in
49 humans alongside the alphacoronaviruses human coronavirus 229E (HCoV-229E)
50 and human coronavirus NL63 (HCoV-NL63, New Haven coronavirus) and the
51 betacoronaviruses human coronavirus OC43 (HCoV-OC43), human coronavirus
52 HKU1 (HCoV-HKU1), severe acute respiratory syndrome coronavirus (SARS-CoV),
53 and Middle East respiratory syndrome coronavirus (MERS-CoV) [Corman et al., 2018;
54 Yin and Wunderink, 2018; Cui et al., 2019; Wu et al., 2020b]. HCoV-229E, HCoV-
55 OC43, HCoV-NL63, and HCoV-HKU1 are endemic in humans and typically cause mild
56 to moderate common cold-like respiratory disease [Channappanavar & Perlman,
57 2017; Corman et al., 2018].

58 Since 2002, SARS-CoV-2 is the third coronavirus, after SARS-CoV and MERS-
59 CoV, that has caused a substantial outbreak associated with significant mortality [Wu
60 et al., 2020b]. According to WHO, the SARS-CoV outbreak resulted in 8,098 confirmed
61 and suspected cases and 774 deaths, equalling a mortality rate of 9.6%
62 (www.who.int). For MERS-CoV, the WHO currently (2nd April 2020) reports 2,494
63 laboratory-confirmed cases and 858 deaths (mortality rate: 34.4%) (www.who.int).
64 However, human-to-human spread of MERS-CoV remains very limited. SARS-CoV-2
65 disease is associated with a lower mortality. Currently, about 5.3% of individuals with
66 confirmed SARS-CoV-2 infection have died, and the risk of severe disease increases
67 with age [Dong et al., 2020; CDC COVID-19 Response Team, 2020]. This mortality
68 level is likely to be an overestimation. A mortality rate of 1% or less may be more
69 realistic, because patients with severe symptoms are more likely to be tested, while
70 mild and asymptomatic cases are likely to go unreported [Borges do Nascimento,
71 2020; Nishiura et al., 2020; Pan et al., 2020; Rothe et al., 2020]. In contrast to SARS-
72 CoV-infected patients, SARS-CoV-2 has been reported to be spread by individuals
73 who are asymptomatic during the incubation period or who do not develop symptoms
74 at all [Li et al., 2020; Nishiura et al., 2020; Nishiura et al., 2020a; Pan et al., 2020;
75 Rothe et al., 2020; Yu et al., 2020]

76 We have developed an approach to identify sequence-associated phenotypic
77 differences between related viruses based on the identification of differentially
78 conserved amino acid sequence positions (DCPs) and in silico modelling of protein
79 structures [Pappalardo et al., 2016; Martell et al., 2019]. Here, we used this method to
80 identify differentially conserved positions that may explain phenotypic differences
81 between SARS-CoV-2 and SARS-CoV [Coronaviridae Study Group of the
82 International Committee on Taxonomy of Viruses, 2020; Lu et al., 2020; Zhou et al.,
83 2020]. These findings were analysed in combination with data from cells infected with
84 a recently derived SARS-CoV-2 isolate [Hoehl et al., 2020]. Our results reveal
85 characteristic differences between SARS-CoV-2 and SARS-CoV. Most importantly,
86 we found that therapeutic concentrations of the protease inhibitor aprotinin interfere
87 with SARS-CoV-2 infection. The efficacy of aprotinin can be further increased by
88 therapeutic concentrations of the proton pump inhibitor omeprazole.

89 Results

90

91 Determination of differentially conserved positions (DCPs)

92 Coronavirus genomes harbour single-stranded positive sense RNA (+ssRNA)
93 of about 30 kilobases in length, which contain six or more open reading frames (ORFs)
94 [Cui et al., 2019; Song et al., 2019; Chen et al., 2020b; Wu et al., 2020b]. The SARS-
95 CoV-2 genome has a size of approximately 29.8 kilobases and was annotated to
96 encode 14 ORFs and 27 proteins [Wu et al., 2020b]. Two ORFs at the 5'-terminus
97 (ORF1a, ORF1ab) encode the polyproteins pp1a and pp1b, which comprise 15 non-
98 structural proteins (nsps), the nsps 1 to 10 and 12-16 [Wu et al., 2020b]. Additionally,
99 SARS-CoV-2 encodes four structural proteins (S, E, M, N) and eight accessory
100 proteins (3a, 3b, p6, 7a, 7b, 8b, 9b, orf14) [Wu et al., 2020b]. This set-up resembles
101 that of SARS-CoV. Notable differences include that there is an 8a protein in SARS-
102 CoV, which is absent in SARS-CoV-2, that 8b is longer in SARS-CoV-2 (121 amino
103 acids) than in SARS-CoV (84 amino acids), and that 3b is shorter in SARS-CoV-2 (22
104 amino acids) than in SARS-CoV (154 amino acids) [Wu et al., 2020b].

105 To identify genomic differences between SARS-CoV-2 and SARS-CoV that
106 may affect the structure and function of the encoded virus proteins, we applied an
107 approach that we have previously used to compare the human pathogenic
108 Ebolaviruses species with Reston virus, an Ebolavirus that does not cause disease in
109 humans [Pappalardo et al., 2016; Martell et al., 2019]. This methodology is based on
110 the determination of differentially conserved positions (DCPs) [Rausell et al., 2010],
111 i.e. amino acid positions that are differently conserved between phenotypically
112 different groups, in our case related viruses. The potential impact of the DCPs on
113 protein structure and function is then determined by in silico modelling [Pappalardo et
114 al., 2016; Martell et al., 2019].

115 For the 22 SARS-CoV-2 virus proteins that could be compared with SARS-CoV,
116 comparison of the two reference sequences identified 1393 positions that encode
117 different amino acids. 1243 (89%) of these positions were DCPs (Table 1), which
118 represents 13% of all residues encoded by the SARS-CoV-2 genome. Most of the
119 amino acid substitutions at DCPs appear to be fairly conservative as demonstrated by
120 the average BLOSUM substitution score of 0.49 (median 0; Supplementary Figure 1)
121 and with 73% of them having a score of 0 or greater (the higher the score the more
122 frequently such amino acid substitutions are observed naturally in evolution). It
123 followed that 45% of DCPs represent conservative changes where amino acid
124 properties are retained (e.g. change between two hydrophobic amino acids), a further
125 30% represented polar - hydrophobic substitutions, while changes between charged
126 amino acids were rare (<10% of DCPs) (Supplementary Table 1).

127 DCPs are enriched in six of the SARS-CoV-2 proteins, spike (S), 3a, p6, nsp2,
128 nsp3 (papain-like protease) and nsp4 with 19.4%, 21.5%, 28.6%, 28.6%, 21.3% and
129 18.8% of their residues being DCPs respectively (Table 1). In contrast, very few DCPs
130 were observed in the envelope (E) protein and most of remaining non-structural
131 proteins encoded by ORF1ab, for example 0.5% of residues in the helicase and 2%
132 of residues in the RNA-directed RNA polymerase, 2'-O-Methyltransferase, nsp8 and
133 nsp9 are DCPs (Table 1).

134 The availability of structures of both SARS-CoV and some SARS-CoV-2
135 proteins, coupled with the ability to model some of the remaining proteins (ref to
136 methods and supplementary table) enabled us to map 525 DCPs onto protein

137 **Table 1.** Specificity Determining Positions (DCPs) identified between SARS-CoV and
138 SARS-CoV-2.

Protein (SARS-CoV)	Protein (SARS-CoV-2)	Sequences Dataset	in	Protein Length (SARS-CoV)	DCPs Identified	% of Residues DCPs
S	S	1326		1255	243	19.4
3a	ORF3a	1377		274	59	21.5
3b		n/a		154		
E	E	1377		76	3	4.0
M	M	1372		221	16	7.2
6	6	1380		63	18	28.6
7a	7a	1376		122	11	9.0
7b	7b	n/a		44	NA	
8a/8b	8	21		39/84	NA	NA
9b		n/a		98	NA	
N	N	1379		422	26	6.2
	ORF10	n/a		n/a		
nsp1	nsp1	1288		180	22	12.2
nsp2	nsp2	1288		636	182	28.6
Nsp3	nsp3	1288		1922	409	21.3
nsp4	nsp4	1288		500	94	18.8
nsp5	nsp5	1288		306	10	3.3
nsp6	nsp6	1288		290	36	12.4
nsp7	nsp7	1288		83	1	1.2
nsp8	nsp8	1288		198	6	3.0
nsp9	nsp9	1288		113	3	2.7
nsp10	nsp10	1288		139	4	2.9
nsp12	nsp12	1281		932	22	2.4
nsp13	nsp13	1281		601	3	0.5
nsp14	nsp14	1281		527	29	5.5
nsp15	nsp15	1281		346	32	9.3
nsp16	nsp16	1281		298	14	4.7
Total					1243	13.1

139 structures (Supplementary Figure 1, Supplementary Table 1). Overall, nearly all of the
140 mapped DCPs occur on the protein surface (92%), with only 40 DCPs buried within
141 the protein, primarily in S and the papain-like protease (nsp3) (Supplementary Table
142 1). Based on our structural analysis, we propose that 45 DCPs are likely to result in
143 structural (or functional) differences between SARS-CoV and SARS-CoV-2 proteins.
144 A further 222 could result in some change, with our analysis suggesting that the
145 remaining 258 DCPs seem unlikely to have a substantial effect on protein structure
146 and function.
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150 **Differentially conserved positions (DCPs) in interferon antagonists**

151 At least 10 SARS-CoV proteins have roles in interferon antagonism [Totura and
152 Baric, 2012]. Two of these proteins, p6 and the papain-like protease (nsp3), are
153 enriched in DCPs, two are depleted in DCPs (nsp7 and nsp16), five have intermediate
154 proportions of DCPs (nsp14, nsp1, nsp15, N and M), while p3b is not encoded by
155 SARS-CoV-2. Initial studies have identified a difference in the interferon inhibition
156 between SARS-CoV and SARS-CoV-2 [Lokugamage et al., 2020], so it is possible
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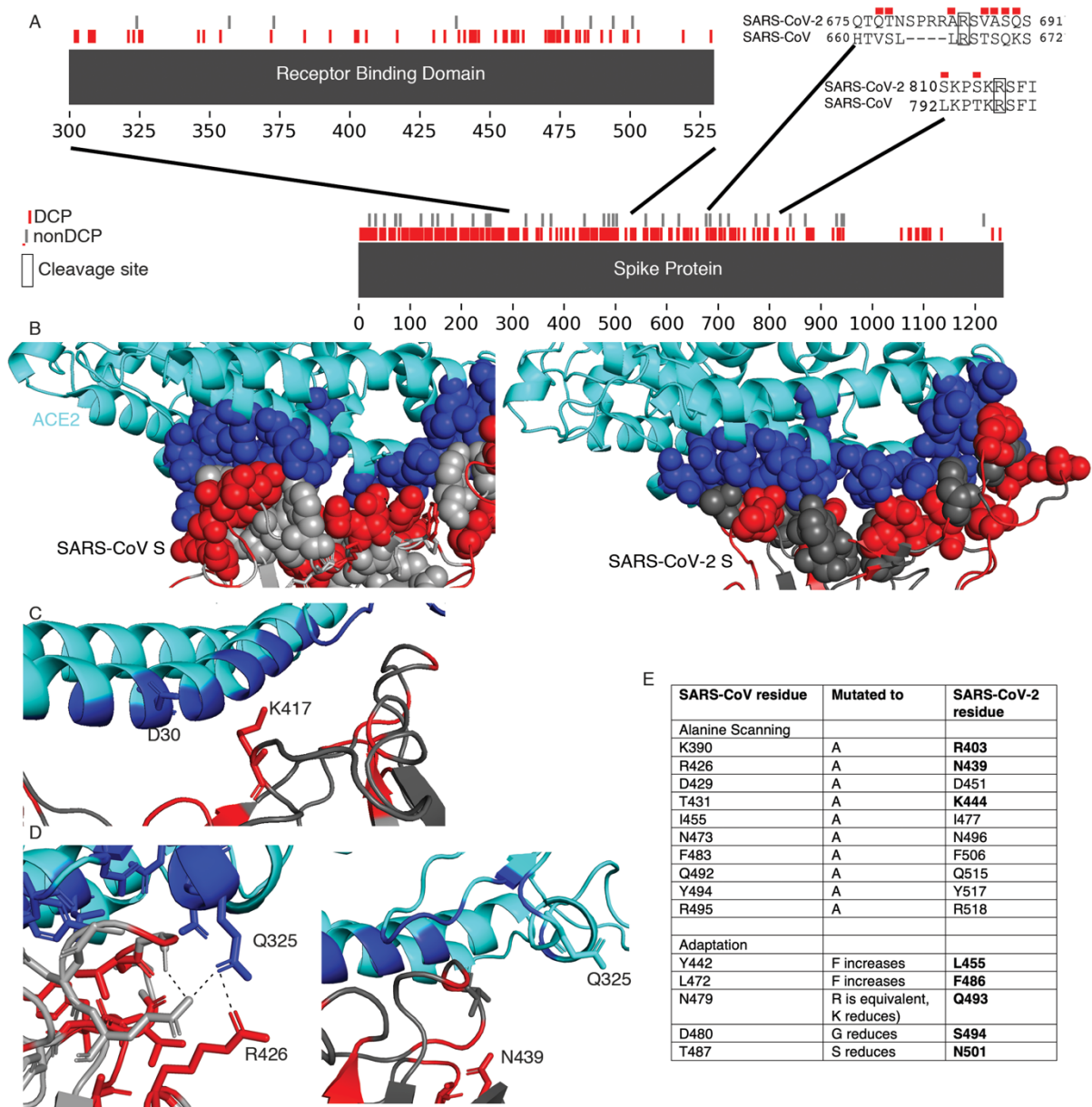
158 that the DCPs identified in these proteins, especially in p6 and the papain-like
159 protease, may have an effect on interferon inhibition.
160

161 **S (Spike) protein**

162 The most interesting changes were detected in the spike (S) protein, which
163 mediates coronavirus entry into host cells [Cui et al., 2019; Chen et al., 2020b]. SARS-
164 CoV-2 S is 77.46% sequence identical to the SARS-CoV S and most of the remaining
165 positions are DCPs (243 residues, 1%) (Table 1). SARS-CoV entry depends on the
166 cleavage of S by transmembrane serine protease 2 (TMPRSS2), the endosomal
167 cysteine protease cathepsin L, and/ or other cellular proteases, with residues R667
168 and R797 being critical cleavage sites [Matsuyama et al., 2010; Simmons et al., 2013;
169 Zhou et al., 2015; Reinke et al., 2017; Iwata-Yoshikawa et al., 2019]. Serine protease
170 inhibitors such as camostat and nafamostat interfere with S cleavage by TMPRSS2
171 and virus uptake [Kawase et al., 2012; Yamamoto et al., 2016; Zhou et al., 2015; Shin
172 & Seong, 2017]. R667 and R797 are conserved in SARS-CoV-2 (R685 and R815).
173 However, there is a four amino acid insertion in SARS-CoV-2 S prior to R685 and
174 many of the residues close to R685 are DCPs (V663=Q677, S664=T678, T669=V687,
175 S670=A688, Q671=S689, DCPs are represented by the SARS CoV residue followed
176 by the SARS-CoV-2 residue) (Figure 1A). There is greater conservation around the
177 R815 cleavage site with only two DCPs in close proximity (L792=S810, T795=S813)
178 (Figure 1A).

179 The SARS-CoV S receptor binding domain (residues 306-527, equivalent to
180 328-550 in SARS-CoV-2) is enriched in DCPs, containing 51 DCPs (23% of residues).
181 Eleven of the 24 SARS-CoV S residues in direct contact with ACE2 were DCPs (Figure
182 1A, Supplementary Table 2). Analysis of the DCPs using the SARS-CoV and SARS-
183 CoV-2 S protein complexes with ACE2 [Song et al., 2018; Yan et al., 2020] identified
184 runs of DCPs (A430-T433, F460-A471) in surface loops forming part of the S-ACE2
185 interface and resulted in different conformations in SARS-CoV-2 S compared to
186 SARS-CoV S (Figure 1B). Two DCPs remove intramolecular hydrogen bonding within
187 the spike protein in SARS-CoV-2 (Supplementary Table 2) and three DCPs
188 (R426=N439, N479=QQ493, Y484=Q498) are residues that form hydrogen bonds
189 with ACE2. For two of these positions, hydrogen bonding with ACE2 is present with
190 both S proteins, but for R426=N439 hydrogen bonding with ACE2 is only observed
191 with SARS-CoV S. N439 in SARS-CoV-2 S is not present in the interface and the
192 sidechain points away from the interface (Figure 1D). Further, analysis of the SARS-
193 CoV-2 S-ACE2 complex highlighted important roles of the V404=K417 DCP, where
194 K417 in SARS-CoV-2 S is able to form a salt bridge with ACE2 D30 (Figure 1C) [Yan
195 et al., 2020].

196 Alanine scanning [Chakraborti et al., 2005] and adaptation experiments [Wan
197 et al., 2020] have identified 16 SARS-CoV S residues associated with determining the
198 binding affinity with ACE2. For all five residues identified from adaptation studies and
199 four of the 11 identified by alanine scanning experiments different amino acids are
200 present in SARS-CoV-2 S (Figure 1E), highlighting the difference in the interaction
201 with ACE2.

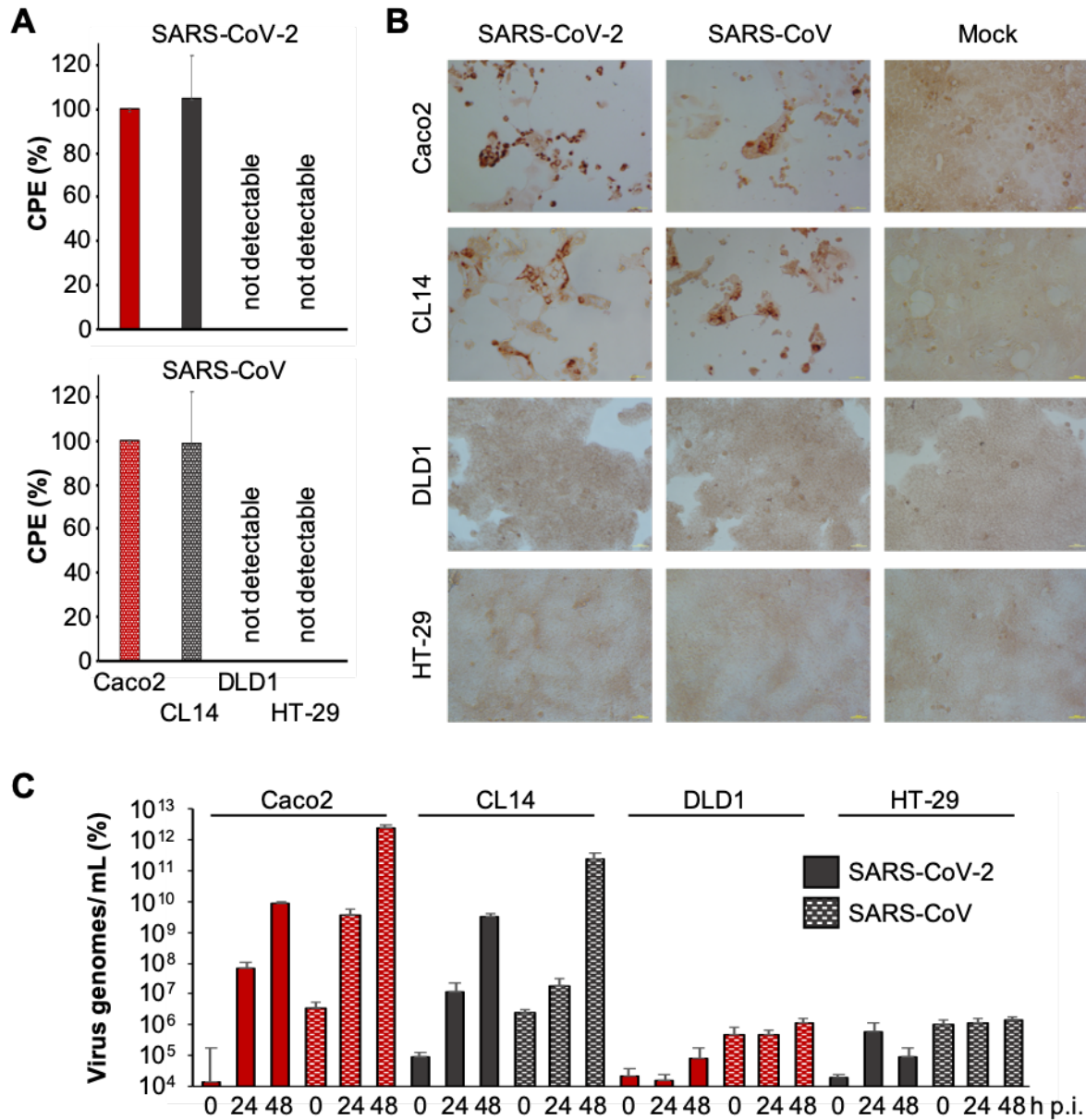


202
 203 **Figure 1.** Differentially conserved positions in the Spike protein. A) A sequence view
 204 of the DCPs present in the Spike protein, with insets showing the receptor binding
 205 domain and the two cleavage sites. B) The S interface with ACE2 (cyan). The ACE2
 206 interface is shown in blue spheres, DCPs in red. C) The V404=K417 DCP. D) The
 207 R426=N439 DCP, the left image shows SARS-CoV S R426, the image on the right
 208 show the equivalent N439 in SARS-CoV-2 S. E) SARS-CoV residues associated with
 209 altering ACE2 affinity and the residues at these positions in SARS-CoV-2 S.

210
 211 **SARS-CoV-2 replication in different cell lines**

212 In further experiments, we investigated to see the extent to which the
 213 substantial number of amino acid positions that are differently conserved between
 214 SARS-CoV and SARS-CoV-2, result in different phenotypes. Infection experiments
 215 using the four colorectal cancer cell lines Caco2, CL14, HT-29, and DLD-1 resulted in
 216 similar susceptibility profiles. Replication of both viruses was detected in Caco2 and
 217 CL14 cells, but not in HT-29 or DLD-1 cells, as shown by cytopathogenic effects (CPE)

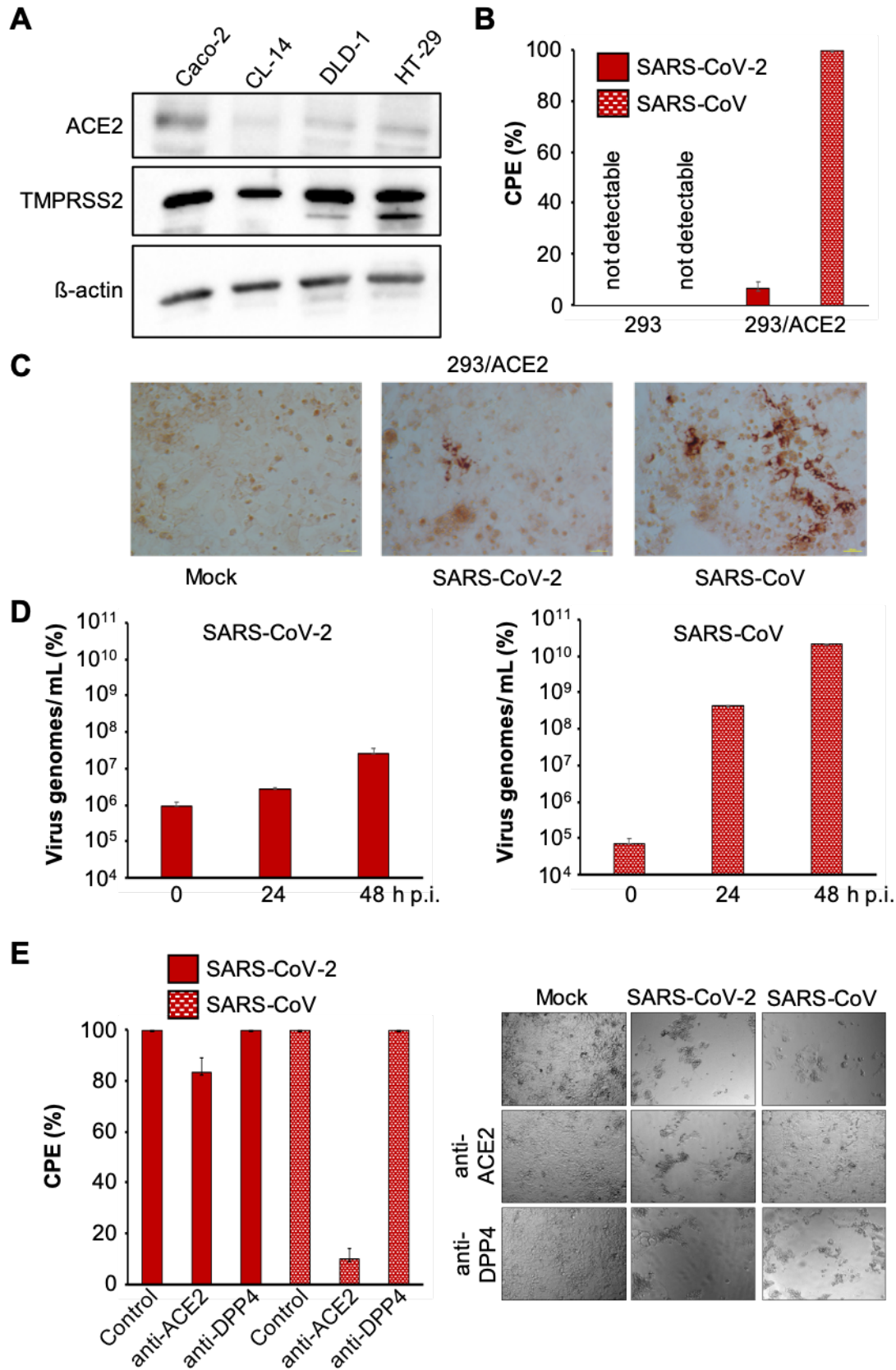
218 (Figure 2A), staining for double-stranded RNA (Figure 2B), and viral genomic RNA
 219 levels (Figure 2C). These findings are in line with previous findings showing that Caco2
 220 and CL14 cells are susceptible to SARS-CoV infection [Cinatl et al., 2004] and the
 221 previous isolation of SARS-CoV-2 cells in Caco2 cells [Hoehl et al., 2020]. Moreover,
 222 we identified CL14 as an additional model to study SARS-CoV-2 infection and
 223 replication.
 224



225
 226 **Figure 2.** SARS-CoV-2 and SARS-CoV susceptibility of colorectal cancer cell lines.
 227 A) Cytopathogenic effect (CPE) formation 48h post infection in MOI 0.01-infected
 228 cells. B) Representative images showing MOI 0.01-infected cells immunostained for
 229 double-stranded RNA 48h post infection. C) Quantification of virus genomes by qPCR
 230 at different time points post infection (p.i.).
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233 **SARS-CoV-2 infection does not correlate with the cellular ACE2 status**

234 Although ACE2 was identified as a SARS-CoV-2 receptor [Hoffmann et al.,
235 2020; Letko et al., 2020; Walls et al., 2020; Wan et al., 2020; Wrapp et al., 2020; Yan
236 et al., 2020; Zhou et al., 2020], there was no correlation between the cellular ACE2
237 levels and the cellular susceptibility to SARS-CoV-2 (and SARS-CoV) (Figure 3A).
238 CL14 cells displayed lower ACE2 levels than both HT-29 and DLD-1 (Figure 3A),
239 although CL14 was, in contrast to HT-29 and DLD-1, permissive to SARS-CoV-2 (and
240 SARS-CoV) infection (Figure 2). This suggests that there are other factors in addition
241 to the cellular ACE2 levels that determine cellular susceptibility to SARS-CoV-2 and
242 SARS-CoV.



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Figure 3. N A) Western blots indicating cellular ACE2 and TMPRSS2 protein levels. B) CPE formation in SARS-CoV and SARS-CoV-2 (MOI 0.01)-infected ACE2-negative 293 cells and 293 cells stably expressing ACE2 cells (293/ACE2) 48h post

248 infection. C) Immunostaining for double-stranded RNA in SARS-CoV-2 and SARS-
249 CoV (MOI 0.01)-infected 293/ACE2 cells 48h post infection. D) Quantification of virus
250 genomes by qPCR in SARS-CoV-2 and SARS-CoV (MOI 0.01)-infected 293/ACE2
251 cells 48h post infection. E) Cytopathogenic effect (CPE) formation in SARS-CoV-2 and
252 SARS-CoV (MOI 0.01)-infected Caco2 cells in the presence of antibodies directed
253 against ACE2 or DPP4 (MERS-CoV receptor) 48h post infection.
254

255 **ACE2 expression mediates 293 cell susceptibility to SARS-CoV but not to SARS-** 256 **CoV-2**

257 Next, we compared SARS-CoV-2 and SARS-CoV replication dependence on
258 ACE2 in an additional model. 293 cells are not susceptible to SARS-CoV infection due
259 to a lack of ACE2 expression. However, 293 cells that stably express ACE2
260 (293/ACE2) support SARS-CoV infection [Kamitani et al., 2006]. As expected,
261 infection of 293 cells with SARS-CoV or SARS-CoV-2 did not result in detectable
262 cytopathogenic effect (CPE) (Figure 3B), but a SARS-CoV-induced CPE was detected
263 in 293/ACE2 cells (Figure 3B). In contrast to SARS-CoV, however, 293/ACE2 cells
264 displayed limited permissiveness to SARS-CoV-2 infection (Figure 3B). Staining for
265 double-stranded RNA (Figure 3C) and detection of viral genomic RNA copies (Figure
266 3D) confirmed reduced SARS-CoV-2 infection of and replication in 293/ACE2 cells
267 relative to SARS-CoV. These findings further suggest that there are differences in the
268 host cell factors that mediate SARS-CoV and SARS-CoV-2 susceptibility and in turn
269 differences in the cell tropisms.
270

271 **Reduced activity of anti-ACE2 antibody against SARS-CoV-2 compared to** 272 **SARS-CoV**

273 Antibodies directed against ACE2 have been shown to inhibit SARS-CoV
274 replication [Li et al., 2003]. In agreement, an anti-ACE2 antibody inhibited SARS-CoV
275 infection in Caco2 cells (Figure 3E). However, the anti-ACE2 antibody displayed
276 limited activity against SARS-CoV-2 infection (Figure 3E). This is in agreement with
277 previous findings indicating a stronger binding affinity of SARS-CoV-2 S to ACE2
278 compared to SARS-CoV S [Walls et al., 2020; Wrapp et al., 2020], which may be more
279 difficult to antagonise using anti-ACE2 antibodies. As anticipated, antibodies directed
280 against DPP4, the MERS-CoV receptor [de Wit et al., 2016; Cui et al., 2019], did not
281 interfere with SARS-CoV or SARS-CoV-2 infection (Figure 3E).
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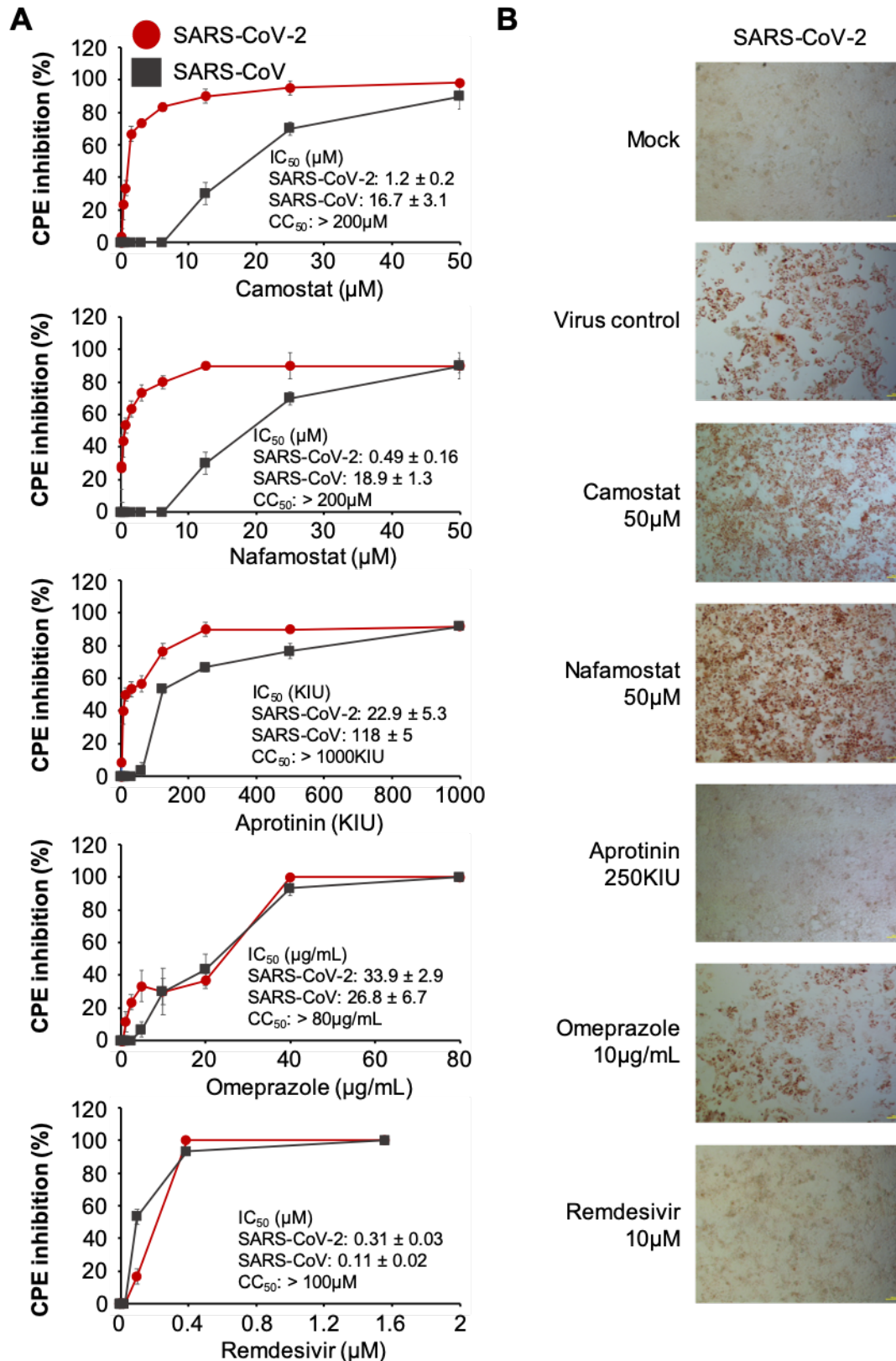
283 **SARS-CoV-2 is more sensitive to TMPRSS2 inhibitors than SARS-CoV**

284 SARS-CoV S and SARS-CoV-2 S are cleaved and activated by TMPRSS2
285 (transmembrane serine protease 2) [Matsuyama et al., 2010; Hoffmann et al., 2020;
286 Matsuyama et al., 2020]. Notably, all four cell lines, which we had tested for
287 susceptibility to SARS-CoV-2 replication, displayed similar TMPRSS2 levels (Figure
288 3A). Hence, cellular permissiveness to SARS-CoV-2 infection is determined by further
289 host cell factors in addition to TMPRSS2 and ACE2.

290 Previous findings had shown that the serine protease inhibitor camostat, which
291 is approved for the treatment of chronic pancreatitis in Japan [Ramsey et al., 2019],
292 inhibits both SARS-CoV and SARS-CoV-2 cell entry via interference with TMPRSS2-
293 mediated S cleavage [Kawase et al., 2012; Zhou et al., 2015; Hoffmann et al., 2020].
294 Camostat inhibited cell entry of VSV pseudotypes bearing SARS-CoV-2 S in a
295 concentration-dependent manner [Hoffmann et al., 2020]. Control experiments using
296 wild-type virus were only performed using a single high camostat concentration of 100
297 μM . Here, we directly compared the concentration-dependent effects of camostat on

298 SARS-CoV-2- and SARS-CoV-induced CPE formation in Caco2 cells (Figure 4A).
299 Camostat displayed nearly 14-fold increased activity against SARS-CoV-2
300 (concentration that inhibits CPE formation by 50%, IC_{50} 1.20 μ M) compared to SARS-
301 CoV (IC_{50} 16.7 μ M) (Figure 4A). Nafamostat is an alternative serine protease inhibitor,
302 which is approved for pancreatitis and [Minakata et al., 2019; Hirota et al., 2020] and
303 has been shown to exert superior effects against MERS-CoV compared to camostat
304 [Yamamoto et al., 2016].

305 Nafamostat displayed higher activity against SARS-CoV-2 CPE formation (IC_{50}
306 0.49 μ M) than camostat, but similar activity against SARS-CoV (18.9 μ M) (Figure 4A).
307 Therapeutic plasma levels for both compounds were described to reach about 0.2 μ M
308 [Hiraku et al., 1982; Cao et al., 2008], which is below the antivirally active
309 concentrations. Moreover, although both compounds inhibited SARS-CoV-2-induced
310 CPE formation, they displayed limited effects on the SARS-CoV-2 replication cycle as
311 indicated by high levels of double-stranded RNA in both nafamostat- and camostat-
312 treated SARS-CoV-2-infected cells (Figure 4B). Hence, both camostat and nafamostat
313 may primarily exert cytoprotective effects in SARS-CoV-2-infected cells, which inhibit
314 syncytium formation and cell lysis, but may not inhibit SARS-CoV-2 replication in the
315 same way.



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Figure 4. Anti-SARS-CoV-2 effects of antiviral drug candidates. A) Concentration-dependent effects of drug candidates on SARS-CoV-2- and SARS-CoV-induced cytopathogenic effect (CPE) formation determined 48h post infection in Caco2 cells infected at an MOI of 0.01. B) Immunostaining for double-stranded RNA in drug-treated SARS-CoV-2 (MOI 0.01)-infected cells 48h post infection. Camostat and

322 nafamostat prevent SARS-CoV-2-mediated cell lysis but are characterised by high
323 levels of double-stranded RNA.

324 Aprotinin is a further serine protease inhibitor that has been previously
325 investigated against influenza viruses [Zhirnov et al., 2011; Shen et al., 2017]. It is
326 used to reduce blood loss during surgery and for pancreatitis [Moggia et al., 2017;
327 Kapadia et al., 2019]. The efficacy of aprotinin is measured in kallikrein inhibitor units
328 (KIU) [Levy et al., 1994; Zhirnov et al., 2011]. Like the other serine protease inhibitors,
329 aprotinin was also more effective against SARS-CoV-2-induced CPE formation (IC_{50}
330 22.9 KIU/mL) than against SARS-CoV (IC_{50} 118 KIU/mL) (Figure 4A). In addition and
331 in contrast to nafamostat and camostat, aprotinin also inhibited double-stranded RNA
332 formation in SARS-CoV-2-infected cells (Figure 4B). Therapeutic aprotinin plasma
333 levels were described to reach 147 ± 61 KIU/mL after the administration of 1,000,000
334 KIU [Levy et al., 2019]. Moreover, an aerosol preparation of aprotinin is approved for
335 the treatment of influenza in Russia [Zhirnov et al., 2011]. Since aprotinin interferes
336 with SARS-CoV-2 in therapeutic concentrations and displays more pronounced direct
337 antiviral effects than camostat and nafamostat, it seems to have a greater potential for
338 the treatment of SARS-CoV-2-infected individuals based on our data.

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341 **Testing of additional antiviral drug candidates**

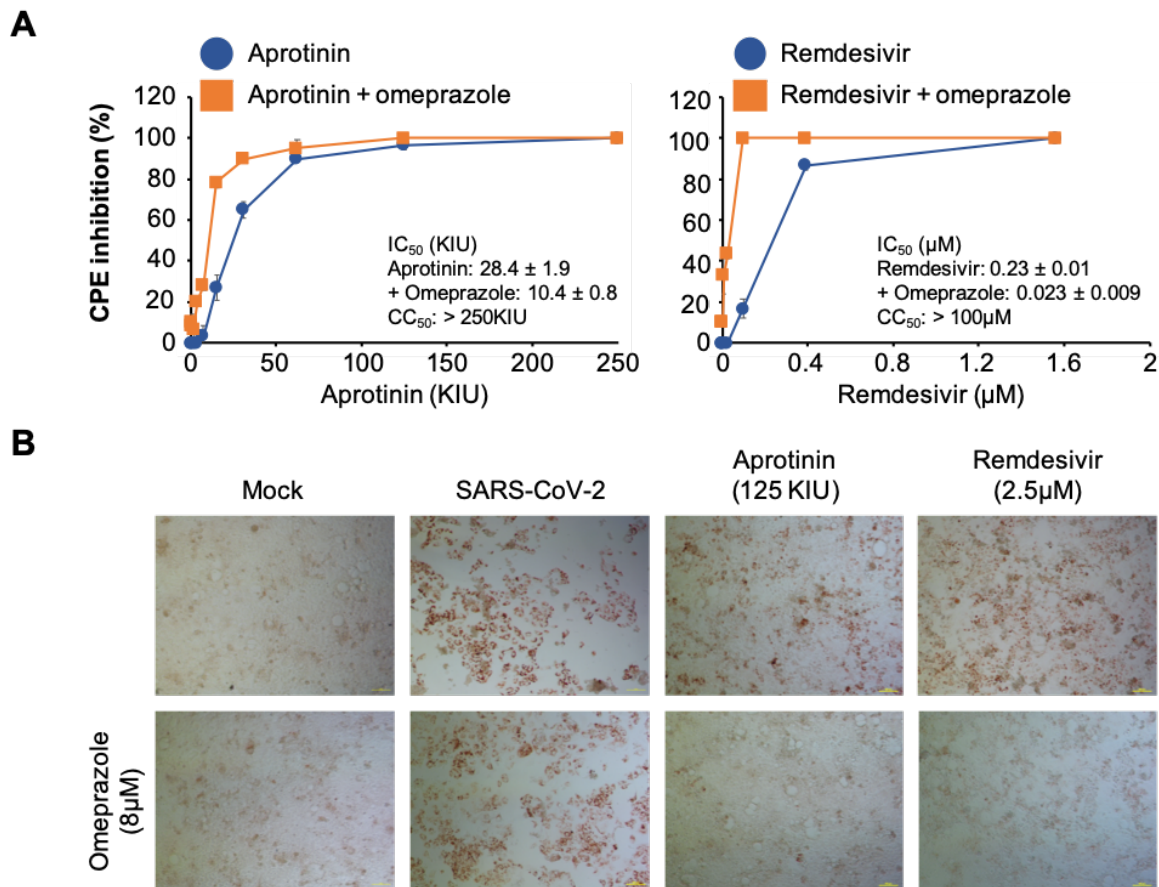
342 To investigate whether there are also differences in the drug sensitivity profiles
343 of SARS-CoV-2 and SARS-CoV to other antiviral drug candidates, we tested two
344 further compounds (Figure 4A). Previous research had shown that
345 hydroxychloroquine and ammonium chloride interfere with SARS-CoV and SARS-
346 CoV-2 replication, as lysosomotropic agents that increase the pH in lysosomes [Talbot
347 and Vance, 1980; Randolph and Stollar, 1990; Touret and de Lamballerie, 2020; Wang
348 et al., 2020; Hoffmann et al., 2020]. Proton pump inhibitors including omeprazole may
349 also inhibit virus replication by lysosomotropic and/ or other mechanisms [Dowall et
350 al., 2016; Strickland et al., 2017; Watanabe et al., 2020]. Thus, we included
351 omeprazole in our study. Moreover, we tested remdesivir, a drug that was developed
352 for the treatment of flavivirus infections and displayed activity against a range of RNA
353 viruses [Beigel et al., 2019; Hoenen et al., 2019]. Most recently, remdesivir was found
354 to inhibit MERS-CoV and SARS-CoV-2 and suggested as a therapy candidate for
355 SARS-CoV-2 infection [de Wit et al., 2020; Sheahan et al., 2020; Wang et al., 2020].
356 Currently (as of 31st March 2020), there are eight active clinical trials investigating
357 remdesivir for SARS-CoV-2-infected individuals (www.clinicaltrials.gov). Omeprazole
358 inhibited both viruses in similar concentrations, and SARS-CoV was more sensitive to
359 remdesivir than to SARS-CoV-2 (Figure 4A). Both omeprazole and remdesivir also
360 inhibited the formation of double-stranded RNA (Figure 4B)

361

362 **Omeprazole increases the anti-SARS-CoV-2 activity of remdesivir and aprotinin**

363 Omeprazole is a well-tolerated drug and a promising candidate for drug
364 repurposing strategies [Ikemura et al., 2017]. However, the omeprazole
365 concentrations that interfered with SARS-CoV-2 CPE formation (IC_{50} 34 μ M) was
366 beyond therapeutic omeprazole plasma concentrations reported to reach about 8 μ M
367 [Shin & Kim, 2013]. We have recently shown that omeprazole increases the antiviral
368 activity of acyclovir [Michaelis et al., 2019]. Here, we combined both aprotinin and
369 remdesivir with a fixed omeprazole concentration of 8 μ M, which resulted in further
370 increased activity against CPE formation (aprotinin 2.7-fold, remdesivir 10-fold)
371 (Figure 5A) and double-stranded RNA formation (Figure 5B). Hence, combinations of

372 aprotinin and remdesivir with omeprazole may represent therapy candidates for the
373 treatment of SARS-CoV-2-associated disease.



374 **Figure 5.** Anti-SARS-CoV-2 effects of omeprazole in combination with aprotinin and
375 remdesivir. A) Effect of omeprazole 8μM on cytopathogenic effect (CPE) formation in
376 combination with aprotinin and remdesivir in SARS-CoV-2 (MOI 0.01)-infected Caco2
377 cells 48h post infection. B) Immunostaining for double-stranded RNA indicating
378 combined effects of aprotinin and remdesivir in combination with omeprazole in SARS-
379 CoV-2 (MOI 0.01)-infected Caco2 cells 48h post infection.
380

381 Discussion

382 Here, we performed an in-silico analysis of the effects of differentially
383 conserved amino acid positions (DCPs) between SARS-CoV-2 and SARS-CoV
384 proteins on virus protein structure and function in combination with a comparison of
385 wild-type SARS-CoV-2 and SARS-CoV in cell culture.

386 Our analysis identified 1243 DCPs, which represents 89% of the amino acid
387 positions that differ between SARS-CoV-2 and SARS-CoV and nearly 13% of all
388 residues encoded by the SARS-CoV genome. 258 of these DCPs (2.6% of all
389 residues) are likely to have a structural and functional impact. The DCPs are not
390 equally distributed between the proteins. DCPs are enriched in S, 3a, p6, nsp2,
391 papain-like protease, and nsp4, but very few DCPs are present in the envelope (E)
392 protein and most of the remaining non-structural proteins encoded by ORF1ab. This
393 indicates that the individual proteins differ in their tolerance to sequence changes and/
394 or their exposure to selection pressure exerted by the host environment.

395 This large proportion of DCPs reflects the differences in the clinical behaviour
396 of SARS-CoV-2 and SARS-CoV. The mortality associated with SARS-CoV is
397 substantially higher than that associated with SARS-CoV-2 (www.who.int) [Dong et
398 al., 2020; Nishiura et al., 2020a; Pan et al., 2020; Rothe et al., 2020]. While SARS-
399 CoV causes a disease of the lower respiratory tract, and infected individuals are only
400 contagious when they experience severe symptoms [de Wit et al., 2016], SARS-CoV-
401 2 is present in the upper respiratory tract and seems to be readily transmitted via
402 droplets and direct contact prior to the onset of symptoms. Moreover, mild but
403 infectious cases may substantially contribute to its spread [Li et al., 2020; Rothe et al.,
404 2020; Yang et al., 2020; Yu et al., 2020]. Further research will be required to elucidate
405 in detail, which DCPs are responsible for which differences in virus behaviour.

406 However, we have already identified a number of differences between SARS-
407 CoV-2 and SARS-CoV, with regard to their cell tropism and drug sensitivity profiles.
408 Both viruses use ACE2 as a receptor and are activated by the transmembrane serine
409 protease TMPRSS2 [Li et al., 2003; Matsuyama et al., 2010; Cui et al., 2019;
410 Hoffmann et al., 2020; Letko et al., 2020; Lu et al., 2020; Matsuyama et al., 2020; Wals
411 et al., 2020; Wan et al., 2020; Wetko et al., 2020; Wrapp et al., 2020; Yan et al., 2020;
412 Zhou et al., 2020]. Our results show, however, that the ACE2 and the TMPRSS2 status
413 are not sufficient to predict cells susceptibility to SARS-CoV-2 or SARS-CoV. We
414 found that the colorectal cancer cell line CL14 supported SARS-CoV-2 replication,
415 although it displayed lower ACE2 levels and similar TMPRSS2 levels to the non-
416 susceptible cell lines DLD-1 and HT29. Hence CL14 represents a novel additional
417 model for the studying of SARS-CoV-2 replication. Notably, attempts to identify SARS-
418 CoV-2 target cells based on the ACE2 status [Luan et al., 2020; Qiu et al., 2020; Xu
419 et al., 2020] need to be considered with caution in the light of our current findings.

420 As previously described [Kamitani et al., 2006], ACE2 expression rendered
421 SARS-CoV non-permissive 293 cells susceptible to SARS-CoV. However, the effects
422 of ACE2 expression had a substantially lower impact on SARS-CoV-2 infection,
423 indicating differences in other host cell determinants of SARS-CoV and SARS-CoV-2
424 susceptibility. Moreover, an anti-ACE2 antibody displayed higher efficacy against
425 SARS-CoV than against SARS-CoV-2. This may be explained by an increased SARS-
426 CoV-2 S affinity to ACE2 compared to SARS-CoV S [Wrapp et al., 2020], which may
427 be more difficult to antagonise.

428 The serine protease inhibitors camostat, nafamostat, and aprotinin inhibited
429 both SARS-CoV-2 and SARS-CoV CPE formation. In contrast to aprotinin, camostat,

430 and nafamostat exerted limited activity against double-stranded RNA formation in
431 SARS-CoV-2-infected cells. This may indicate that camostat and nafamostat rather
432 exert cytoprotective effects that prevent cells from virus-induced lysis but less
433 pronounced antiviral activity. The mechanisms underlying the enhanced anti-SARS-
434 CoV-2 activity of aprotinin remain unclear. Differences in the interference with
435 additional proteases involved in SARS-CoV-2 replication may be responsible. Notably,
436 aprotinin had been identified in the past as a protease inhibitor with pronounced
437 antiviral activity, which may interfere with viral proteases in addition to cellular ones
438 [Hayashi et al., 1991; Aleshin et al., 2007; Zhirnov et al., 2011; Lin et al., 2017].

439 Notably, SARS-CoV-2 was more sensitive to aprotinin than SARS-CoV, which
440 may be at least in part explained by the DCPs observed in the vicinity of the cleavage
441 sites in S. Effective aprotinin concentrations were in the range of clinically achievable
442 concentrations. Moreover, aprotinin aerosols, which may result in increased local drug
443 concentrations in the lungs are approved for the treatment of influenza viruses in
444 Russia [Zhirnov et al., 2011]. Remdesivir, a broad spectrum antiviral agent under
445 investigation in clinical trials for the treatment of SARS-CoV-2 patients
446 (www.clinicaltrials.gov), exerted stronger effects against SARS-CoV than against
447 SARS-CoV-2.

448 Therapeutic concentrations of the proton pump inhibitor omeprazole further
449 increased the activity of aprotinin and remdesivir. Omeprazole may interfere with the
450 acidification of the lysosomes, which is required to support coronavirus replication
451 [Shen et al., 2017]. However, other, so far unknown, mechanisms may also contribute
452 to this. Notably, omeprazole and other proton pump inhibitors have recently been
453 shown to increase the anti-herpes simplex virus activity of acyclovir [Michaelis et al.,
454 2019].

455 In conclusion, our in-silico study revealed a substantial number of differentially
456 conserved amino acid positions in the SARS-CoV-2 and SARS-CoV proteins. In
457 agreement, cell culture experiments identified differences in the cell tropism and drug
458 sensitivity profiles of these two viruses. Our data also show that cellular ACE2 levels
459 do not reliably indicate cell susceptibility to SARS-CoV-2. Hence, ACE2 expression
460 studies are not sufficient to predict the SARS-CoV-2 cell tropism. Differences in the
461 drug sensitivity profiles between SARS-CoV-2 and SARS-CoV, the most closely
462 related coronavirus known to have caused disease in humans, indicate that
463 approaches to identify anti-SARS-CoV-2 drugs will require testing against this virus.
464 Finally, and probably most importantly during an ongoing pandemic, we have shown
465 that the approved drug aprotinin inhibits SARS-CoV-2 infection in clinically achievable
466 concentrations. The efficacy of aprotinin (and of remdesivir, which is investigated
467 against SARS-CoV-2 in clinical trials) can be further enhanced by therapeutic
468 concentrations of the proton pump inhibitor omeprazole. Hence, our study has
469 identified novel candidate therapies based on approved drugs that can be readily
470 tested in a clinical setting.

471 **Methods**

472

473 **Structural analysis**

474 Full genome sequences for SARS-CoV-2 were obtained from the National
475 Center for Biotechnology Information (NCBI) 4 and the GISAID resource. A total of
476 1266 full length genome sequences were available as of 27/03/2020. Fifty-three
477 SARS-CoV genome sequences were downloaded from VIPR [Pickett et al., 2012;
478 Pickett et al., 2012A] restricted to sequences with a collection year between 2003-
479 2004 and a human or unknown host. Where the host was unknown the genome
480 information was further checked to see if it was appropriate. Open Reading Frames
481 (ORFs) were extracted using EMBOSS getorf [Rice et al., 2000]. These ORFs were
482 matched to known proteins using BLAST, and fragments and mismatches were
483 discarded. To match the ORF1ab non-structural proteins, a BLAST database of the
484 sequences from the SARS non-structural proteins was generated and the SARS-CoV-
485 2 ORF1ab searched against it. After each ORF was assigned to a known protein they
486 were aligned using ClustalO [Sievers et al., 2011] with default settings. Sequences
487 that fell below 50% coverage were removed from analysis.

488 SDPs were identified by calculating the Jensen-Shannon divergence [Capra &
489 Singh, 2007] score for each position in the multiple sequence alignment in each
490 species. Highly conserved alignment positions where the conservation score was >0.8
491 for both species were retained. Any of alignment positions where the same amino acid
492 occurred in both species were then removed. The remaining residues, were
493 considered SDPs.

494 All available SARS-CoV-2 and SARS-CoV protein structures were downloaded
495 from the protein Databank (PDB) [Armstrong et al., 2020]. Where structures were not
496 available they were modelled using Phyre2 [Kelley et al., 2015] (Supplementary Table
497 4). SDPs were mapped onto protein structures using PyMOL from structures obtained
498 from the Protein Databank (PDB). To model the complex between the SARS-CoV-2
499 spike protein and ACE2, a model of the SARS-CoV-2 spike protein was built using
500 Phyre2 based on the SARS-CoV structure (PDB:6acg) as some of the residues
501 involved in binding by the SARS-CoV spike protein were not resolved in the SARS-
502 CoV-2 spike protein structure. This homology model was docked to ACE2 using
503 HADDOCK with constraints based on the likely interface residues equivalent to the
504 SARS-CoV complex.

505

506 **Cell culture**

507 The Caco2 cell line was obtained from DSMZ (Braunschweig, Germany). The
508 cells were grown at 37°C in minimal essential medium (MEM) supplemented with 10%
509 foetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml of streptomycin. 293
510 cells (PD-02-01; Microbix Biosystems Inc.) and 293/ACE2 cells [Kamitani et al., 2006]
511 (kindly provided by Shinji Makino, UTMB, Galveston, Texas) were cultured in
512 Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 50 IU/ mL
513 penicillin, and 50µg/ mL streptomycin. Selection of 293/ACE2 cells constitutively
514 expressing human angiotensin-converting enzyme 2 (ACE2) was performed by
515 addition of 12 µg/ mL blasticidin. All culture reagents were purchased from Sigma
516 (Munich, Germany). Cells were regularly authenticated by short tandem repeat (STR)
517 analysis and tested for mycoplasma contamination.

518

519 **Virus infection**

520 The isolate SARS-CoV-2/1/Human/2020/Frankfurt was derived from an
521 individual, who had been evacuated from the Hubei province in China, transferred to
522 University Hospital Frankfurt, and tested positive for SARS-CoV [Hoehl et al., 2020]
523 and cultivated in Caco2 cells as previously described for SARS-CoV strain FFM-1
524 [Cinatl et al., 2004]. Virus titres were determined as TCID₅₀/ml in confluent cells in 96-
525 well microtitre plates [Cinatl et al., 2003; Cinatl et al., 2005].
526

527 **Western blot**

528 Cells were lysed using Triton-X-100 sample buffer, and proteins were
529 separated by SDS-PAGE. Detection occurred by using specific antibodies against β -
530 actin (1:2500 dilution, Sigma-Aldrich, Munich, Germany), ACE2, and TMPRSS2 (both
531 1:1000 dilution, abcam, Cambridge, UK). Protein bands were visualised by laser-
532 induced fluorescence using infrared scanner for protein quantification (Odyssey, Li-
533 Cor Biosciences, Lincoln, NE, USA).
534

535 **Receptor blocking experiments**

536 To investigate whether ACE2 or DPP4 receptors are involved in SARS-CoV-2
537 internalisation and replication, Caco2 cells were pre-treated for 30 min at 37°C with
538 goat antibody directed against the human ACE2 or DPP4 ectodomain (R&D Systems,
539 Wiesbaden-Nordenstadt, Germany). Then, cells were washed three times with PBS
540 and infected with SARS-CoV-2 at MOI 0.01. Cytopathogenic effects were monitored
541 48h post infection.
542

543 **Antiviral assay**

544 Confluent cell cultures were infected with SARS-CoV-2 in 96-well plates at MOI
545 0.01 in the absence or presence of drug. Cytopathogenic effect (CPE) was assessed
546 visually 48h post infection [Cinatl et al., 2003].
547

548 **Viability assay**

549 Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-
550 diphenyltetrazolium bromide (MTT) assay modified after Mosman [Mosmann, 1983],
551 as previously described [Onafuye et al., 2019]. Confluent cell cultures in 96-well plates
552 were incubated with drug for 48h. Then, 25 μ L of MTT solution (2 mg/mL (w/v) in PBS)
553 were added per well, and the plates were incubated at 37 °C for an additional 4 h.
554 After this, the cells were lysed using 200 μ L of a buffer containing 20% (w/v) sodium
555 dodecylsulfate and 50% (v/v) N,N-dimethylformamide with the pH adjusted to 4.7 at
556 37 °C for 4 h. Absorbance was determined at 570 nm for each well using a 96-well
557 multiscanner. After subtracting of the background absorption, the results are
558 expressed as percentage viability relative to control cultures which received no drug.
559 Drug concentrations that inhibited cell viability by 50% (IC₅₀) were determined using
560 CalcuSyn (Biosoft, Cambridge, UK).
561

562 **qPCR**

563 SARS-CoV-2 and SARS-CoV RNA from cell culture supernatant samples was
564 isolated using AVL buffer and the QIAamp Viral RNA Kit (Qiagen) according to the
565 manufacturer's instructions.

566 Absorbance-based quantification of the RNA yield was performed using the
567 Genesys 10S UV-Vis Spectrophotometer (Thermo Scientific). RNA was subjected to

568 OneStep qRT-PCR analysis using the Luna Universal One-Step RT-qPCR Kit (New
569 England Biolabs) and a CFX96 Real-Time System, C1000 Touch Thermal Cycler.
570 Primers were adapted from the WHO protocol²⁹ targeting the open reading frame for
571 RNA-dependent RNA polymerase (RdRp): RdRP_SARSr-F2 (GTG ARA TGG TCA
572 TGT GTG GCG G) and RdRP_SARSr-R1 (CAR ATG TTA AAS ACA CTA TTA GCA
573 TA) using 0.4 μ M per reaction. Standard curves were created using plasmid DNA
574 (pEX-A128-RdRP) harbouring the corresponding amplicon regions for RdRP target
575 sequence according to GenBank Accession number NC_045512. For each condition
576 three biological replicates were used. Mean and standard deviation were calculated
577 for each group.

578

579 **Immunostaining for double-stranded RNA**

580 Immunostaining was performed as previously described [Cinatl et al., 1995],
581 using a monoclonal antibody directed against double-stranded RNA (1:150 dilution,
582 SCICONS J2, mouse, IgG2a, kappa chain, English & Scientific Consulting Kft., Szirák,
583 Hungary) 48h post infection.

584

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588

589 **Data availability**

590 All data are provided in the manuscript and its supplements.

591

592 **Author contributions**

593 D.B., J.E.M, K.M., S.G.M., M.N.W., and J.C. performed experiments. V.K.
594 provided essential materials. All authors analysed data. M.N.W., M.M., and J.C.
595 planned, conducted, and supervised the study. M.M. wrote the first manuscript draft.
596 All authors were involved in the drafting of and approved the final manuscript version.

597

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