

1 **Glycopeptide antibiotic teicoplanin inhibits cell entry of SARS-CoV-2**  
2 **by suppressing the proteolytic activity of cathepsin L**

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4 **Running Head:** Teicoplanin inhibits cell entry of SARS-CoV-2

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35

36 **Abstract**

37       Since the outbreak of the coronavirus disease 2019 (COVID-19) caused by  
38 severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the public health  
39 worldwide has been greatly threatened. The development of an effective treatment for  
40 this infection is crucial and urgent but is hampered by the incomplete understanding  
41 of the viral infection mechanism and the lack of specific antiviral agents. We  
42 previously reported that teicoplanin, a glycopeptide antibiotic that has been  
43 commonly used in the clinic to treat bacterial infection, significantly restrained the  
44 cell entry of Ebola virus, SARS-CoV and MERS-CoV by specifically inhibiting the  
45 activity of cathepsin L (CTSL). Here, we found that the cleavage sites of CTSL on the  
46 Spike of SARS-CoV-2 were highly conserved among all the variants. The treatment  
47 with teicoplanin suppressed the proteolytic activity of CTSL on Spike and prevented  
48 the cellular infection of different pseudotyped SARS-CoV-2 viruses. Teicoplanin  
49 potently prevented the entry of authentic SARS-CoV-2 into the cellular cytoplasm  
50 with an  $IC_{50}$  of 2.038  $\mu$ M for the Wuhan-Hu-1 reference strain and an  $IC_{50}$  of 2.116  
51  $\mu$ M for the SARS-CoV-2 (D614G) variant. The pre-treatment of teicoplanin also  
52 prevented SARS-CoV-2 infection in hACE2 mice. In summary, our data reveal that  
53 CTSL is required for both SARS-CoV-2 and SARS-CoV infection and demonstrate  
54 the therapeutic potential of teicoplanin for universal anti-CoVs intervention.

55

56 **Keywords**

57 teicoplanin, SARS-CoV-2, Spike, cathepsin L, viral entry

58

## 59 **Importance**

60 Disease prevention and treatment are two important countermeasures to end the  
61 coronavirus disease 2019 (COVID-19). However, severe acute respiratory syndrome  
62 coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, evolves all the time,  
63 resulting in the emerging of many epidemic SARS-CoV-2 mutants, which  
64 significantly impairs the effectiveness of early strain-based vaccines and antibodies.  
65 Developing universal vaccines and broad-spectrum antiviral drugs are essential to  
66 confront SARS-CoV-2 mutants including those may emerge in the future. Our study  
67 reported here showed that the cleavage sites of cellular cathepsin L (CTSL) are highly  
68 conserved among all the SARS-CoV-2 mutants and SARS-CoV. The CTSL inhibitor  
69 teicoplanin not only inhibited the cell entry of two live SARS-CoV-2 strains and  
70 various pseudotyped viruses but also prevented live virus infection in animal models.  
71 Based on our previous finding that teicoplanin also inhibited SARS-CoV and MERS-  
72 CoV infection, we believe that teicoplanin possesses the potential to become a  
73 universal anti-CoVs drug.

74

75

## 76 **Introduction**

77        Coronaviruses (CoVs) are enveloped, positive sense single-stranded RNA viruses  
78 (1, 2). Many members of the coronavirus family are life-threatening human pathogens  
79 and can cause severe respiratory diseases, such as severe acute respiratory syndrome-  
80 associated coronavirus (SARS-CoV) emerged in 2003 and middle east respiratory  
81 syndrome coronavirus (MERS-CoV) emerged in 2012 (3-9). Since December 2019, a  
82 novel coronavirus has emerged and spread globally, resulting in millions of  
83 pneumonia cases around the world (10-14). This novel coronavirus, named SARS-  
84 CoV-2, belongs to the beta-coronavirus according to the sequence released (13, 15).  
85 Evolutionary analyses have shown that SARS-CoV-2 shares 79% homology with  
86 SARS-CoV and 50% homology with MERS-CoV (15-17). During the last two years,  
87 many independent dominant SARS-CoV-2 variants have emerged locally and  
88 circulated globally, which included B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma),  
89 B.1.429 (Epsilon), B.1.525 (Eta), B.1.526 (Iota), B.1.617.1 (Kappa), B.1.617.2  
90 (Delta), B.1.621 (Mu) and C.37 (Lambda) (18-26). Given the high frequency of  
91 mutation, the high infectious rate and the lack of effective treatment for SARS-CoV-2,  
92 it is urgent to develop an efficient antiviral drug for SARS-CoV-2 and its mutants.

93        The Spike (S) glycoproteins, which cover on the surface of virions, mediate the  
94 viral entry into host cells and determine the host range of coronaviruses (27-29). The  
95 infection of both SARS-CoV and SARS-CoV-2 is initiated by the attachment of the S  
96 protein to the host receptor angiotensin-converting enzyme 2 (ACE2) (29-31),  
97 followed by the S protein priming by cellular proteases such as TMPRSS2 (31-34).  
98 The viruses are then transported into host cells through the early endosomes and late  
99 endosomes, and subsequently endo / lysosomes. For SARS-CoV, the primed S  
100 proteins are further cleaved by other proteases such as cysteine proteinase cathepsin L

101 (CTSL) within endocytic vesicles to complete the activation (35-38). The activated S  
102 proteins then mediate the fusion of viral and cellular membranes, resulting in the  
103 release of SARS-CoV genome into the cytoplasm.

104 We previously found that teicoplanin, a commonly used clinical glycopeptide  
105 antibiotic, potently suppressed the cellular entry of Ebola virus, SARS-CoV, and  
106 MERS-CoV (39). Further mechanism investigation revealed that teicoplanin blocked  
107 the virus entry by specifically inhibiting the proteolytic activity of CTSL, indicating  
108 the potential of teicoplanin as an effective drug for CTSL-dependent viral infection.  
109 In this study, we investigated the role of CTSL in SARS-CoV-2 entry and tested the  
110 inhibitory effect of teicoplanin and homologs on the viral entry process. We found that  
111 the cleavage sites of CTSL were highly conserved among the S sequences of various  
112 epidemic SARS-CoV-2 mutants and SARS-CoV. The loss of CTSL significantly  
113 crippled SARS-CoV-2 infection, while the overexpression of CTSL significantly  
114 increased the infectivity of SARS-CoV-2. Meanwhile, teicoplanin and dalbavancin,  
115 but not vancomycin, exhibited remarkable inhibitory activity toward the entry of  
116 SARS-CoV-2. Teicoplanin was able to inhibit the entry of all the major epidemic  
117 SARS-CoV-2 mutants. Further mechanism study indicated that teicoplanin inhibited  
118 SARS-CoV-2 entry by inhibiting the proteolytic activity of CTSL on S proteins. More  
119 importantly, teicoplanin inhibited the entry of authentic SARS-CoV-2 viruses with an  
120  $IC_{50}$  lower than 5  $\mu$ M (2.038  $\mu$ M for the original strain, 2.116  $\mu$ M for the D614G  
121 variant). The pre-treatment of teicoplanin also prevented the infection of authentic  
122 SARS-CoV-2 in mice models. Combined with our previous finding that teicoplanin  
123 inhibited the entry of SARS-CoV and MERS-CoV, our study reported here indicated  
124 that the CTSL inhibitor teicoplanin could be a universal anti-CoVs drug.

125

## 126 **Results**

### 127 **SARS-CoV-2 infection depended on the activity of CTSL.**

128 The proteolytic processing of the S protein is essential for SARS-CoV entry and  
129 fusion. Many host proteases, including TMPRSS2 and CTSL, are involved in the  
130 priming and activation of the SARS-CoV S protein, and some of which also have  
131 been identified and experimentally validated in SARS-CoV-2 infection (31, 32, 40-  
132 45). To systematically identify cellular proteases and receptors which mediated the  
133 entry and fusion of SARS-CoV-2 to target cells, we knocked down ten major  
134 proteases and receptors in HEK293T cells, which included CTSL, CTSB, CTSK,  
135 TMPRSS2, TMPRSS11A, TMPRSS11D, Furin, PLG, DPP4 and ACE2.  
136 Subsequently, these cells were infected by pseudotyped SARS-CoV-2 S / HIV-1  
137 viruses which harbored an integrated *luciferase* gene. The expression of luciferase  
138 indicated the entry and expression of pseudotyped virus. We found that the absence of  
139 CTSL, TMPRSS2, Furin or ACE2 significantly decreased the pseudotyped SARS-  
140 CoV-2 virus infection (**Fig. 1A**). The ACE2 protein has been identified as the major  
141 receptor of SARS-CoV-2 (17). Both TMPRSS2 and Furin also have been found to be  
142 essential for efficient infection of SARS-CoV-2 (31, 42). To determine whether CTSL  
143 is also involved in SARS-CoV-2 S protein activation, we compared the cleavage sites  
144 of CTSL in the gene sequences encoding the SARS-CoV and SARS-CoV-2 S proteins.  
145 After alignment, we found that the cleavage sites of CTSL were well-conserved  
146 between SARS-CoV and SARS-CoV-2 S proteins (**Fig. 1B**). Moreover, the cleavage  
147 sites of CTSL on S proteins were also highly conserved among all the major epidemic  
148 SARS-CoV-2 variants including D614 (Wuhan-Hu-1), G614 (SYSU-IHV), B.1.1.7  
149 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.429 (Epsilon), B.1.525 (Eta), B.1.526  
150 (Iota), B.1.617.1 (Kappa), B.1.617.2 (Delta), B.1.621 (Mu) and C.37 (Lambda) (18-26)

151 **(Fig. 1C)**. Previously, CTSL has been found to play pivotal roles in SARS-CoV  
152 infection by cleaving and activating S proteins (38, 41, 46). We speculated that CTSL  
153 might also participate in SARS-CoV-2 entry and fusion. Thus, we overexpressed  
154 CTSL proteins in HEK293T cells which were subsequently infected by pseudotyped  
155 SARS-CoV-2 S / HIV-1 viruses. We found that the infectivity of pseudotyped virus to  
156 HEK293T cells was linearly and positively correlated with the expression level of  
157 CTSL **(Fig. 1D)**. We also co-overexpressed ACE2 with CTSL in HEK293T cells. We  
158 found that the co-overexpression of CTSL significantly increased the infectivity of  
159 pseudotyped virus compared with ACE2-overexpression only **(Fig. 1E)**. These results  
160 indicated that the effective infection of SARS-CoV-2 to host cells depended on the  
161 proteolytic activity of CTSL.

162

### 163 **Teicoplanin specifically inhibited the entry of SARS-CoV-2.**

164 Previously, we have found that teicoplanin, a glycopeptide antibiotic which  
165 inhibited CTSL activity, suppressed the entry of SARS-CoV, MERS-CoV and Ebola  
166 viruses (39). We speculated that teicoplanin might also be able to block the entry of  
167 SARS-CoV-2. Thus, we conducted the pseudotyped virus entry upon drug treatment  
168 assay. We generated a highly sensitive HEK293T-hACE2 cell line which  
169 constitutively expressed high level of hACE2 receptors. HEK293T-hACE2 cells were  
170 co-incubated with teicoplanin and pseudotyped SARS-CoV-2 S / HIV-1 virus. The  
171 infectivity of pseudotyped virus, which was represented by the amounts of luciferase  
172 within HEK293T-hACE2 cells, was measured 48 hours post infection **(Fig. 2A)**. To  
173 exclude the possibility that teicoplanin inhibited the early events of the pseudotyped  
174 HIV-1 life cycle, pseudotyped VSV-G / HIV-1 viruses bearing vesicular stomatitis  
175 virus (VSV) glycoproteins were also packaged and treated as the negative control.

176 Pseudotyped SARS-CoV S / HIV-1 viruses bearing SARS-CoV S were packaged and  
177 treated as the positive control. The results showed that teicoplanin effectively  
178 inhibited the entry of both SARS-CoV-2 and SARS-CoV pseudotyped viruses in a  
179 dose-dependent manner, whereas teicoplanin treatment did not affect the infection of  
180 pseudotyped VSV-G / HIV-1 viruses (**Fig. 2B and 2C**).

181 Teicoplanin homologs including dalbavancin also have specific inhibitory effects  
182 on CTSL based on our previous study (39). While vancomycin, another glycopeptide  
183 antibiotic which was clinically used for Gram-positive bacterial infections, did not  
184 show inhibitory activity on CTSL. Therefore, we further tested whether dalbavancin  
185 and vancomycin could inhibit the entry of SARS-CoV-2. Similar to teicoplanin,  
186 dalbavancin effectively inhibited both SARS-CoV-2 and SARS-CoV pseudotyped  
187 viruses entering into HEK293T-hACE2 cells in a dose-dependent manner, but it did  
188 not affect pseudotyped VSV-G / HIV-1 viruses infection (**Fig. 2D and 2E**). In  
189 contrast, vancomycin did not show any inhibitory activity on the infection of  
190 pseudotyped SARS-CoV-2, SARS-CoV or VSV-G viruses (**Fig. 2F and 2G**). Taken  
191 together, these results indicated that CTSL inhibitors teicoplanin and its homolog  
192 dalbavancin could suppress the entry of SARS-CoV-2.

193

194 **Teicoplanin inhibited the entry of SARS-CoV-2 by inhibiting the activity of**  
195 **CTSL.**

196 To further confirm that teicoplanin inhibited the entry of SARS-CoV-2, we  
197 investigated the antiviral activity of teicoplanin on authentic (live) SARS-CoV-2. We  
198 obtained two authentic SARS-CoV-2 strains. One was SARS-CoV-2 D614 virus  
199 (Wuhan-Hu-1) which was provided by Guangdong Provincial Center for Disease  
200 Control and Prevention (GDCDC). The other was SARS-CoV-2 G614 virus (SYSU-



201 IHV) which was isolated by us from the sputum sample of an infected patient (47).  
202 We found that teicoplanin effectively inhibited the entry of both authentic strains with  
203 a half maximal inhibitory concentration (IC<sub>50</sub>) of 2.038 μM for the Wuhan-Hu-1  
204 reference strain and an IC<sub>50</sub> of 2.116 μM for the SARS-CoV-2 (D614G) variant (**Fig.**  
205 **3A and 3B**). Given that the serum concentrations of teicoplanin in patients are at least  
206 15 mg / L (8.78 μM) after the loading dose treatment for most Gram-positive bacterial  
207 infections, our data indicated that teicoplanin was able to potently suppress the entry  
208 of SARS-CoV-2 of both the original Wuhan-Hu-1 strain and the D614G mutation  
209 strain at a relatively low and safe dose (39).

210 To elucidate whether the target of teicoplanin was the virus itself, or the host cell,  
211 or both, we conducted drug / virus pre-treatment assay. In the first group, HEK293T-  
212 hACE2 cells were pre-treated with different concentrations of teicoplanin followed by  
213 infecting with pseudotyped SARS-CoV-2 (drug pre-treatment group). In the second  
214 group, cells were co-incubated with both teicoplanin and pseudotyped virus (drug-  
215 virus co-treatment group). In the third group, cells were pre-infected with  
216 pseudotyped virus followed by treating with teicoplanin (virus pre-treatment group).  
217 We found that the infectivity of pseudotyped SARS-CoV-2 in both drug pre-treatment  
218 group and drug-virus co-treatment group was negatively correlated with the  
219 concentration of teicoplanin, whereas the infectivity of pseudotyped virus in virus pre-  
220 treatment group was almost unchanged upon the treatment of different concentrations  
221 of teicoplanin (**Fig. 3C**). These results indicated that teicoplanin targeted host cells  
222 rather than viral particles.

223 Our previous report has revealed that teicoplanin targets on CTSL directly within  
224 host cells (39). To provide direct evidence that teicoplanin inhibiting SARS-CoV-2  
225 entry via inhibiting the activity of CTSL as well, we conducted *in vitro* CTSL

226 enzymatic inhibition assay. The *in vitro* purified CTSL proteins were firstly activated  
227 in CTSL assay buffer, followed by incubating with SARS-CoV S or SARS-CoV-2 S.  
228 In another group, pre-activated CTSL proteins were co-incubated with different S  
229 proteins and teicoplanin. Then the CTSL- and teicoplanin-treated S proteins were  
230 proceeded to SDS-PAGE and silver staining. We found that CTSL proteins were able  
231 to effectively cleave both SARS-CoV S and SARS-CoV-2 S (**Fig. 3D and 3E**).  
232 However, the co-treatment of teicoplanin with CTSL proteins inhibited the enzymatic  
233 activity of CTSL on both S proteins, resulting in the presence of more full-length S  
234 proteins (**Fig. 3D and 3E**). Our above results further confirmed that teicoplanin  
235 inhibited the entry of SARS-CoV-2 by directly inhibiting the proteolytic activity of  
236 CTSL within host cells.

237

#### 238 **Teicoplanin inhibited the entry of various SARS-CoV-2 mutants.**

239 Since December 2019, many SARS-CoV-2 mutants have emerged locally and  
240 spread worldwide, such as B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.429  
241 (Epsilon), B.1.525 (Eta), B.1.526 (Iota), B.1.617.1 (Kappa), B.1.617.2 (Delta),  
242 B.1.621 (Mu) and C.37 (Lambda) (18-26). We have found that the cleavage sites of  
243 CTSL on S proteins of these mutants were highly conserved (**Fig. 1C**). Thus, we  
244 speculated that teicoplanin-mediated inhibition of CTSL activity might also cripple  
245 the cell entry of SARS-CoV-2 mutants. To evaluate whether teicoplanin still was able  
246 to inhibit the infection of these variants, we constructed different S-expressing  
247 plasmids which were derived from various SARS-CoV-2 mutants (**Fig. 4A**). Similar  
248 to the package of pseudotyped SARS-CoV-2 (D614) S / HIV-1 viruses, we packaged  
249 ten different pseudotyped SARS-CoV-2 viruses based on the above S mutants.  
250 HEK293T-hACE2 cells were co-incubated with different pseudotyped viruses and

251 two-fold serially diluted teicoplanin, followed by the measurement of the luciferase  
252 activity which could represent viral infectivity. The IC<sub>50</sub> of teicoplanin against  
253 different pseudotyped viruses was calculated based on the percentages of viral  
254 inhibition. We found that all the IC<sub>50</sub> of teicoplanin against the entry of these viruses  
255 were below 5 μM (3.002 μM for B.1.351 / Beta, 3.117 μM for P.1 / Gamma, 3.056  
256 μM for B.1.429 / Epsilon, 2.041 μM for B.1.525 / Eta, 1.963 μM for B.1.526 / Iota,  
257 2.188 μM for B.1.617.1 / Kappa, 2.300 μM for B.1.617.2 / Delta, 1.998 μM for  
258 B.1.621 / Mu, 2.306 μM for C.37 / Lambda), except that the IC<sub>50</sub> of teicoplanin  
259 against pseudotyped SARS-CoV-2 (B.1.1.7 / Alpha) S / HIV-1 viruses was 5.423 μM  
260 (**Fig. 4B-4K**). Taken together, our above results indicated that the CTSL inhibitor  
261 teicoplanin was able to inhibit the entry of different SARS-CoV-2 variants.

262

### 263 **Teicoplanin prevented SARS-CoV-2 infection in hACE2 mice.**

264 To evaluate whether the treatment of teicoplanin could protect individuals from  
265 SARS-CoV-2 infection, we conducted mice infection experiments upon teicoplanin  
266 treatment. We utilized K18-hACE2 mice which were generated by knocking in the  
267 human K18 promoter-driven human ACE2 within the mouse Hipp11 (H11) “safe-  
268 harbor” locus. hACE2 mice were intraperitoneally administrated with 100 mg / kg  
269 body weight teicoplanin or equal volume of saline, followed by intranasally  
270 challenging with  $1 \times 10^5$  focus-forming units (FFU) of authentic SARS-CoV-2 D614  
271 virus (n=4 in each group). These mice were euthanized 5 days post infection (**Fig.**  
272 **5A**). Lung tissues of each mice were proceeded to SARS-CoV-2 viral RNA  
273 quantification, hematoxylin & eosin (HE) and immunohistochemistry (IHC) analysis.  
274 We found that lung tissues of hACE2 mice in saline group harbored large amounts of  
275 viral RNA copies ( $1.9 \times 10^4$ ,  $2.3 \times 10^5$ ,  $1.2 \times 10^5$ , and  $8.8 \times 10^4$  copies per ml) (**Fig. 5B**).

276 While lung tissues of hACE2 mice in teicoplanin group harbored only few numbers of  
277 viral RNA copies (less than 10 copies per ml in average) (**Fig. 5B**). HE and IHC  
278 assays also revealed that lung tissues in mice from saline group were severely  
279 damaged upon SARS-CoV-2 challenge, which were interspersed with thickened  
280 alveolar septa, collapsed alveoli and Nucleoprotein (N) protein-expressing cells (**Fig.**  
281 **5C**). Whereas no pathological changes and N-expressing cells were observed in  
282 teicoplanin treatment group (**Fig. 5C**). These results indicated that teicoplanin  
283 treatment was able to prevent the infection of authentic SARS-CoV-2 virus in hACE2  
284 mice.

285

## 286 **Discussion**

287 To date, many drugs have been tested for treatment of COVID-19. Remdesivir  
288 showed some efficacy in COVID-19 patients, but many severe side effects were  
289 observed (48). Among patients hospitalized in metropolitan New York with COVID-  
290 19, treatment with hydroxychloroquine and / or azithromycin failed to significantly  
291 improve in-hospital mortality (49). Recently, Merck Sharp and Dohme (MSD) and its  
292 partner Ridgeback Biotherapeutics reported that the antiviral drug molnupiravir (MK-  
293 4482, EIDD-2801) reduced the risk of hospitalization or death by 50% compared to  
294 placebo for patients with mild or moderate COVID-19 based on their Phase III study  
295 (NCT04575597). Although previous reports also showed that molnupiravir could  
296 prevent SARS-CoV-2 infection and transmission in animal models, more clinical data  
297 of long-term monitoring need to be collected to investigate its potential side effects in  
298 the future clinical trials (50, 51). Specific treatment for COVID-19 is still lacking and  
299 urgently needed. Host cell entry is the first step of the viral life cycle and is an ideal  
300 process to develop potential drugs. In this study, we identified that teicoplanin could

301 inhibit the entry of SARS-CoV-2 with an  $IC_{50}$  of lower than 2.5  $\mu$ M. Teicoplanin not  
302 only exhibited remarkable inhibitory activity on various pseudotyped SARS-CoV-2 S  
303 / HIV-1 mutants entry, but also potently restrained the infection of authentic SARS-  
304 CoV-2 viruses including the original D614 reference strain and the later G614 variant.  
305 This inhibitory effect was also confirmed by animal experiment. Therefore, these  
306 findings may provide a novel therapeutic treatment to improve current antiviral  
307 therapy.

308 During the invasion phase, SARS-CoV-2 firstly binds to its receptor hACE2 on the  
309 surface of host cells. The interaction between the receptor-binding domain (RBD) of  
310 the S protein and hACE2 triggers conformational changes within the S protein, which  
311 renders the S protein susceptible to be activated by the host cell protease TMPRSS2  
312 (31, 32, 40). Subsequently, the SARS-CoV-2 virus enters to the early endosome of the  
313 host cell through endocytosis or macropinocytosis. During the maturation process of  
314 the early endosome, the endosome gradually acidifies, which facilitates the entry of  
315 viruses into cells. The antiviral drug chloroquine, which can increase the endosomal  
316 pH to block virus infection, has been found to inhibit the entry of both SARS-CoV  
317 and SARS-CoV-2 (17, 52, 53). During the entry and fusion of SARS-CoV, the  
318 cysteine proteinase CTSL within the late endosome can further cleave the S protein  
319 and activate the membrane fusion, resulting in the release of viral genome (37, 38).

320 Several CRIPSR-mediated knock-out and animal infection experiments have  
321 highlighted the importance of CTSL in SARS-CoV-2 infection (43-45). Here we  
322 showed that knocking down CTSL potently inhibited SARS-CoV-2 entry. The  
323 overexpression of CTSL significantly increased the infectivity of pseudotyped SARS-  
324 CoV-2 viruses. Moreover, our data also demonstrated that teicoplanin was able to  
325 potently inhibit the infection of both authentic SARS-CoV-2 viruses and different

326 pseudotyped SARS-CoV-2 mutants by inhibiting the enzymatic activity of CTSL and  
327 preventing S proteins further activation. Without complete activation of S proteins,  
328 the SARS-CoV-2 virus was gradually degraded within the endosome (**Fig. 5D**). Based  
329 on all these findings, we believe that the endosomal proteinase CTSL plays vital roles  
330 in the infection of SARS-CoV, MERS-CoV, SARS-CoV-2, and possibly other  
331 coronaviruses. Therefore, CTSL and its inhibitor teicoplanin provide important  
332 therapeutic potential for developing universal anti-CoVs intervention.

333 Teicoplanin is a glycopeptide antibiotic which is mainly used for serious infection  
334 caused by Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus*  
335 (54-56). As a commonly used clinical antibiotic, teicoplanin is well known for its low  
336 toxicity, mild side effects, long half-life in blood plasma, convenient administration,  
337 and high safety. Clinically, the serum concentration of teicoplanin is at least 15 mg / L  
338 (8.78  $\mu$ M) after the completion of the loading dose treatment for most Gram-positive  
339 bacterial infections (39). In this study, we found that teicoplanin, and its homolog  
340 dalbavancin, could inhibit the entry of SARS-CoV-2 in HEK293T-hACE2 cells  
341 expressing the key receptor ACE2. Importantly, teicoplanin was able to inhibit  
342 authentic SARS-CoV-2 viruses entry with an  $IC_{50}$  of 2.038  $\mu$ M for the Wuhan-Hu-1  
343 reference strain and an  $IC_{50}$  of 2.116  $\mu$ M for the SARS-CoV-2 (D614G) variant,  
344 which indicated that teicoplanin inhibited authentic viruses at a relatively low and safe  
345 dose. Moreover, our data showed that the pre-treatment of teicoplanin was able to  
346 prevent authentic SARS-CoV-2 infection in hACE2 mice. Given that the principles of  
347 antiviral therapy are to prevent virus infection and use extensively as early as possible,  
348 it is reasonable to recommend the use of teicoplanin for SARS-CoV-2 in the early  
349 infection stage. Therefore, teicoplanin could potentially function as a dual inhibitor  
350 for both SARS-CoV-2 and co-infected Gram-positive bacteria.

351

## 352 **Materials and Methods**

### 353 **Cell lines and viruses.**

354 HEK293T and Vero E6 cells were maintained in DMEM (ThermoFisher)  
355 supplemented with 10% FBS (ThermoFisher), 100 units/ml penicillin, and 100 µg/ml  
356 streptomycin (ThermoFisher) at 37 °C and 5% CO<sub>2</sub>. The HEK293T-hACE2 cell line  
357 was generated by infecting HEK293T cells with lentiviruses which expressed human  
358 angiotensin-converting enzyme 2 (hACE2). The hACE2-positive cells were sorted by  
359 fluorescence activated cell sorting (FACS) and confirmed by western blot with  
360 antibodies against hACE2. The HEK293T-hACE2 cells were maintained as wildtype  
361 HEK293T cells. All cells have been tested for mycoplasma by PCR-based assay and  
362 confirmed to be mycoplasma-free (Mycoplasma-F: 5'-  
363 GGGAGCAAACAGGATTAGTATCCCT-3'; Mycoplasma-R:5'-  
364 TGCACCATCTGTCACCTCTGTTACCCTC-3').

365 The plasmid expressing the Spike (S) of SARS-CoV-2 (D614; Wuhan-Hu-1,  
366 GISAID: EPI\_ISL\_402125) was purchased from Generay Biotech company  
367 (Shanghai, China) and inserted into the pcDNA3.1 vector. SARS-CoV-2 S / HIV-1  
368 pseudotyped viruses were packaged by co-transfecting a lentiviral construct pHIV-  
369 Luciferase (Addgene plasmid # 21375), a packaging construct psPAX2 (Addgene  
370 plasmid # 12260) and a plasmid expressing S proteins into HEK293T cells. The  
371 culture medium was replaced with fresh DMEM 6 hours post transfection. The  
372 pseudotyped viruses-containing supernatant was collected 48 hours post transfection  
373 and filtered through 0.45 µm filters. The amounts of pseudotyped viruses were  
374 quantified by RT-qPCR assay with primers against the long term repeat (LTR)  
375 (HIVTotal-F: 5'-CTGGCTAACTAGGGAACCCACTGCT-3'; HIVTotal-R: 5'-

376 GCTTCAGCAAGCCGAGTCCTGCGTC-3'). Pseudotyped viruses including SARS-  
377 CoV S / HIV-1 and VSV-G / HIV-1 were packaged and quantified as pseudotyped  
378 SARS-CoV-2 S / HIV-1 viruses. Different pseudotyped SARS-CoV-2 S / HIV-1  
379 mutants were also packaged and quantified as above, the S proteins of which included  
380 those of G614 virus (SYSU-IHV, EPI\_ISL\_444969), B.1.1.7 (Alpha, GISAID:  
381 EPI\_ISL\_581117), B.1.351 (Beta, EPI\_ISL\_678597), P.1 (Gamma,  
382 EPI\_ISL\_792683), B.1.429 (Epsilon, EPI\_ISL\_1675148), B.1.525 (Eta,  
383 EPI\_ISL\_1093465), B.1.526 (Iota, EPI\_ISL\_1080752), B.1.617.1 (Kappa,  
384 EPI\_ISL\_1372093), B.1.617.2 (Delta, EPI\_ISL\_1337507), B.1.621 (Mu,  
385 EPI\_ISL\_1220045) and C.37 (Lambda, EPI\_ISL\_1534645).

386 Patient-derived authentic SARS-CoV-2 D614 virus (Wuhan-Hu-1) was obtained  
387 from Guangdong Provincial Center for Disease Control and Prevention. Authentic  
388 SARS-CoV-2 G614 virus (SYSU-IHV) was isolated from the sputum sample of a  
389 female admitted in Guangzhou Eighth People's Hospital who was infected at  
390 Guangzhou by an African traveler in April 2020. Vero E6 cells were utilized to  
391 propagate these viruses.

392

### 393 **Pseudotyped virus infection assay.**

394 Pseudotyped viruses including SARS-CoV S / HIV-1, SARS-CoV-2 S / HIV-1,  
395 VSV-G / HIV-1 and 10 different SARS-CoV-2 mutants were packaged as above. For  
396 viral infection in siRNA-mediated gene knock-down experiment, targeted genes in  
397 HEK293T cells were firstly knocked down by a mixture of three different siRNAs,  
398 followed by the infection of pseudotyped SARS-CoV-2 S / HIV-1 virus 24 hours post  
399 transfection. Another 24 hours later, siRNA- and virus-treated cells were lysed and



400 measured for the amounts of luciferase which could represented the percentages of  
401 virus-infected cells.

402 For virus infection in protein overexpression experiment, HEK293T cells were  
403 firstly transfected with CTSL-expressing or hACE2-expressing plasmids, followed by  
404 infecting with pseudotyped SARS-CoV-2 S / HIV-1 virus. The amounts of luciferase  
405 within each group were measured utilizing luminometer (Promega) 48 hours post  
406 infection, and represented as relative luminescence units.

407 For virus infection in drug treatment experiment, HEK293T-hACE2 cells were  
408 incubated with serially diluted drugs including teicoplanin (Selleck, S1399),  
409 dalbavancin (Selleck, S4848) and vancomycin (Selleck, S2575), and different  
410 pseudotyped viruses. The amounts of luciferase within each group were measured 48  
411 hours post infection.

412

#### 413 **Authentic virus infection assay.**

414 HEK293T-hACE2 cells were seeded in 12-well plates. 24 hours post seeding, cells  
415 were co-incubated with authentic SARS-CoV-2 D614 (Wuhan-Hu-1) virus and two-  
416 fold serially diluted teicoplanin. Another 48 hours post incubation, the supernatant in  
417 each group was collected and proceeded to RNA extraction with RNeasy Mini Kit  
418 (QIAGEN, 74104) according to the manufacturer's instruction. SARS-CoV-2 viral  
419 RNA copies were determined by one-step SARS-CoV-2 RNA detection kit (PCR-  
420 Fluorescence Probing) (Da An Gene Co., DA0931) with the following primers and  
421 probes: N-F (5'-CAGTAGGGGAAGTTCTCCTGCT-3'), N-R (5'-  
422 CTTTGCTGCTGCTTGACAGA-3') and N-P (5'-FAM-  
423 CTGGCAATGGCGGTGATGCTGC-BHQ1-3'). The half maximal inhibitory  
424 concentration (IC50) of teicoplanin against SARS-CoV-2 D614 virus was calculated

425 by GraphPad software (San Diego, USA) according to these viral RNA copies within  
426 each group. The IC<sub>50</sub> of teicoplanin against authentic SARS-CoV-2 G614 virus  
427 (SYSU-IHV) was determined similarly. Authentic SARS-CoV-2 virus infection  
428 assays were conducted in the BSL-3 facility of Sun Yat-sen University.

429

#### 430 **Drug or virus pre-treatment assay.**

431 To determine whether teicoplanin targeted virus directly or targeted host cell  
432 indirectly, HEK293T-hACE2 cells were treated with drug or virus in three different  
433 ways. In the first group, HEK293T-hACE2 cells were pre-treated with 0  $\mu$ M, 12.5  $\mu$ M,  
434 25  $\mu$ M and 50  $\mu$ M teicoplanin respectively. Four hours post drug treatment, cells were  
435 infected with pseudotyped SARS-CoV-2 S / HIV-1 virus. Another 48 hours later, the  
436 amounts of luciferase within each group were monitored and represented as relative  
437 luminescence units. In the second group, HEK293T-hACE2 cells were co-treated with  
438 different concentrations of teicoplanin and pseudotyped SARS-CoV-2 S / HIV-1 virus  
439 simultaneously. The amounts of luciferase were measured 48 hours post co-treatment.  
440 In the third group, cells were pre-infected with pseudotyped SARS-CoV-2 S / HIV-1  
441 virus. Four hours post infection, cells were treated with different concentrations of  
442 teicoplanin. Another 48 hours later, cells were lysed and measured for the amounts of  
443 luciferase.

444

#### 445 ***In vitro* CTSL enzymatic inhibition assay.**

446 To evaluate CTSL enzymatic activity upon teicoplanin treatment *in vitro*, the  
447 purified 250 ng CTSL proteins (Sino Biological Inc., 10486-H08H) were added into  
448 CTSL assay buffer (400 mM NaAc, 4 mM EDTA, 8 mM DTT and pH 5.5) and  
449 incubated in ice for 15 min for activation. To evaluate the inhibition of teicoplanin on

450 CTSL activity, the purified 250 ng CTSL proteins were also co-incubated with 50  $\mu$ M  
451 teicoplanin (Selleck, S1399). After activation, 2  $\mu$ g *in vitro* purified SARS-CoV S  
452 (40634-V08B) or SARS-CoV-2 S (40589-V08B1) were added into each group. S  
453 protein only group (without CTSL and teicoplanin) was set as control group. The S-  
454 CTSL-teicoplanin mixtures were incubated at 37 °C for 1.5 hours. The enzymatic  
455 reaction was stopped by adding with SDS-PAGE loading buffer and followed by  
456 boiling at 100 °C for 10 min. Digested proteins were proceeded to SDS-PAGE and  
457 analyzed by silver staining (Sigma-Aldrich, PROTSIL2-1KT).

458

#### 459 **Animal infection.**

460 Eight-week-old specific-pathogen-free (SPF) transgenic hACE2 mice (C57BL/6)  
461 were purchased from GemPharmatech Co., Ltd (Cat No.: T037657). All mice were  
462 housed in SPF facilities at Laboratory Animal Center of Sun Yat-sen University.  
463 Animal experiments were conducted in strict compliance with the guidelines and  
464 regulations of Laboratory Monitoring Committee of Guangdong Province of China.  
465 The Ethics Committees of Guangdong Provincial People's Hospital and Sun Yat-sen  
466 University approved animal experiments.

467 Six hours before viral challenge, four hACE2 mice were intraperitoneally  
468 administrated with 100 mg/kg body weight teicoplanin (dissolved in saline). Four  
469 hACE2 mice in control group were intraperitoneally administrated with equal volume  
470 of saline. Six hours later, all the mice were intranasally challenged with  $1 \times 10^5$  focus-  
471 forming units (FFU) of authentic SARS-CoV-2 D614 virus. Another 5 days later,  
472 mice were euthanized to harvest lung tissues. Viral RNA copies in lung tissues were  
473 quantified by one-step SARS-CoV-2 RNA detection kit (PCR-Fluorescence Probing)  
474 (Da An Gene Co., DA0931). Authentic SARS-CoV-2 challenge studies were

475 approved by the Ethics Committee of Zhongshan School of Medicine of Sun Yat-sen  
476 University on Laboratory Animal Care (Assurance Number: SYSU-IACUC-2021-  
477 B0020).

478

#### 479 **Histopathology and immunohistochemistry.**

480 Lung tissues of authentic SARS-CoV-2 infected mice were fixed in 4%  
481 paraformaldehyde for at least two days. These lung tissues were embedded in paraffin  
482 and proceeded to histopathology and immunohistochemistry analysis (Nanjing  
483 FreeThinking Biotechnology Co., Ltd). For histopathology analysis, sections (3-4  $\mu\text{m}$ )  
484 of lung tissues were stained with hematoxylin and eosin (H&E). For  
485 immunohistochemistry analysis, sections of lung tissues were deparaffinized and  
486 rehydrated with xylene and gradient alcohol. Antigens were retrieved in citric acid  
487 buffer (pH 6.0) and quenched with 3%  $\text{H}_2\text{O}_2$ . After blocking with BSA, sections were  
488 incubated with rabbit anti-SARS-CoV-2 Nucleoprotein (N) for 24 hours at 4 °C,  
489 followed by incubating with goat anti-rabbit IgG secondary antibody (HRP-  
490 conjugated) and staining with 3,3'-diaminobenzidine. Antibody-conjugated sections  
491 were stained with hematoxylin, followed by dehydrating with gradient ethanol.  
492 Samples were covered by neutral balsam and imaged with HS6 microscope (Sunny  
493 Optical Technology Co., Ltd).

494

#### 495 **Sequence data collection and alignment.**

496 The genome sequences of SARS-CoV and SARS-CoV-2 were collected from the  
497 GenBank database (<https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/>) and the  
498 GISAID's EpiCoV database (<https://www.gisaid.org/>). The sequences of SARS-CoV  
499 circulating in 2003 contain 6 strains (accession numbers: AY278488, AY545918,

500 AY545917, AY394977, AY394978, and AY394979). The sequences of SARS-CoV-2  
501 include 12 variants: D614 (Wuhan-Hu-1, GISAID: EPI\_ISL\_402125), G614 (SYSU-  
502 IHV, EPI\_ISL\_444969), B.1.1.7 (Alpha, GISAID: EPI\_ISL\_581117), B.1.351 (Beta,  
503 EPI\_ISL\_678597), P.1 (Gamma, EPI\_ISL\_792683), B.1.429 (Epsilon,  
504 EPI\_ISL\_1675148), B.1.525 (Eta, EPI\_ISL\_1093465), B.1.526 (Iota,  
505 EPI\_ISL\_1080752), B.1.617.1 (Kappa, EPI\_ISL\_1372093), B.1.617.2 (Delta,  
506 EPI\_ISL\_1337507), B.1.621 (Mu, EPI\_ISL\_1220045) and C.37 (Lambda,  
507 EPI\_ISL\_1534645). The S gene sequences were obtained from the genome of SARS-  
508 CoV and SARS-CoV-2 according to the annotation in the GenBank database. The  
509 sequence datasets were aligned using the ClustalW program implemented in MEGA  
510 X software. Consensus sequences were created using BioEdit software  
511 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) based on the multiple alignment of  
512 SARS-CoV and SARS-CoV-2. The amino acid sequence logos were generated by  
513 WebLogo.

514

### 515 **Statistical analysis.**

516 All the measurements in this study have been performed for at least three times by  
517 at least two lab technicians or students. Detailed statistical information including  
518 statistical tests, sample numbers, mean values, standard errors of the mean (SEM) and  
519 p-values have been shown in the main text and figure legends. Statistical analysis was  
520 conducted with Graphpad Prism 8.0 or Microsoft Excel. Triplicate and quadruplicate  
521 data were presented as mean  $\pm$  SEM. A value of  $p \geq 0.05$  was considered to be not  
522 statistically significant and represented as “ns”. A value of  $p < 0.05$  was considered to  
523 be statistically significant and represented as asterisk (\*). Value of  $p < 0.01$  was  
524 considered to be more statistically significant and represented as double asterisks (\*\*).

525 Value of  $p < 0.001$  was considered to be the most statistically significant and  
526 represented as triple asterisks (\*\*\*). When comparing mean differences between  
527 groups which were split by one independent variable, one-way ANOVA with Tukey's  
528 multiple comparison test or Dunnett's multiple comparison test was conducted. When  
529 comparing mean differences between groups which were split by two independent  
530 variables, two-way ANOVA with Tukey's multiple comparisons test or Dunnett's  
531 multiple comparisons test was conducted. For data with a normal distribution, we  
532 used Student's *t* test.

533

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551

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553

## 554 **References**

- 555 1. Cui J, Li F, Shi ZL. 2019. Origin and evolution of pathogenic coronaviruses. *Nat Rev*  
556 *Microbiol* 17:181-192.
- 557 2. Chen Y, Liu Q, Guo D. 2020. Emerging coronaviruses: Genome structure, replication,  
558 and pathogenesis. *J Med Virol* 92:418-423.
- 559 3. Chan JF, To KK, Tse H, Jin DY, Yuen KY. 2013. Interspecies transmission and  
560 emergence of novel viruses: lessons from bats and birds. *Trends Microbiol* 21:544-55.
- 561 4. Lu G, Wang Q, Gao GF. 2015. Bat-to-human: spike features determining 'host jump'  
562 of coronaviruses SARS-CoV, MERS-CoV, and beyond. *Trends Microbiol* 23:468-78.
- 563 5. de Wit E, van Doremalen N, Falzarano D, Munster VJ. 2016. SARS and MERS:  
564 recent insights into emerging coronaviruses. *Nat Rev Microbiol* 14:523-34.
- 565 6. Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, Wong SS, Leung SY, Chan  
566 KH, Yuen KY. 2005. Severe acute respiratory syndrome coronavirus-like virus in  
567 Chinese horseshoe bats. *Proc Natl Acad Sci U S A* 102:14040-5.
- 568 7. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. 2012.  
569 Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl*  
570 *J Med* 367:1814-20.
- 571 8. Peiris JS, Yuen KY, Osterhaus AD, Stohr K. 2003. The severe acute respiratory  
572 syndrome. *N Engl J Med* 349:2431-41.
- 573 9. Peiris JS, Lai ST, Poon LL, Guan Y, Yam LY, Lim W, Nicholls J, Yee WK, Yan WW,  
574 Cheung MT, Cheng VC, Chan KH, Tsang DN, Yung RW, Ng TK, Yuen KY, group Ss.  
575 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet*  
576 361:1319-25.
- 577 10. Horton R. 2020. Offline: 2019-nCoV outbreak-early lessons. *Lancet* 395:322.
- 578 11. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, Wang W, Song H, Huang B, Zhu N, Bi Y,  
579 Ma X, Zhan F, Wang L, Hu T, Zhou H, Hu Z, Zhou W, Zhao L, Chen J, Meng Y,  
580 Wang J, Lin Y, Yuan J, Xie Z, Ma J, Liu WJ, Wang D, Xu W, Holmes EC, Gao GF,  
581 Wu G, Chen W, Shi W, Tan W. 2020. Genomic characterisation and epidemiology of  
582 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet*  
583 395:565-574.
- 584 12. Rothe C, Schunk M, Sothmann P, Bretzel G, Froeschl G, Wallrauch C, Zimmer T,  
585 Thiel V, Janke C, Guggemos W, Seilmaier M, Drosten C, Vollmar P, Zwirgmaier K,  
586 Zange S, Wolfel R, Hoelscher M. 2020. Transmission of 2019-nCoV infection from  
587 an Asymptomatic Contact in Germany. *N Engl J Med* 382:970-971.

- 588 13. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R,  
589 Niu P, Zhan F, Ma X, Wang D, Xu W, Wu G, Gao GF, Tan W, China Novel  
590 Coronavirus I, Research T. 2020. A Novel Coronavirus from Patients with Pneumonia  
591 in China, 2019. *N Engl J Med* doi:10.1056/NEJMoa2001017.
- 592 14. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z,  
593 Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J,  
594 Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. 2020. Clinical features of patients  
595 infected with 2019 novel coronavirus in Wuhan, China. *Lancet* doi:10.1016/S0140-  
596 6736(20)30183-5.
- 597 15. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, Hu Y, Tao ZW, Tian JH, Pei YY,  
598 Yuan ML, Zhang YL, Dai FH, Liu Y, Wang QM, Zheng JJ, Xu L, Holmes EC, Zhang  
599 YZ. 2020. A new coronavirus associated with human respiratory disease in China.  
600 *Nature* 579:265-269.
- 601 16. Chan JF, Kok KH, Zhu Z, Chu H, To KK, Yuan S, Yuen KY. 2020. Genomic  
602 characterization of the 2019 novel human-pathogenic coronavirus isolated from a  
603 patient with atypical pneumonia after visiting Wuhan. *Emerg Microbes Infect* 9:221-  
604 236.
- 605 17. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang  
606 CL, Chen HD, Chen J, Luo Y, Guo H, Jiang RD, Liu MQ, Chen Y, Shen XR, Wang X,  
607 Zheng XS, Zhao K, Chen QJ, Deng F, Liu LL, Yan B, Zhan FX, Wang YY, Xiao GF,  
608 Shi ZL. 2020. A pneumonia outbreak associated with a new coronavirus of probable  
609 bat origin. *Nature* 579:270-273.
- 610 18. Candido DS, Claro IM, Jesus JGd, Souza WM, Moreira FRR, Dellicour S, Mellan TA,  
611 Plessis Ld, Pereira RHM, Sales FCS, Manuli ER, Thézé J, Almeida L, Menezes MT,  
612 Voloch CM, Fumagalli MJ, Coletti TM, Silva CAMd, Ramundo MS, Amorim MR,  
613 Hoeltgebaum HH, Mishra S, Gill MS, Carvalho LM, Buss LF, Prete CA, Ashworth J,  
614 Nakaya HI, Peixoto PS, Brady OJ, Nicholls SM, Tanuri A, Rossi ÁD, Braga CKV,  
615 Gerber AL, Guimarães APdC, Gaburo N, Alencar CS, Ferreira ACS, Lima CX, Levi  
616 JE, Granato C, Ferreira GM, Francisco RS, Granja F, Garcia MT, Moretti ML,  
617 Perroud MW, Castiñeiras TMPP, Lazari CS, et al. 2020. Evolution and epidemic  
618 spread of SARS-CoV-2 in Brazil. *Science* 369:1255-1260.
- 619 19. Cherian S, Potdar V, Jadhav S, Yadav P, Gupta N, Das M, Rakshit P, Singh S,  
620 Abraham P, Panda S, Team N. 2021. SARS-CoV-2 Spike Mutations, L452R, T478K,  
621 E484Q and P681R, in the Second Wave of COVID-19 in Maharashtra, India.  
622 *Microorganisms* 9:1542.
- 623 20. Kimura I, Kosugi Y, Wu J, Yamasoba D, Butlertanaka EP, Tanaka YL, Liu Y,  
624 Shirakawa K, Kazuma Y, Nomura R, Horisawa Y, Tokunaga K, Takaori-Kondo A,  
625 Arase H, Consortium TGtPJ, Saito A, Nakagawa S, Sato K. 2021. SARS-CoV-2  
626 Lambda variant exhibits higher infectivity and immune resistance. *bioRxiv*  
627 doi:10.1101/2021.07.28.454085:2021.07.28.454085.
- 628 21. Laiton-Donato K, Franco-Muñoz C, Álvarez-Díaz DA, Ruiz-Moreno HA, Usme-Ciro  
629 JA, Andrés Prada D, Reales-González J, Corchuelo S, Herrera-Sepúlveda MT,  
630 Naizaque J, Santamaría G, Rivera J, Rojas P, Hernández Ortiz J, Cardona A, Malo D,  
631 Prieto-Alvarado F, Gómez FR, Wiesner M, Ospina Martínez ML, Mercado-Reyes M.



- 632 2021. Characterization of the emerging B.1.621 variant of interest of SARS-CoV-2.  
633 medRxiv doi:10.1101/2021.05.08.21256619:2021.05.08.21256619.
- 634 22. McCallum M, Bassi J, Marco AD, Chen A, Walls AC, Iulio JD, Tortorici MA,  
635 Navarro M-J, Silacci-Fregni C, Saliba C, Agostini M, Pinto D, Culap K, Bianchi S,  
636 Jaconi S, Cameroni E, Bowen JE, Tilles SW, Pizzuto MS, Guastalla SB, Bona G,  
637 Pellanda AF, Garzoni C, Van Voorhis WC, Rosen LE, Snell G, Telenti A, Virgin HW,  
638 Piccoli L, Corti D, Veessler D. 2021. SARS-CoV-2 immune evasion by variant  
639 B.1.427/B.1.429. bioRxiv doi:10.1101/2021.03.31.437925:2021.03.31.437925.
- 640 23. Meng B, Kemp SA, Papa G, Datir R, Ferreira IATM, Marelli S, Harvey WT, Lytras S,  
641 Mohamed A, Gallo G, Thakur N, Collier DA, Mlcochova P, Robson SC, Loman NJ,  
642 Connor TR, Golubchik T, Martinez Nunez RT, Ludden C, Corden S, Johnston I,  
643 Bonsall D, Smith CP, Awan AR, Bucca G, Torok ME, Saeed K, Prieto JA, Jackson  
644 DK, Hamilton WL, Snell LB, Moore C, Harrison EM, Goncalves S, Fairley DJ,  
645 Loose MW, Watkins J, Livett R, Moses S, Amato R, Nicholls S, Bull M, Smith DL,  
646 Barrett J, Aanensen DM, Curran MD, Parmar S, Aggarwal D, Shepherd JG, Parker  
647 MD, et al. 2021. Recurrent emergence of SARS-CoV-2 spike deletion H69/V70 and  
648 its role in the Alpha variant B.1.1.7. *Cell Reports* 35:109292.
- 649 24. Ozer EA, Simons LM, Adewumi OM, Fowotade AA, Omoruyi EC, Adeniji JA, Dean  
650 TJ, Zayas J, Bhimalli PP, Ash MK, Godzik A, Schneider JR, Mamede JI, Taiwo BO,  
651 Hultquist JF, Lorenzo-Redondo R. 2021. Coincident rapid expansion of two SARS-  
652 CoV-2 lineages with enhanced infectivity in Nigeria. medRxiv  
653 doi:10.1101/2021.04.09.21255206:2021.04.09.21255206.
- 654 25. Tegally H, Wilkinson E, Giovanetti M, Iranzadeh A, Fonseca V, Giandhari J, Doolabh  
655 D, Pillay S, San EJ, Msomi N, Mlisana K, von Gottberg A, Walaza S, Allam M,  
656 Ismail A, Mohale T, Glass AJ, Engelbrecht S, Van Zyl G, Preiser W, Petruccione F,  
657 Sigal A, Hardie D, Marais G, Hsiao N-y, Korsman S, Davies M-A, Tyers L, Mudau I,  
658 York D, Maslo C, Goedhals D, Abrahams S, Laguda-Akingba O, Alisoltani-Dehkordi  
659 A, Godzik A, Wibmer CK, Sewell BT, Lourenço J, Alcantara LCJ, Kosakovsky Pond  
660 SL, Weaver S, Martin D, Lessells RJ, Bhiman JN, Williamson C, de Oliveira T. 2021.  
661 Detection of a SARS-CoV-2 variant of concern in South Africa. *Nature* 592:438-443.
- 662 26. Zhou H, Dcosta BM, Samanovic MI, Mulligan MJ, Landau NR, Tada T. 2021.  
663 B.1.526 SARS-CoV-2 variants identified in New York City are neutralized by  
664 vaccine-elicited and therapeutic monoclonal antibodies. bioRxiv  
665 doi:10.1101/2021.03.24.436620:2021.03.24.436620.
- 666 27. Gallagher TM, Buchmeier MJ. 2001. Coronavirus spike proteins in viral entry and  
667 pathogenesis. *Virology* 279:371-4.
- 668 28. Ou X, Liu Y, Lei X, Li P, Mi D, Ren L, Guo L, Guo R, Chen T, Hu J, Xiang Z, Mu Z,  
669 Chen X, Chen J, Hu K, Jin Q, Wang J, Qian Z. 2020. Characterization of spike  
670 glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with  
671 SARS-CoV. *Nature Communications* 11:1620.
- 672 29. Shang J, Wan Y, Luo C, Ye G, Geng Q, Auerbach A, Li F. 2020. Cell entry  
673 mechanisms of SARS-CoV-2. *Proceedings of the National Academy of Sciences*  
674 117:11727-11734.

- 675 30. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M,  
676 Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. 2003. Angiotensin-  
677 converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature*  
678 426:450-4.
- 679 31. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S,  
680 Schiergens TS, Herrler G, Wu N-H, Nitsche A, Müller MA, Drosten C, Pöhlmann S.  
681 2020. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by  
682 a Clinically Proven Protease Inhibitor. *Cell* 181:271-280.e8.
- 683 32. Glowacka I, Bertram S, Muller MA, Allen P, Soilleux E, Pfefferle S, Steffen I,  
684 Tsegaye TS, He Y, Gnirss K, Niemeyer D, Schneider H, Drosten C, Pöhlmann S.  
685 2011. Evidence that TMPRSS2 activates the severe acute respiratory syndrome  
686 coronavirus spike protein for membrane fusion and reduces viral control by the  
687 humoral immune response. *J Virol* 85:4122-34.
- 688 33. Shulla A, Heald-Sargent T, Subramanya G, Zhao J, Perlman S, Gallagher T. 2011. A  
689 transmembrane serine protease is linked to the severe acute respiratory syndrome  
690 coronavirus receptor and activates virus entry. *J Virol* 85:873-82.
- 691 34. Reinke LM, Spiegel M, Plegge T, Hartleib A, Nehlmeier I, Gierer S, Hoffmann M,  
692 Hofmann-Winkler H, Winkler M, Pöhlmann S. 2017. Different residues in the SARS-  
693 CoV spike protein determine cleavage and activation by the host cell protease  
694 TMPRSS2. *PLoS One* 12:e0179177.
- 695 35. Matsuyama S, Ujike M, Morikawa S, Tashiro M, Taguchi F. 2005. Protease-mediated  
696 enhancement of severe acute respiratory syndrome coronavirus infection. *Proc Natl*  
697 *Acad Sci U S A* 102:12543-7.
- 698 36. Belouzard S, Chu VC, Whittaker GR. 2009. Activation of the SARS coronavirus  
699 spike protein via sequential proteolytic cleavage at two distinct sites. *Proc Natl Acad*  
700 *Sci U S A* 106:5871-6.
- 701 37. Huang IC, Bosch BJ, Li F, Li W, Lee KH, Ghiran S, Vasilieva N, Dermody TS,  
702 Harrison SC, Dormitzer PR, Farzan M, Rottier PJM, Choe H. 2006. SARS  
703 Coronavirus, but Not Human Coronavirus NL63, Utilizes Cathepsin L to Infect  
704 ACE2-expressing Cells. *J Biol Chem* 281:3198-3203.
- 705 38. Simmons G, Gosalia DN, Rennekamp AJ, Reeves JD, Diamond SL, Bates P. 2005.  
706 Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry.  
707 *Proc Natl Acad Sci U S A* 102:11876-11881.
- 708 39. Zhou N, Pan T, Zhang J, Li Q, Zhang X, Bai C, Huang F, Peng T, Zhang J, Liu C, Tao  
709 L, Zhang H. 2016. Glycopeptide Antibiotics Potently Inhibit Cathepsin L in the Late  
710 Endosome/Lysosome and Block the Entry of Ebola Virus, Middle East Respiratory  
711 Syndrome Coronavirus (MERS-CoV), and Severe Acute Respiratory Syndrome  
712 Coronavirus (SARS-CoV). *J Biol Chem* 291:9218-32.
- 713 40. Simmons G, Zmora P, Gierer S, Heurich A, Pöhlmann S. 2013. Proteolytic activation  
714 of the SARS-coronavirus spike protein: cutting enzymes at the cutting edge of  
715 antiviral research. *Antiviral Res* 100:605-14.
- 716 41. Bosch BJ, Bartelink W, Rottier PJ. 2008. Cathepsin L functionally cleaves the severe  
717 acute respiratory syndrome coronavirus class I fusion protein upstream of rather than  
718 adjacent to the fusion peptide. *J Virol* 82:8887-90.

- 719 42. Johnson BA, Xie X, Bailey AL, Kalveram B, Lokugamage KG, Muruato A, Zou J,  
720 Zhang X, Juelich T, Smith JK, Zhang L, Bopp N, Schindewolf C, Vu M,  
721 Vanderheiden A, Winkler ES, Swetnam D, Plante JA, Aguilar P, Plante KS, Popov V,  
722 Lee B, Weaver SC, Suthar MS, Routh AL, Ren P, Ku Z, An Z, Debbink K, Diamond  
723 MS, Shi P-Y, Freiberg AN, Menachery VD. 2021. Loss of furin cleavage site  
724 attenuates SARS-CoV-2 pathogenesis. *Nature* 591:293-299.
- 725 43. Wei J, Alfajaro MM, DeWeirdt PC, Hanna RE, Lu-Culligan WJ, Cai WL, Strine MS,  
726 Zhang S-M, Graziano VR, Schmitz CO, Chen JS, Mankowski MC, Filler RB,  
727 Ravindra NG, Gasque V, de Miguel FJ, Patil A, Chen H, Oguntuyo KY, Abriola L,  
728 Surovtseva YV, Orchard RC, Lee B, Lindenbach BD, Politi K, van Dijk D, Kadoch C,  
729 Simon MD, Yan Q, Doench JG, Wilen CB. 2021. Genome-wide CRISPR Screens  
730 Reveal Host Factors Critical for SARS-CoV-2 Infection. *Cell* 184:76-91.e13.
- 731 44. Zhu Y, Feng F, Hu G, Wang Y, Yu Y, Zhu Y, Xu W, Cai X, Sun Z, Han W, Ye R, Qu D,  
732 Ding Q, Huang X, Chen H, Xu W, Xie Y, Cai Q, Yuan Z, Zhang R. 2021. A genome-  
733 wide CRISPR screen identifies host factors that regulate SARS-CoV-2 entry. *Nature*  
734 *Communications* 12:961.
- 735 45. Zhao M-M, Yang W-L, Yang F-Y, Zhang L, Huang W-J, Hou W, Fan C-F, Jin R-H,  
736 Feng Y-M, Wang Y-C, Yang J-K. 2021. Cathepsin L plays a key role in SARS-CoV-2  
737 infection in humans and humanized mice and is a promising target for new drug  
738 development. *Signal Transduction and Targeted Therapy* 6:134.
- 739 46. Belouzard S, Millet JK, Licitra BN, Whittaker GR. 2012. Mechanisms of coronavirus  
740 cell entry mediated by the viral spike protein. *Viruses* 4:1011-33.
- 741 47. Ma X, Zou F, Yu F, Li R, Yuan Y, Zhang Y, Zhang X, Deng J, Chen T, Song Z, Qiao Y,  
742 Zhan Y, Liu J, Zhang J, Zhang X, Peng Z, Li Y, Lin Y, Liang L, Wang G, Chen Y,  
743 Chen Q, Pan T, He X, Zhang H. 2020. Nanoparticle Vaccines Based on the Receptor  
744 Binding Domain (RBD) and Heptad Repeat (HR) of SARS-CoV-2 Elicit Robust  
745 Protective Immune Responses. *Immunity* 53:1315-1330.e9.
- 746 48. Grein J, Ohmagari N, Shin D, Diaz G, Asperges E, Castagna A, Feldt T, Green G,  
747 Green ML, Lescure F-X, Nicastri E, Oda R, Yo K, Quiros-Roldan E, Studemeister A,  
748 Redinski J, Ahmed S, Bernett J, Chelliah D, Chen D, Chihara S, Cohen SH,  
749 Cunningham J, D'Arminio Monforte A, Ismail S, Kato H, Lapadula G, L'Her E,  
750 Maeno T, Majumder S, Massari M, Mora-Rillo M, Mutoh Y, Nguyen D, Verweij E,  
751 Zoufaly A, Osinusi AO, DeZure A, Zhao Y, Zhong L, Chokkalingam A, Elboudwarej  
752 E, Telep L, Timbs L, Henne I, Sellers S, Cao H, Tan SK, Winterbourne L, Desai P, et  
753 al. 2020. Compassionate Use of Remdesivir for Patients with Severe Covid-19. *N*  
754 *Engl J Med* doi:10.1056/NEJMoa2007016.
- 755 49. Rosenberg ES, Dufort EM, Udo T, Wilberschied LA, Kumar J, Tesoriero J, Weinberg  
756 P, Kirkwood J, Muse A, DeHovitz J, Blog DS, Hutton B, Holtgrave DR, Zucker HA.  
757 2020. Association of Treatment With Hydroxychloroquine or Azithromycin With In-  
758 Hospital Mortality in Patients With COVID-19 in New York State. *JAMA*  
759 doi:10.1001/jama.2020.8630.
- 760 50. Cox RM, Wolf JD, Plemper RK. 2021. Therapeutically administered ribonucleoside  
761 analogue MK-4482/EIDD-2801 blocks SARS-CoV-2 transmission in ferrets. *Nature*  
762 *Microbiology* 6:11-18.

- 763 51. Wahl A, Gralinski LE, Johnson CE, Yao W, Kovarova M, Dinnon KH, Liu H,  
764 Madden VJ, Krzystek HM, De C, White KK, Gully K, Schäfer A, Zaman T, Leist SR,  
765 Grant PO, Bluemling GR, Kolykhalov AA, Natchus MG, Askin FB, Painter G,  
766 Browne EP, Jones CD, Pickles RJ, Baric RS, Garcia JV. 2021. SARS-CoV-2 infection  
767 is effectively treated and prevented by EIDD-2801. *Nature* 591:451-457.
- 768 52. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, Shi Z, Hu Z, Zhong W, Xiao G.  
769 2020. Remdesivir and chloroquine effectively inhibit the recently emerged novel  
770 coronavirus (2019-nCoV) in vitro. *Cell Res* 30:269-271.
- 771 53. Vincent MJ, Bergeron E, Benjannet S, Erickson BR, Rollin PE, Ksiazek TG, Seidah  
772 NG, Nichol ST. 2005. Chloroquine is a potent inhibitor of SARS coronavirus  
773 infection and spread. *Virology Journal* 2:69.
- 774 54. Parenti F, Beretta G, Berti M, Arioli V. 1978. Teichomycins, new antibiotics from  
775 *Actinoplanes teichomyceticus* Nov. Sp. I. Description of the producer strain,  
776 fermentation studies and biological properties. *J Antibiot (Tokyo)* 31:276-83.
- 777 55. Borghi A, Coronelli C, Faniuolo L, Allievi G, Pallanza R, Gallo GG. 1984.  
778 Teichomycins, new antibiotics from *Actinoplanes teichomyceticus* nov. sp. IV.  
779 Separation and characterization of the components of teichomycin (teicoplanin). *J*  
780 *Antibiot (Tokyo)* 37:615-20.
- 781 56. Shea KW, Cunha BA. 1995. Teicoplanin. *Med Clin North Am* 79:833-44.

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784

785 **Figure Legends**

786 **Figure 1. SARS-CoV-2 infection depended on the activity of CTSL.**

787 (A) CTSL, CTSB, CTSK, TMPRSS2, TMPRSS11A, TMPRSS11D, Furin, PLG,  
788 DPP4 and ACE2 in HEK293T cells were knocked down by siRNAs. These cells were  
789 infected with pseudotyped SARS-CoV-2 viruses 24 hours post transfection. The  
790 intracellular luciferase activity was measured after another 24 hours. The fold change  
791 of luciferase expression in each group was normalized to si-negative control (siNC)  
792 group (n=3). (B) Sequence alignment based on the consensus S protein sequences of  
793 SARS-CoV and SARS-CoV-2. The overall height of the stack indicated the sequence  
794 conservation at that position, while the height of symbols within the stack indicated  
795 the relative frequency (Y-axis) of each amino acid at that position (X-axis). (C) The  
796 multiple alignments were created based on the region containing the cleavage site of  
797 cathepsin L (CTSL) (SIIAYTMSLGA) in the S protein of SARS-CoV-2. The S  
798 proteins of 12 different SARS-CoV-2 variants including D614 (Wuhan-Hu-1), G614  
799 (SYSU-IHV), B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.429 (Epsilon),  
800 B.1.525 (Eta), B.1.526 (Iota), B.1.617.1 (Kappa), B.1.617.2 (Delta), B.1.621 (Mu)  
801 and C.37 (Lambda) were involved. The identity/similarity shading with the color  
802 refers to the chemistry of each amino acid at that position. (D) HEK293T cells in 96-  
803 well plate were transfected with two-fold serially diluted CTSL-expressing plasmids,  
804 ranging from 0.39 ng to 200 ng. Cells were infected with pseudotyped SARS-CoV-2 S  
805 /HIV-1 viruses 24 hours post transfection. Another 48 hours post infection, cells were  
806 lysed and measured for the amounts of luciferase which were represented by

807 luminescence units (n=3). Western blot with antibodies against CTSL was conducted  
808 to confirm the expression of CTSL plasmids.  $\beta$ -Actin was immunoblotted as internal  
809 control. (E) HEK293T cells were transfected with different amounts of ACE2-  
810 expressing plasmids. Another groups of cells were co-transfected with CTSL-  
811 expressing plasmids. These cells were infected with pseudotyped SARS-CoV-2 S /  
812 HIV-1 viruses 24 hours post transfection. The amounts of luciferase within each group  
813 were measured 48 hours post infection and represented as luminescence units (n=3).  
814 The expression of ACE2 and CTSL was confirmed by western blot. Data in (A) and  
815 (D-E) represented as mean  $\pm$  SEM in triplicate. P-values in (A) and (D) were  
816 calculated by one-way ANOVA with Dunnett's multiple comparison test which  
817 compared the mean of each group with the mean of the control group. P-values in (E)  
818 were calculated by one-way ANOVA with Tukey's multiple comparison test which  
819 compared the mean of each group with the mean of every other group. ns =  $p \geq 0.05$ ,  
820 \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

821

822 **Figure 2. Teicoplanin specifically inhibited the entry of SARS-CoV-2.**

823 (A) Schematic of the pseudotyped virus entry upon drug treatment assay. To package  
824 different pseudotyped viruses, psPAX2 plasmids and pHIV-Luciferase plasmids were  
825 co-transfected into HEK293T cells with pcDNA3.1-SARS-CoV S, pcDNA3.1-SARS-  
826 CoV-2 S and pCMV-VSV-G plasmids respectively. HEK293T-hACE2 cells were  
827 incubated with drugs and different pseudotyped viruses. The amounts of luciferase  
828 within cells were measured 48 hours post infection. (B) Chemical structure of

829 teicoplanin. (C) HEK293T-hACE2 cells were treated with 0  $\mu$ M, 25  $\mu$ M and 50  $\mu$ M  
830 teicoplanin respectively, followed by infecting with different pseudotyped viruses  
831 including SARS-CoV S / HIV-1, SARS-CoV-2 S / HIV-1 and VSV-G / HIV-1. The  
832 intracellular luciferase activity was measured after 48 hours post infection (n=3). (D)  
833 Chemical structure of dalbavancin. (E) HEK293T-hACE2 cells were treated as in (C),  
834 except that the drug was replaced with dalbavancin (n=3). (F) Chemical structure of  
835 vancomycin. (G) HEK293T-hACE2 cells were treated as in (C), except that the drug  
836 was replaced with vancomycin (n=3). Data in (C), (E) and (G) represented as mean  $\pm$   
837 SEM in triplicate. P-values were calculated by two-way ANOVA with Dunnett's  
838 multiple comparisons test which compared the mean of each group with the mean of  
839 the control group. ns =  $p \geq 0.05$ , \*\*\* $p < 0.001$ .

840

841 **Figure 3. Teicoplanin inhibited the entry of SARS-CoV-2 by inhibiting the**  
842 **activity of CTSL.**

843 (A) HEK293T-hACE2 cells were co-incubated with authentic SARS-CoV-2 D614  
844 (Wuhan-Hu-1) virus and two-fold serially diluted teicoplanin. At 48 hours post  
845 incubation, the supernatant in each group was collected and proceeded to RNA  
846 extraction. Viral RNA copies in supernatant were quantified by one-step SARS-CoV-2  
847 RNA detection kit. The  $IC_{50}$  of teicoplanin against SARS-CoV-2 D614 virus was  
848 calculated according to these viral RNA copies within each group (n=3). (B) The  $IC_{50}$   
849 of teicoplanin against authentic SARS-CoV-2 G614 (SYSU-IHV) virus was  
850 determined as in (A) (n=3). (C) In the first group, HEK293T-hACE2 cells were pre-



851 treated with 0  $\mu$ M, 12.5  $\mu$ M, 25  $\mu$ M and 50  $\mu$ M teicoplanin respectively. Four hours  
852 later, cells were infected with pseudotyped SARS-CoV-2 S / HIV-1 virus. In the  
853 second group, cells were co-treated with different concentrations of teicoplanin and  
854 pseudotyped SARS-CoV-2 S / HIV-1 virus simultaneously. In the third group, cells  
855 were pre-infected with pseudotyped SARS-CoV-2 S / HIV-1 virus. Four hours post  
856 infection, cells were treated with different concentrations of teicoplanin. The amounts  
857 of luciferase within cells were quantified 48 hours post infection. The fold changes of  
858 luciferase expression within each sample were calculated by normalizing to those in  
859 cells treated with 0  $\mu$ M teicoplanin (n=3). **(D-E)** The *in vitro* purified 250 ng CTSL  
860 proteins were added into CTSL assay buffer for activation. In another group, CTSL  
861 were also co-incubated with 50  $\mu$ M teicoplanin. After activation, the *in vitro* purified  
862 SARS-CoV S or SARS-CoV-2 S proteins were added into each group. S protein only  
863 group was set as control group. The digested proteins were proceeded to SDS-PAGE  
864 and analyzed by silver staining. Data in **(A-C)** represented as mean  $\pm$  SEM in  
865 triplicate. Inhibition curves in **(A-B)** were generated by log (inhibitor) vs. response  
866 nonlinear fit. P-values in **(C)** were calculated by two-way ANOVA with Dunnett's  
867 multiple comparisons test which compared the mean of each group with the mean of  
868 the control group. ns =  $p \geq 0.05$ , \* $p < 0.05$ , \*\*\* $p < 0.001$ .

869

870 **Figure 4. Teicoplanin inhibited the entry of various SARS-CoV-2 mutants.**

871 **(A)** Schematics of the Spike proteins of 12 different SARS-CoV-2 mutants which  
872 included D614 (Wuhan-Hu-1), G614 (SYSU-IHV), B.1.1.7 (Alpha), B.1.351 (Beta),



873 P.1 (Gamma), B.1.429 (Epsilon), B.1.525 (Eta), B.1.526 (Iota), B.1.617.1 (Kappa),  
874 B.1.617.2 (Delta), B.1.621 (Mu) and C.37 (Lambda). The mutation sites were shown  
875 alongside each backbone and indicated in red. **(B-K)** HEK293T-hACE2 cells were co-  
876 incubated with different pseudotyped SARS-CoV-2 S / HIV-1 viruses and serially  
877 diluted teicoplanin. The amounts of luciferase within each group were measured 48  
878 hours post infection and represented as luminescence units. The IC<sub>50</sub> of teicoplanin  
879 against these pseudotyped SARS-CoV-2 mutants was calculated based on the amounts  
880 of luciferase within each group (n=3). Data in **(B-K)** represented as mean ± SEM in  
881 triplicate. Inhibition curves were generated by log (inhibitor) vs. response nonlinear  
882 fit.

883

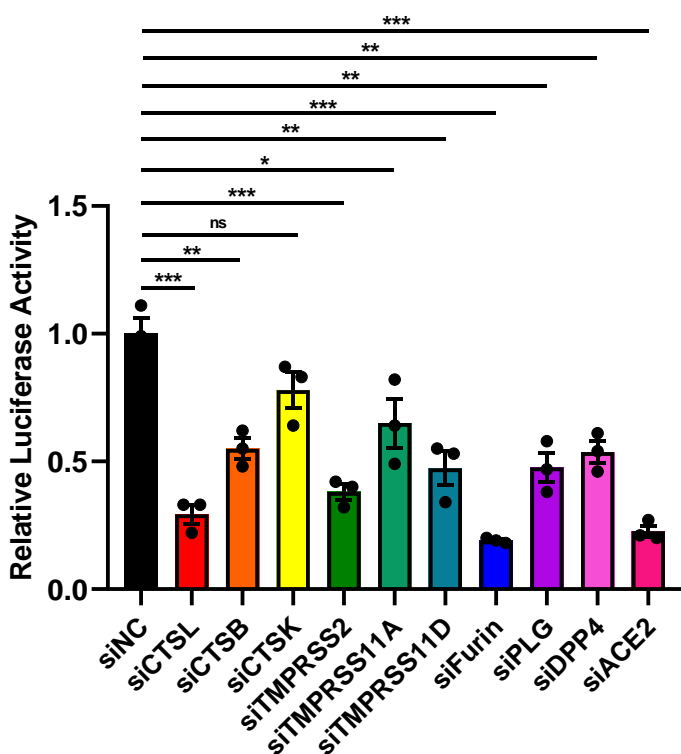
884 **Figure 5. Teicoplanin prevented SARS-CoV-2 infection in hACE2 mice.**

885 **(A)** Schematic of mice experiment procedure. hACE2 mice were intraperitoneally  
886 administrated with 100 mg/kg body weight teicoplanin or saline (n=4 in each group).  
887 Six hours later, each mice was challenged with  $1 \times 10^5$  FFU of authentic SARS-CoV-2  
888 D614 viruses. Another 5 days later, mice were euthanized to harvest lung tissues  
889 which were proceeded to viral RNA detection, HE and IHC. **(B)** Viral RNA copies in  
890 lung tissues of virus-challenged mice were quantified by one-step SARS-CoV-2 RNA  
891 detection kit and plotted as log<sub>10</sub> copies per ml (n=4). **(C)** Lung tissues of mice from  
892 saline group and teicoplanin group were proceeded to HE staining and IHC with  
893 antibodies against N proteins. **(D)** Schematic of teicoplanin inhibiting the entry of  
894 SARS-CoV-2. The SARS-CoV-2 virus binds to the cellular receptor ACE2 by its

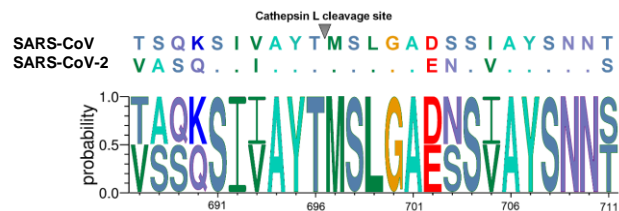
895 Spike (S) proteins which cover the surface of the virus. The S-ACE2 binding event  
896 initiates the proteolytic process of TMPRSS2 to S protein on the cellular membrane,  
897 followed by the entry of virus to the early endosome. Then, the virion is transported  
898 into the late endosome where the S protein is further activated by cleavage with  
899 cysteine proteinase CTSL. After S activation by CTSL, the viral genome is released to  
900 the cytoplasm where viruses replicate and assemble. Teicoplanin can effectively block  
901 the proteolytic activity of CTSL, rendering the S protein unable to be activated.  
902 Without S activation, The SARS-CoV-2 virus is dissolved in the endosome gradually.  
903 Scale bars in (C) represented 100  $\mu\text{m}$ . Data in (B) represented as mean  $\pm$  SEM in  
904 quadruplicate. P-value was calculated by Student's *t* test. \*\*\* $p < 0.001$ .  
905

# Figure 1. SARS-CoV-2 infection depended on the activity of CTSL.

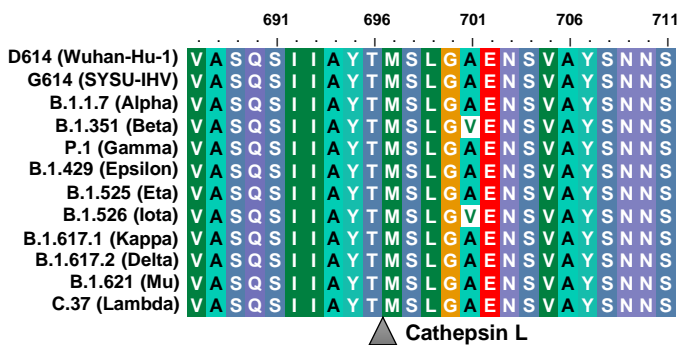
## A The influence of target gene knockdown on SARS-CoV-2 pseudotyped virus infection



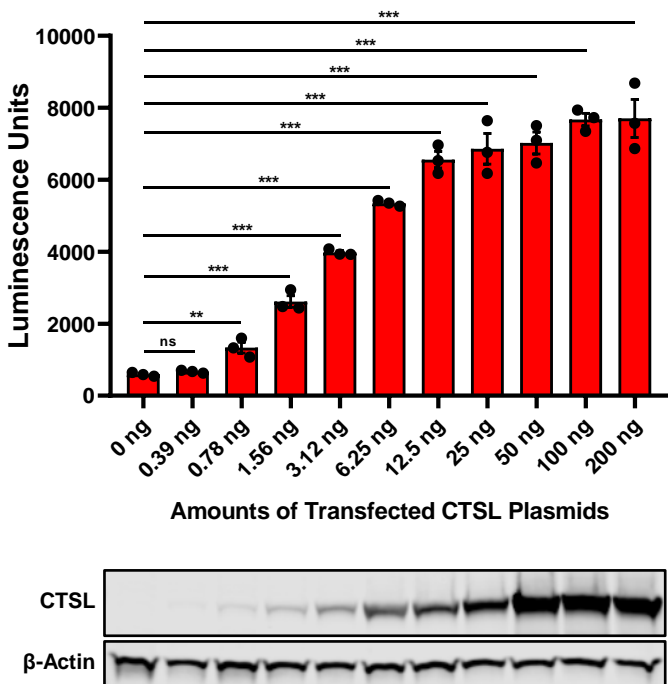
## B



## C

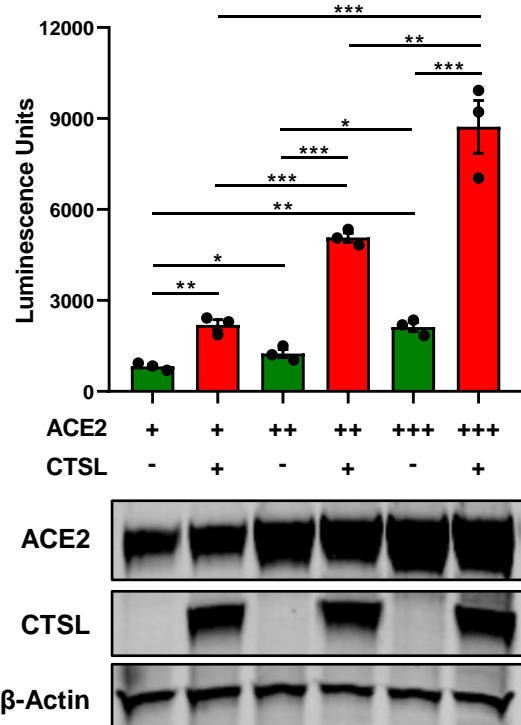


## D CTSL Overexpression in HEK293T Cells



## E

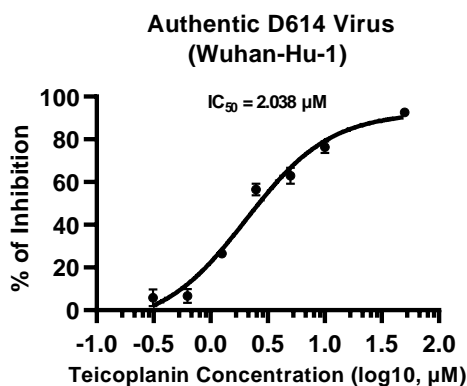
### CTSL and ACE2 co-overexpression in HEK293T Cells



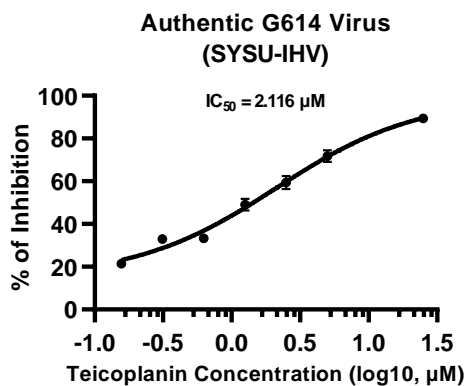


### Figure 3. Teicoplanin inhibited the entry of SARS-CoV-2 by inhibiting the activity of CTSL.

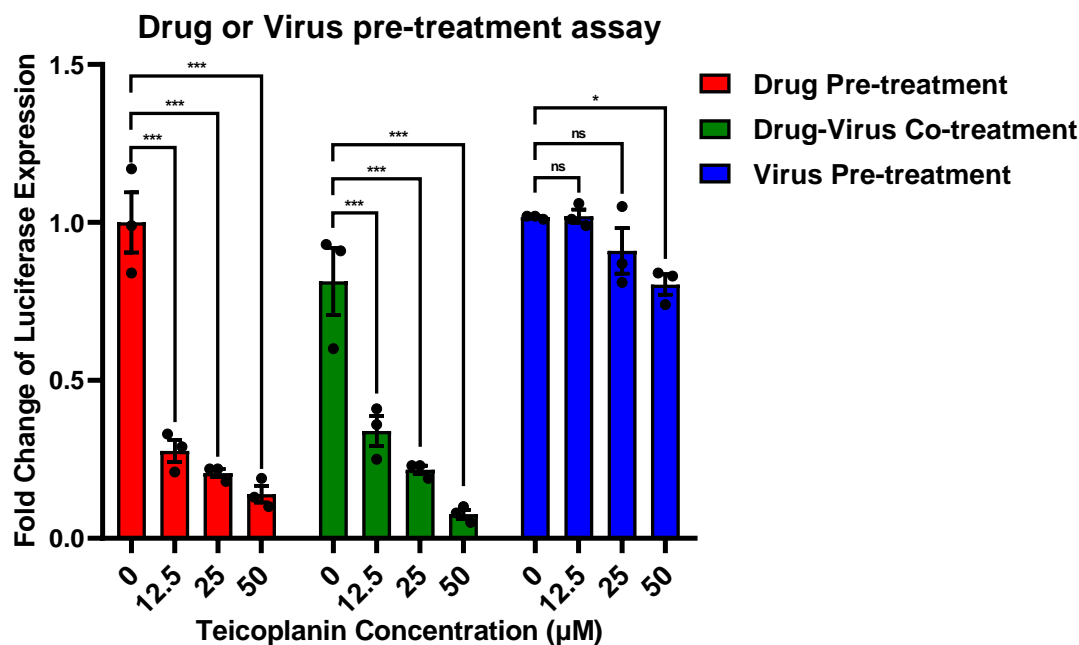
A



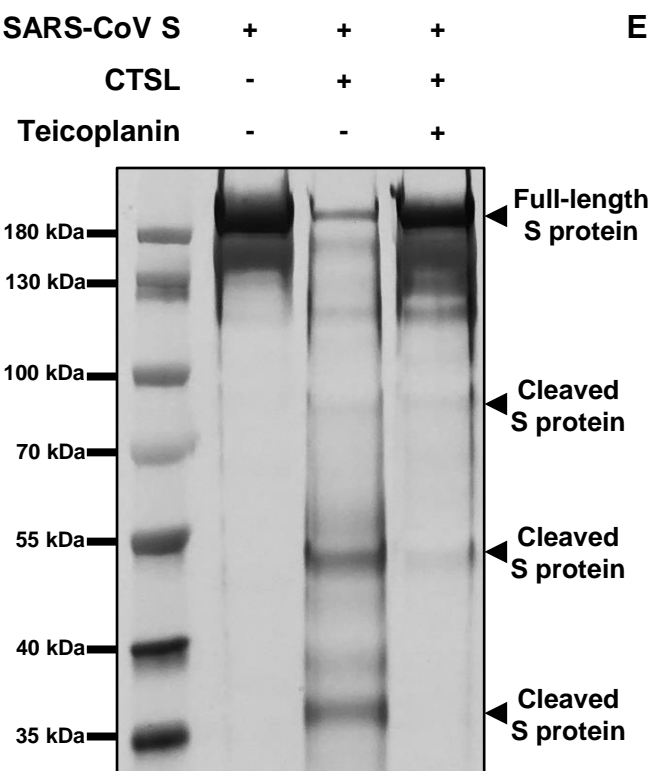
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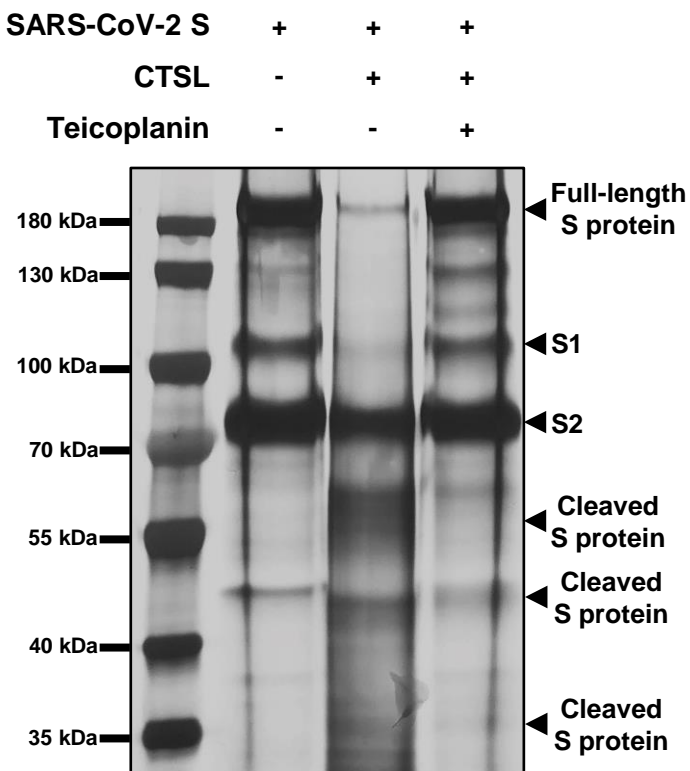
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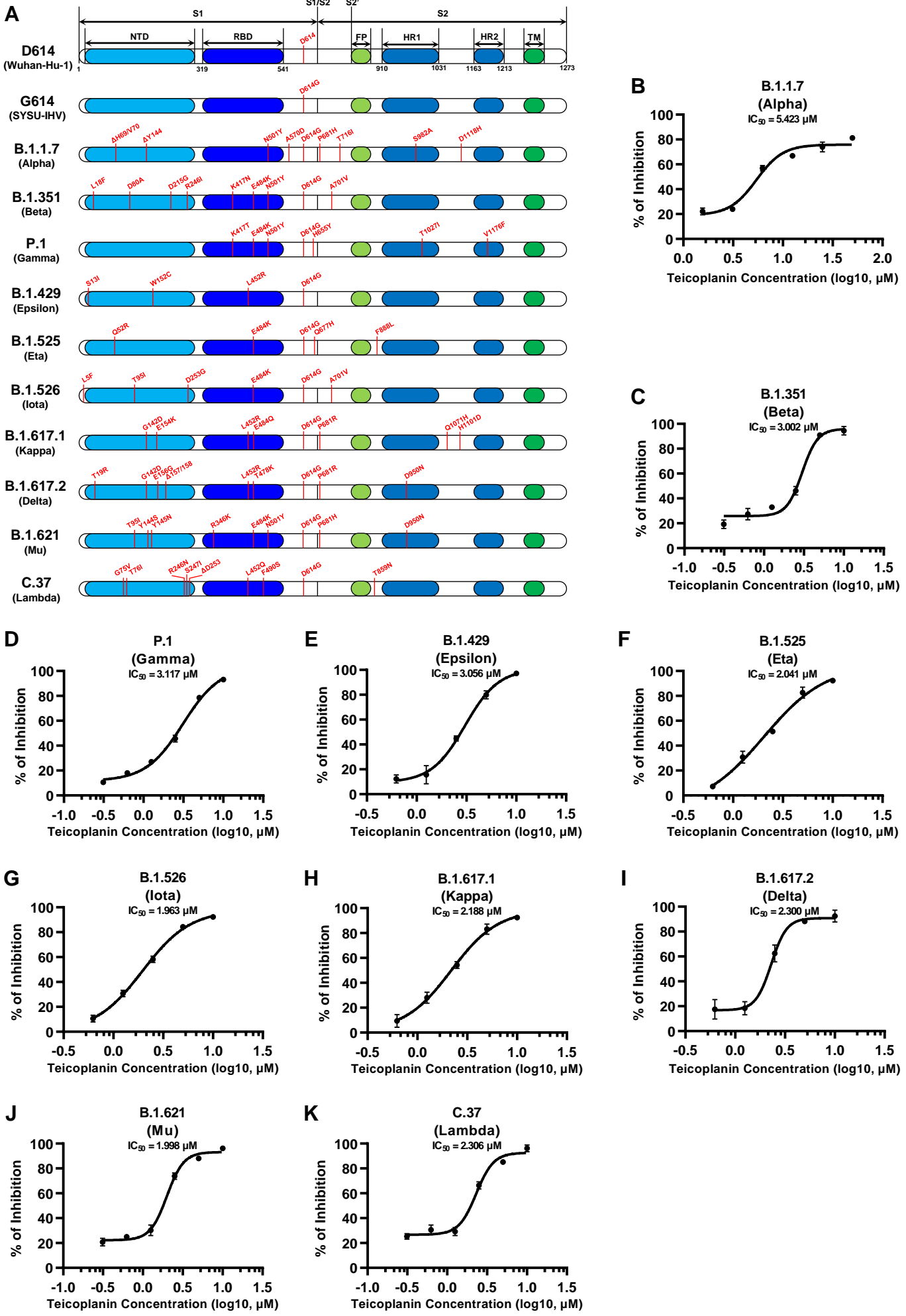
D



E



# Figure 4. Teicoplanin inhibited the entry of various SARS-CoV-2 mutants.



# Figure 5. Teicoplanin prevented SARS-CoV-2 infection in hACE2 mice.

