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1 A photoactivable natural product with broad antiviral activity against enveloped  
2 viruses including highly pathogenic coronaviruses

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27

28 **Abstract**

29 The SARS-CoV-2 outbreak has highlighted the need for broad-spectrum antivirals against  
30 coronaviruses (CoVs). Here, pheophorbide a (Pba) was identified as a highly active antiviral  
31 molecule against HCoV-229E after bioguided fractionation of plant extracts. The antiviral  
32 activity of Pba was subsequently shown for SARS-CoV-2 and MERS-CoV, and its mechanism of  
33 action was further assessed, showing that Pba is an inhibitor of coronavirus entry by directly  
34 targeting the viral particle. Interestingly, the antiviral activity of Pba depends on light exposure,  
35 and Pba was shown to inhibit virus-cell fusion by stiffening the viral membrane as demonstrated  
36 by cryo-electron microscopy. Moreover, Pba was shown to be broadly active against several  
37 other enveloped viruses, and reduced SARS-CoV-2 and MERS-CoV replication in primary  
38 human bronchial epithelial cells. Pba is the first described natural antiviral against SARS-CoV-2  
39 with direct photosensitive virucidal activity that holds potential for COVID-19 therapy or  
40 disinfection of SARS-CoV-2 contaminated surfaces.

41

42

## 43 **Introduction**

44 The COVID-19 pandemic has highlighted the lack of specific antiviral compounds available  
45 against coronaviruses (CoVs). COVID-19 is caused by the severe acute respiratory syndrome  
46 coronavirus 2 (SARS-CoV-2), the third identified human CoV causing severe pneumonia (1–3).  
47 Before 2003, coronaviruses were known to cause severe diseases in animals, but human CoVs,  
48 such as HCoV-229E and OC-43, were mainly associated with common colds, and only rarely  
49 with severe outcomes (4). The severe acute respiratory syndrome coronavirus (SARS-CoV)  
50 outbreak in 2003 was the first emergence of a highly pathogenic human CoV. The second highly  
51 pathogenic coronavirus, identified in 2012 in Saudi Arabia, is the Middle-East respiratory  
52 syndrome coronavirus (MERS-CoV), which is still endemically present up to date. SARS-CoV-2  
53 is highly related to SARS-CoV (5). The tremendous efforts of the scientific community  
54 worldwide to counteract the COVID-19 pandemic have rapidly led to the development of highly  
55 efficient vaccines that are now administered worldwide. Unfortunately, the emergence of SARS-  
56 CoV-2 variants in different regions of the world might render the vaccines less efficient and may  
57 necessitate a booster vaccination every year which might not be achievable for billions of human  
58 beings in all parts of the world. Therefore, to get rid of this pandemic and face future epidemics,  
59 it is assumed that not only vaccination is necessary but also the availability of efficient antiviral  
60 treatments. Before the emergence of SARS-CoV-2, no specific antiviral was commercially  
61 available for treatment of CoV infections. Due to the urgent need for antivirals against SARS-  
62 CoV-2, many researchers have focused their investigations on repurposing available drugs.  
63 Unfortunately, until now, none of them has been able to significantly reduce severe outcomes in  
64 patients. High content screening *in vitro* of approved drugs identified some potential interesting  
65 antiviral molecules that have still to be tested in clinic on patients with COVID-19 (6–8).  
66 Protease and polymerase inhibitors are also widely investigated in *in vitro* studies (9). Recently,  
67 White *et al.* identified plitidepsin, an inhibitor of the host protein eEF1A as a potential antiviral

68 agent for SARS-CoV-2 (10). To date, only synthetic neutralizing monoclonal antibodies have  
69 been approved for emergency usage in newly infected patients.

70 Coronavirus are members of the *Coronaviridae* family within the order *Nidovirales*. CoVs are  
71 enveloped viruses with a positive single-stranded RNA genome of around 30 kb. The genome  
72 encodes 4 structural proteins, the nucleocapsid (N), the spike (S), the envelope (E), and the  
73 membrane (M) proteins. The S protein is important for the interaction of the viral particle with  
74 the cellular host receptor, being angiotensin-converting enzyme 2 (ACE2) for SARS-CoVs (11,  
75 12), dipeptidyl peptidase 4 (DPP4) for MERS-CoV (13) and aminopeptidase N (APN) for HCoV-  
76 229E (14). Once attached, the virus releases its genome into the cytosol by fusion of the viral  
77 envelope with a host membrane. This fusion process is mediated by the S protein, a class I fusion  
78 protein, and can occur either at the plasma- or at the endosomal membrane. Viral class I fusion  
79 proteins are typically synthesized as inactive precursor proteins and require proteolytical  
80 activation by cellular proteases to acquire their fusion competent state. The host-cell protease  
81 TMPRSS2 has been shown to be necessary for plasma membrane fusion of many coronaviruses  
82 including SARS-CoV-2 (12, 15, 16), whereas cathepsins are often involved in fusion processes at  
83 endosomal membranes (17).

84 It is estimated that about 80% of the global population rely on traditional medicine to treat  
85 infectious diseases. Plants are a natural source of compounds with a structural diversity that is  
86 much higher than those obtained by chemical synthesis. Many of these compounds have proven  
87 their antiviral activity *in vitro*. To date, some reports describe the antiviral activity of natural  
88 compounds on coronavirus including SARS-CoV-2, but many of them are *in silico* analyses  
89 without any *in vitro* or *in vivo* evidence (18, 19).

90 Here we show that pheophorbide a (Pba) isolated from *Mallotus oppositifolius* (Geiseler)  
91 Müll.Arg. (Mo, *Euphorbiaceae*) leave crude extract after bioguided fractionation has antiviral  
92 activity against various CoVs, including HCoV-229E, MERS-CoV and SARS-CoV-2, but also

93 against other enveloped viruses, such as yellow fever virus (YFV), hepatitis C virus (HCV) and  
94 Sindbis virus (SINV). Moreover, we demonstrate that Pba is an antiviral photosensitizer directly  
95 acting on the viral particle, thereby impairing the virus-cell fusion step.

96

## 97 **Results**

### 98 **Pheophorbide a (Pba) isolated from the crude methanolic extract from *Mallotus*** 99 ***oppositifolius* (Geiseler) Müll.Arg. is highly active against HCoV-229E**

100 Fifteen plants methanolic extracts from the Ivorian pharmacopeia, which were initially screened  
101 for their anti-HCV activity (Bamba *et al.*, submitted), were tested against HCoV-229E-Luc, a  
102 luciferase recombinant version of HCoV-229E, which allows for a rapid and easy screening of  
103 diverse molecules *in vitro*. Seven of the fifteen extracts significantly reduced HCoV-229E  
104 infection (Figure 1A), whereas none of the extracts showed cytotoxicity in Huh-7 cells at the  
105 tested concentrations (Bamba *et al.*, submitted). The *Mallotus oppositifolius* (Mo) crude extract  
106 was the most active and therefore selected for further analyses. A bioguided fractionation was  
107 performed to find out the active compound(s) in this plant. This revealed that the methylene  
108 chloride (MC) partition was the most active on HCoV-229E (Figure 1B) and was therefore  
109 chosen for fractionation by centrifugal partition chromatography (CPC), leading to 10 fractions  
110 (F1-F10). Among these fractions, F7 was the most active and subjected to further fractionation by  
111 preparative high performance liquid chromatography (HPLC), leading to 9 subfractions (F7.1-  
112 F7.9). F7.7 was the most active of them and seemed to contain only one molecule. This dark  
113 green product was analysed by LC-UV-MS, which revealed only one peak ( $m+H = 593,3$ ) with  
114 two maxima of absorption in the visible light (409 and 663 nm). This information was used for a  
115 Dictionary of Natural Product search (<https://dnp.chemnetbase.com/>), indicating that this  
116 molecule could be pheophorbide a (Pba). F7.7 purity and chemical structure was further  
117 confirmed by nuclear magnetic resonance (NMR)(data not shown).

118 To confirm that Pba was the active compound of the Mo extract, a dose-response experiment was  
119 performed with both the pure compound isolated in F7.7 and commercial Pba against HCoV-  
120 229E-Luc in Huh-7 cells. As shown in Figure 1C, IC<sub>50</sub> values were comparable for both natural  
121 and commercial Pba (0.51 μM and 0.54 μM, respectively), confirming that Pba was indeed the  
122 active substance of Mo.

123

#### 124 **Pba is active against several human CoVs at non-cytotoxic concentrations**

125 Prior to the assessment of its broad-spectrum activity against various CoVs, the cytotoxicity of  
126 Pba was determined in different cells lines. As Pba is known to be a photosensitizer it was  
127 assumed that light could affect its toxicity. MTS assays were performed in similar conditions  
128 than the infection procedures. Cells were incubated with Pba at different concentrations and taken  
129 out of the incubator after 1 h to change the medium and left for 10 min under light exposure in  
130 the biosafety cabinet (BSC), after which they were replaced in the incubator for 23 h. In parallel,  
131 plates were kept for 24 h in the dark. Pba did not exhibit any toxicity at concentrations up to  
132 120 μM when left in the incubator for 24 h without light exposure, whereas it showed some  
133 toxicity when the cells were shortly exposed to light, with CC<sub>50</sub> values of  $4.4 \pm 1.2$  μM,  $5.8 \pm 1.9$   
134 μM and  $5.5 \pm 2.0$  μM for Huh-7, Vero-E6 cells, and Vero-81, respectively (Figure 2 and Figure S1).  
135 Taken together, these results show that the cytotoxicity of Pba in cell culture depends on light  
136 exposure.

137 The antiviral activity of Pba was tested on different human CoVs. It was first confirmed on  
138 HCoV-229E by measuring infectious titers (Figure 2) with an IC<sub>50</sub> value of 0.1 μM, resulting in a  
139 selectivity index of 44. Similarly, infection inhibition assays were performed with various non-  
140 cytotoxic concentrations of Pba against SARS-CoV-2 and MERS-CoV. As shown in Figure 2,  
141 Pba had a strong antiviral effect against both highly pathogenic coronaviruses at non-toxic

142 concentrations, with IC<sub>50</sub> values of 0.18 μM for both SARS-CoV-2 and MERS-CoV, resulting in  
143 a selectivity index of 32 and 24, respectively.

144

#### 145 **Pba is an inhibitor of coronavirus entry by direct action on the particle**

146 Coronaviruses fusion is triggered by proteolytic cleavage of the spike protein. Depending on  
147 cellular proteases available, fusion can occur after endocytosis of the virus or directly at the cell  
148 surface. It has been demonstrated that HCoV-229E and SARS-CoV-2 fusion at the plasma  
149 membrane depends on the expression of the TMPRSS2 protease (12, 20), and for many  
150 coronaviruses the entry via fusion at the plasma membrane has been shown to be the most  
151 relevant pathway *in vivo* (21). To determine if Pba was able to inhibit both entry pathways, its  
152 antiviral activity was tested in Huh-7, and Huh-7-TMPRSS2 cells, the latter being obtained after  
153 transduction with a TMPRSS2 lentiviral expression vector. Two inhibitors of the entry pathway  
154 were used as a control, E64D as inhibitor of the endocytic pathway and Camostat as inhibitor of  
155 the TMPRSS2 protease. As shown in Figure S2, E64D specifically inhibited HCoV-229E  
156 infection in Huh-7 cells whereas Camostat inhibited infection in Huh-7-TMPRSS2 cells.  
157 Complete inhibition of infection was observed when a combination of the two inhibitors was  
158 used, showing that both entry pathways can be used *in vitro*. No difference in antiviral activity of  
159 Pba against HCoV-229E infection was observed in the presence or absence of TMPRSS2 (Figure  
160 3A). Similar results were obtained with SARS-CoV-2 in Vero cells (our unpublished  
161 observation). These results show that Pba exhibits antiviral activity whatever the entry pathway  
162 used.

163 To gain insights into the mechanism of action of Pba, a time-of-addition assay with Pba was  
164 performed during HCoV-229E or SARS-CoV-2 infection. Therefore, Pba was added at different  
165 time points before, during or after inoculation. All experiments were performed under BSC's  
166 light exposure. For both viruses, no inhibition of infection was observed when Pba was added to



167 the cells before inoculation (pre-treatment of the cells), whereas a strong inhibition of infection  
168 was noticed when Pba was present during the virus inoculation step (Figure 3B and 3C). When  
169 Pba was added only after inoculation, its inhibitory effect rapidly dropped and normal levels of  
170 infectivity were observed again when Pba was added more than 2 h after inoculation.  
171 Chloroquine is a well-known inhibitor of SARS-CoV-2 replication *in vitro*, as it inhibits virus-  
172 cell fusion after endocytosis by preventing endosomal acidification. As shown in Figure 3C, the  
173 Pba and chloroquine inhibition curves were rather similar, showing that Pba could inhibit virus  
174 entry. It is worth noting that normal levels of infectivity were observed when Pba was added  
175 more than 2 h after inoculation, whereas chloroquine-treated cells remained at around 60% of  
176 normal levels which may be due to inhibition of the second round of entry since our experimental  
177 conditions are compatible with reinfection. Taken together, these results suggest that Pba is  
178 inhibiting virus entry.

179 Since no antiviral activity was observed with cells treated with Pba prior to inoculation, we  
180 wondered whether Pba targets the virus instead of the cells. To test this hypothesis, HCoV-229E-  
181 Luc was pre-incubated for 30 min at a concentration 10 times higher than that used during  
182 inoculation. For this experiment, HCoV-229E-Luc was pre-incubated with Pba at 2  $\mu\text{M}$  for 30  
183 min, then diluted to reach a concentration of Pba of 0.2  $\mu\text{M}$  for inoculation, a concentration  
184 which does not severely impact HCoV-229E-Luc infection as shown above. In parallel, cells  
185 were directly inoculated with HCoV-229E-Luc at 0.2 and 2  $\mu\text{M}$  as a control. The results clearly  
186 show that when HCoV-229E-Luc was pre-treated with Pba at high concentration (2  $\mu\text{M}$ ) before  
187 inoculation at low concentration (0.2  $\mu\text{M}$ ), the antiviral activity was much stronger than when  
188 inoculation was performed in the presence of 0.2  $\mu\text{M}$  Pba without any pre-treatment (Figure 3D).  
189 Taken together, these results indicate that Pba inhibits HCoV entry by a direct effect on the viral  
190 particle.

191

192 **Pba is an inhibitor of viral fusion**

193 Virus entry can be divided into two different steps, firstly the viral attachment to the cell surface,  
194 and secondly the fusion of the virus envelope with cellular membranes. To further define the  
195 mode of action of Pba, experiments were performed with HCoV-229E, which can be manipulated  
196 in a lower containment facility. To determine a potential effect at the attachment step, Huh-7-  
197 TMPRSS2 cells were incubated with HCoV-229E in the presence or absence of Pba at 4°C for 1  
198 h. These conditions block endocytosis but allow virus attachment to the cell surface. Cells were  
199 rinsed with PBS and the amount of virions attached to the surface was determined by  
200 quantification of viral genomes by qRT-PCR. As shown in Figure 4A, only a slight and non-  
201 significant decrease in RNA levels was observed in the presence of Pba, indicating that Pba  
202 barely affect virus attachment. The action of Pba on the fusion step was investigated in virus-cell  
203 fusion assay by using trypsin as an exogenous protease to induce coronavirus membrane fusion at  
204 the plasma membrane (22, 23). Huh-7 cells were treated with NH<sub>4</sub>Cl to inhibit fusion in the  
205 endocytic pathway and viruses were bound at the cell surface at 4°C. Then, fusion was induced  
206 by a short trypsin treatment at 37°C. Entry at the cell surface was more efficient in presence of  
207 trypsin compared to the control (Figure 4B), which is consistent with other reports (22, 23). In  
208 addition, Pba at both 0.5 and 1 μM strongly inhibited infection levels in trypsin-mediated fusion  
209 conditions in a similar range compared to the inhibition seen with the control. Taken together,  
210 these results indicate that Pba inhibits entry at the fusion step and not by preventing attachment.  
211 Two viral structures are involved in the virus-cell fusion, namely the spike protein and the viral  
212 membrane itself. To find out if Pba targets the spike, a cell-cell fusion assay was performed. For  
213 this, the SARS-CoV-2 spike protein was transiently expressed by plasmid transfection in Vero-81  
214 cells. At 6 h post-transfection, the medium was replaced with medium containing either DMSO  
215 or 1 μM Pba until 24 h p.t.. As shown in Figure 4C, spike induced cell-cell fusion (apparent as  
216 syncytium formation) occurred equally well in control (DMSO)- and Pba-treated conditions,

217 indicating that Pba cannot prevent cell-cell fusion induced by the viral spike protein. Taken  
218 together these results are not in favour of an effect of Pba on the viral spike fusion protein.

219  
220 **The antiviral activity of Pba is increased upon light exposure and targets the viral**  
221 **membrane**

222 Given that Pba is photoactivable, we wondered whether its antiviral effect is light-dependent. We  
223 therefore inoculated Huh-7 cells with HCoV-229E in the presence of Pba under different light  
224 exposure conditions. As shown in Figure S3, the antiviral activity of Pba increased upon light  
225 exposure. However, even in the dark, antiviral activity was observed at 10  $\mu$ M. A typical feature  
226 of photosensitizers is that the concentration required for its biological properties decreases upon  
227 increase of time of exposure to the light. To see whether Pba behaves as other photosensitizers,  
228 HCoV-229E was pre-incubated with Pba at various concentrations (0.02, 0.2 and 2  $\mu$ M) and  
229 exposed to the normal white light of the laminar flow cabinet for different durations (ranging  
230 from 5 to 80 min). It is important to note that light alone was not responsible of the antiviral  
231 activity because there was no antiviral effect after a long exposure time (80 min) with Pba at  
232 inactive concentration (0.02  $\mu$ M). As shown in Figure 5A, results clearly showed that with a  
233 same concentration of Pba, increased inhibitory effect could be observed with longer light  
234 exposure times. Similar results were observed with SARS-CoV-2 (Figure 5B). Together, these  
235 data indicate that the anti-coronavirus properties of Pba depend on its dynamic photoactivation.

236 Photodynamic inactivation (PDI) of microorganisms typically results from the onset of reactive  
237 oxygen species (ROS), including free radicals or singlet oxygen species ( $^1\text{O}_2$ ), generated when  
238 the light-activated photosensitizer falls back to its ground state, thereby transferring its energy to  
239 molecular oxygen (resulting in the onset of  $^1\text{O}_2$ ) or initiating photochemical reactions with ROS  
240 generation. Two mechanisms of activation have been described, either type I reactions in which  
241 the photosensitizer activates a substrate that generates reactive oxygen species (ROS), or type II

242 reactions in which the photosensitizer directly generates singlet oxygen ( $^1\text{O}_2$ ). Subsequently,  
243 these species can damage various micro-organism structures, such as nucleic acids, proteins or  
244 lipids (24, 25). To determine if the antiviral activity of Pba also depends on ROS or  $^1\text{O}_2$   
245 generation, infection was performed in the presence of quenchers that are able to trap these  
246 generated oxygen species. Two  $^1\text{O}_2$  quenchers were used, a water-soluble analogue of vitamin E,  
247 Trolox, and  $\text{NaN}_3$  (Figure 5C). HCoV-229E was mixed with the quenchers Trolox and  $\text{NaN}_3$  both  
248 at 10 mM, and Pba was added at the inoculation step. Then the cells were rinsed and fresh culture  
249 medium was added for 6 h. The results clearly showed that both Trolox and  $\text{NaN}_3$  were able to  
250 reduce the action of Pba (Figure 5C), indicating that the antiviral activity of Pba is mediated by  
251 the generation of  $^1\text{O}_2$  after photoactivation.

252 Vigant *et al.* clearly demonstrated that the generation of  $^1\text{O}_2$  by the lipophilic photosensitiser  
253 LJ001 induces the phosphorylation of unsaturated phospholipid of viral membranes, and changes  
254 the biophysical properties of viral membranes, thereby affecting membrane fluidity and/or  
255 increasing rigidity (24). As a result, the change of fluidity and/or rigidity of the viral membrane  
256 impairs its ability to undergo virus-cell fusion. We therefore wondered whether a similar action  
257 of the photosensitizer on the lipids of the viral envelope might also explain our observation that  
258 Pba is able to inhibit HCoV-229E fusion by targeting the viral particle. We hypothesized that the  
259 Pba-induced membrane rigidity may render the virus less sensitive to a shrinkage effect induced  
260 by an osmotic stress. Therefore, HCoV-229E was incubated with Pba either in the dark or under  
261 light exposure for 30 min, and subjected to osmotic shock with 400 mM NaCl before fixation  
262 with 4% PFA. Fixed viral particles were visualized by cryo-electron microscopy (CryoEM). As  
263 shown in Figure 6, intact virions with their characteristic spikes at the surface can be observed in  
264 untreated conditions (control). The addition of Pba either in the dark or under light condition did  
265 not affect the overall morphology of virions in normal medium conditions. Interestingly, when  
266 intact virions were subjected to an osmotic stress by increasing NaCl concentration from 100 mM

267 to a final concentration of 400 mM, the virions shape was altered due to a shrinkage of the viral  
268 membrane. When virions incubated with Pba in the dark were subjected to osmotic shock, similar  
269 alteration of viral shape was observed. However, in the presence of Pba and under light  
270 condition, no membrane deformation was observed suggesting that the virus was more resistant  
271 to osmotic shock. These results clearly show that light-activated Pba modify the mechanical  
272 properties of the viral envelope by increasing its stiffness. This Pba-induced increase in the  
273 envelope rigidity likely prevents the membrane deformation needed to undergo virus-cell fusion.  
274 Knowing that there might be an effect of the light, the spike-induced cell-cell fusion assay as  
275 shown in Figure 4C was repeated with short light exposure of the spike-transfected cells every 2  
276 h after addition of Pba. Even with regular exposure to the light, no effect of Pba was seen on the  
277 spike-mediated cell-cell fusion (data not shown), further excluding that Pba additionally targets  
278 the spike protein.

279

280

### 281 **Pba is a broad-spectrum antiviral that targets viral membranes of several enveloped viruses**

282 In contrast to viral proteins, viral membranes are derived from the host cell and hence are not  
283 virus specific. This suggests that Pba might have broader antiviral activity against enveloped  
284 viruses. Indeed, antiviral activities against other viruses have already been reported for Pba or  
285 related molecules. These include HCV, HIV (Human immunodeficiency virus), and IAV  
286 (Influenza A virus) (26–28). To confirm the broad-spectrum activity of Pba, its antiviral activity  
287 was tested on pseudotyped viral particles with envelope proteins of VSV (vesicular stomatitis  
288 virus), HCV, SARS-CoV-2, and MERS-CoV. Pseudoparticles were pre-treated with 0.5, 1 or 2  
289  $\mu$ M Pba and exposed or not to the light for 30 min prior to inoculation. The results clearly  
290 showed that Pba inhibited pseudoparticle infection regardless of the nature of the viral envelope  
291 protein, but only under light conditions (Figure 7A). Next, the antiviral activity of Pba was tested

292 for coxsackievirus (CVB4, non-enveloped), Sindbis virus (SINV, enveloped), hepatitis C virus  
293 (HCV, enveloped), and yellow fever virus (YFV, enveloped). Whereas Pba was not active on the  
294 non-enveloped virus CVB4, it showed a clear antiviral effect against the 3 enveloped viruses  
295 HCV, YFV and SINV (Figure 7B). Taken together, these results confirm the light-dependent  
296 activity of Pba on enveloped viruses and suggest that the lipid membrane is the most likely target  
297 of the compound.

298  
299 **Other chlorophyll-derived products and photosensitizers possess a light-dependent anti-**  
300 **coronaviral activity**

301 Pba is a break-down product of chlorophyll. Chlorophyll is metabolized into different compounds  
302 including Pba, pyropheophorbide a (pyroPba), and chlorin e6. We wondered if these products  
303 would also have antiviral activity. Furthermore, we selected nine porphyrins or  
304 metalloporphyrins structurally related to Pba (N-methyl protoporphyrin IX, N-methyl  
305 mesoporphyrin IX, Zn-protoporphyrin IX, tin-mesoporphyrin IX, temoporfin, phthalocyanine,  
306 hemin chloride, HPPH, and 5,15-DPP), and one photosensitizer without related structure (Rose  
307 Bengale) to determine if similar antiviral activity against coronaviruses could be identified. The  
308 toxicity and antiviral activity of these compounds were investigated (Figure S4). Chlorophyll b,  
309 phthalocyanine and 5,15-DPP were not active at the tested concentrations. Three molecules,  
310 hemin chloride, temoporfin and Rose Bengale had a moderate antiviral activity. The antiviral  
311 activity of the six most active compounds was tested both under normal white light conditions  
312 and in the dark, clearly showing that, similar to Pba, the antiviral activity of most molecules  
313 tested was also light-dependent (Figure S3). Interestingly, N-methyl protoporphyrin IX and N-  
314 methyl mesoporphyrin IX showed an antiviral activity in dark conditions, but much lower than  
315 under light exposure. PyroPba was toxic at tested concentrations, thus dose-response experiments  
316 with lower concentrations were performed to determine precise CC<sub>50</sub> and IC<sub>50</sub>. This was also

317 done for all the active compounds. The  $CC_{50}$  and  $IC_{50}$  of the different compounds were compared  
318 and only pyroPba was more active against HCoV-229E than Pba (Table 1) with an  $IC_{50}$  of 0.35  
319  $\mu\text{M}$ . However, this compound is also more toxic with a  $CC_{50}$  of 2.67  $\mu\text{M}$  and a selective index of  
320 7.6. Thus, our results show that porphyrin-related compounds have antiviral activity against  
321 HCoV-229E but that Pba and pyroPba are the most active under normal white light exposure.

322

323 **Table 1. Inhibitory and cytotoxic concentrations, and selective index of photosensitizers**  
324 **against HCoV-229E**

325

Molecules	$IC_{50}$ ( $\mu\text{M}$ )	$CC_{50}$ ( $\mu\text{M}$ ) 24 h	SI
Pba	0.54	6.08	11.2
PyroPba	0.35	2.67	7.6
Chlorin e6	0.72	21.4	29.7
HPPH	1.14	8.13	7.1
N-methyl protoporphyrin IX	1.21	ND > 80	>66.1
N-methyl mesoporphyrin IX	1.25	ND > 80	>64
Zn protoporphyrin IX	0.79	16.64	21.0
Rose Bengale	2.86	ND > 80	27.9

326

327

328 **Pba reduces SARS-CoV-2 and MERS-CoV replication in human primary airway epithelial**  
329 **cells**

330 As shown above, Pba is able to inhibit SARS-CoV-2 and MERS-CoV infection under white light  
331 exposure in cell culture. To determine if Pba could be used *in vivo*, its antiviral activity was tested  
332 in a preclinical model, the human primary airway epithelial cells. These cells, Mucilair<sup>TM</sup>, are  
333 primary bronchial epithelial cells reconstituted in a 3D structure to mimic bronchial epithelium  
334 with an air-liquid interface. Mucilair<sup>TM</sup> cells were inoculated with SARS-CoV-2 or MERS-CoV  
335 in the presence of Pba at 0.25 or 2.5  $\mu\text{M}$ . Remdesivir at 5  $\mu\text{M}$  was used as a positive control. At  
336 72 h post-inoculation, viral titers were determined and viral RNA levels were quantified. Viral  
337 RNA levels of SARS-CoV-2 and MERS-CoV were significantly decreased in cells in the

338 presence of Pba at 0.25 and 2.5  $\mu\text{M}$ , respectively (Figure 8A). Similarly, viral titers of both  
339 viruses were decreased of more than 1xLog10 in the presence of Pba at 2.5  $\mu\text{M}$  (Figure 8B). In  
340 parallel, the toxicity of Pba was measured in Mucilair<sup>TM</sup> by measuring lactate deshydrogenase  
341 (LDH) secretion and trans-epithelial electrical resistance. Cells were incubated with the molecule  
342 in the same condition as the infection assay. Cells treated with lysis buffer were used as a control.  
343 No toxicity was observed up to 72h in the cells treated with Pba compared to the control (Figure  
344 8C and 8D). In cells treated with lysis buffer, no LDH secretion was observed at 48h and 72h  
345 because the cells were dead. Taken together, these results confirm the antiviral activity of Pba  
346 against highly pathogenic human CoVs and its potential activity *in vivo*.

347

## 348 **Discussion**

349 By screening plant extracts for their antiviral activity against coronaviruses, the present study  
350 identified Pba as the active antiviral compound in the crude methanol extract from  
351 *M. opositifolius* after bioguided fractionation. It was demonstrated that Pba is active against  
352 various human CoVs, including SARS-CoV-2, HCoV-229E and MERS-CoV, as well as other  
353 enveloped viruses, including HCV, SINV and YFV, and various pseudotyped particles.  
354 Furthermore, we characterized Pba as a broad-spectrum antiviral photosensitizer causing PDI of  
355 all tested enveloped viruses by production of singlet oxygen species that most probably increase  
356 the rigidity of the lipid bilayer of the viral envelope.

357 Pba is a product of chlorophyll breakdown which is abundantly present in various plants (such as  
358 spinach) and marine algae. In general, the chlorophyll content in plants may vary and depends on  
359 the season, the part of the plant, the maturity of the organs and many other factors (29, 30).  
360 Chlorophyll is degraded into Pba by chlorophyllase and some plants with high chlorophyllase  
361 content may contain more Pba (31, 32). Pba is well documented for its potential as anti-cancer



362 agent in photodynamic therapy (PDT). It is known to have a low toxicity, to selectively  
363 accumulate in tumours and to have a high adsorption at 665 nm (33, 34).

364 For many years, photosensitizers have mainly been used as antitumor therapy, for which many  
365 photosensitizers have already been proven to be clinically safe and some are currently approved  
366 for use in humans (35). Although reports on the PDI of viruses go back to 1960, where it was  
367 shown that some photosensitive dyes, such as methylene blue, had an antiviral effect, it is only in  
368 the last decades that photosensitizers have gained considerable interest as antimicrobial (bacteria,  
369 fungi, and viruses) agents, due to their strong antimicrobial effects and low toxicity in normal  
370 tissue (36, 37). A major advantage of those molecules is that, due to their direct damaging effect  
371 on the micro-organism, they are insensitive to the onset of resistance of the microorganism  
372 against the compound, the latter being a major problem in today's research on antivirals and  
373 antibiotics. With the present study, we add Pba to the list of photosensitizers with considerable  
374 antiviral effects, at least against enveloped viruses. This antiviral effect is not new, since the  
375 antiviral activity of Pba has already been demonstrated before against several enveloped viruses,  
376 including HCV, Influenza A, herpes simplex-2 virus, and HIV-1 (26–28). Zhang et al (28) also  
377 showed a direct action on the viral particle, which is in line with our results. In our study, we  
378 show that the coronavirus' envelope remains intact after treatment with Pba up to 10  $\mu$ M for 30  
379 min in the light, that Pba treatment does not affect attachment, but that the virus-cell fusion is  
380 impaired probably due to the rigidification of the virus membrane upon light exposure. In  
381 addition, there seemed to be a slight antiviral effect at high concentrations (more than 10  $\mu$ M)  
382 even in dark conditions. In contrast to our results, some papers show that Pba has a virucidal  
383 activity by damaging the virus envelope, thereby inhibiting attachment (28). For HCV and HIV-  
384 1, Pba and chlorophyll a derivatives were applied after virus infection and were shown to act on  
385 replication corresponding to a mode of action different than the one reported in our study.  
386 Furthermore, none of those reports showed a mechanism of action dependent on light exposure,

387 and hence did not show that the antiviral effect is mediated by PDI of the particle. It is important  
388 to note that under usual experimental conditions, cell cultures were not protected from light, thus  
389 is likely that the vast majority of the reports on antiviral activity of Pba were performed under  
390 light exposure. Due to the lack of control conditions in the dark or information on the time of  
391 (unnoticed) light exposure in the above mentioned reports on Pba, it is very hard to compare our  
392 data with the literature at this moment. More in depth studies will be needed to understand and  
393 compare the action of Pba on different viruses, in both light and dark conditions.

394 It is interesting to note that Pba (or highly related compounds such as pyroPba) has been isolated  
395 from different plant species and different organisms including marine algae (38), rendering Pba a  
396 very attractive antiviral due to its high availability. Moreover, it has been postulated that Pba can  
397 transport a metal ion like zinc inside the cell and that zinc can inhibit viral replication (39) which  
398 might be another advantage to use these molecules as antivirals. In our study, it was confirmed  
399 that neither natural nor commercial Pba used in our experiments contained a metal ion, so future  
400 studies should be performed with zinc Pba to demonstrate this hypothesis.

401 Here, we show that Pba inhibits virus-cell fusion, probably by targeting and photodynamically  
402 damaging the viral membrane. With the help of cryo-EM, we demonstrated that the treatment of  
403 virions with Pba and exposed to the light did not affect their shape despite an osmotic shock. This  
404 is an indirect demonstration of an increased rigidity of the viral envelope upon Pba treatment.  
405 This feature was already demonstrated using a biophysical approach with lipophilic  
406 photosensitizers with antiviral activity (24, 40). It was postulated that the increased rigidity  
407 impairs membrane bending required for viral fusion (41). Contrary to the cell membrane, the  
408 viral envelope is not able to undergo regeneration, which renders the PDI virus specific and  
409 insensitive to the onset of resistance. Very recently, Tummino *et al.* suggested that many  
410 repurposed drugs targeted as SARS-CoV-2 antivirals are cationic amphiphilic drugs (CAD),

411 which induce phospholipolysis (42). CAD are host target antivirals that have an action on cell  
412 phospholipids, provoke phospholipidosis and disrupt double membrane vesicles necessary for  
413 viral replication. The mechanism of action of the CAD is different from the one of Pba. Pba is a  
414 virus target antiviral, and it was never reported to have a phospholipidosis activity. Pba most  
415 probably induces an oxidation of the viral envelope phospholipids and, as it was shown by  
416 cryoEM in our study, does not induce phospholipidosis.

417  
418 In the present study, several other compounds structurally related to Pba and to other known  
419 photosensitizers were also screened for their anti-coronavirus activity in order to find out whether  
420 other compounds would have a more potent effect. Several of those compounds, including  
421 pyroPba, chlorin e6, or HPPH, N-methyl protoporphyrin IX, N-methyl mesoporphyrin IX, and  
422 Zn-protoporphyrin IX had a light-dependent antiviral effect, but only pyroPba turned out to be  
423 more active than Pba. However, pyroPba was also more toxic, and hence the final selectivity  
424 index was not higher than that of Pba. Several chlorophyll derivates like pyroPba and pheophytin  
425 a have already been demonstrated to be active against IAV, herpes simplex-2 virus, HCV, RSV  
426 (respiratory syncytial virus), and SARS-CoV-2 (26, 27, 43). The authors studied the mechanism  
427 of action of pyroPba against IAV and showed that the molecule targets the membrane of the virus  
428 and not the surface glycoproteins, a mechanism which is consistent with the one that we observed  
429 for Pba in our study. A requirement for photo-activation of pyroPba was not investigated nor  
430 mentioned by Chen *et al.* (43). Chlorophyllides, chlorophyll precursors, are another class of  
431 chlorophyll derivates that have been shown to have antiviral activity against several viruses  
432 including hepatitis B virus (44–46). Other porphyrins have already been described for their  
433 antiviral activity (25, 47). Interestingly, three photosensitizers which have been shown to be  
434 active against VSV, including N-methyl protoporphyrin IX, N-methyl mesoporphyrin IX, and  
435 Zn-protoporphyrin IX, were also identified in our screen (48). The authors clearly demonstrated

436 that these compounds inactivated VSV after photoactivation via singlet oxygen release. We did  
437 not demonstrate the mechanism of action of these three molecules against HCoV-229E but we  
438 also demonstrated that they are active after photoactivation. Very recently, protoporphyrin IX and  
439 verteporfin were identified as inhibitors of SARS-CoV-2 (49, 50). Both studies showed that  
440 protoporphyrin IX is active at an early step of infection, probably the entry step. Gu *et al.*  
441 postulated that the interaction of the compounds with ACE2 might impair the interaction of the  
442 virus with its receptor (49). Lu *et al.* showed that protoporphyrin IX is active against several  
443 enveloped viruses but that the activity of protoporphyrin IX against IAV is not dependent on light  
444 activation (50). Chlorin e6 is one of the most active compounds against HCoV-229E identified in  
445 this study. The antiviral activity of chlorin e6 against enveloped viruses such as HBV (Hepatitis  
446 B virus), HCV, HIV, DENV (dengue virus), MARV (Marburg virus), TCRV (tacaribe virus) and  
447 JUNV (Junin virus) has been already demonstrated (46). Interestingly the authors also showed  
448 that the molecule is inactive against non-enveloped viruses, suggesting that it targets the viral  
449 envelope.

450 As mentioned above, many other photosensitizers have been studied for their antiviral activity  
451 (25, 41), and for some of them, the PDI was clearly demonstrated as the mechanism of action (41,  
452 51). In light of the current SARS-CoV-2 pandemic, photosensitizers have received renewed  
453 attention as antiviral strategies to face this pandemic, and the use of those substances for the  
454 treatment of COVID-19 or the inactivation of SARS-CoV-2 on surfaces or in water has been  
455 postulated (52, 53). Pba might have some advantages above the already described  
456 photosensitizers, as it is a highly available natural product and active under normal light  
457 conditions. Importantly, it does not require a very specific wavelength-dependent illumination  
458 treatment, at least not when applied on surfaces/mucosae exposed to the environmental light, with  
459 an absorption at 667 nm. However, the light-dependency of such molecules might render their  
460 application as therapeutic agents for internal organs (such as lungs for SARS-CoV-2) more

461 challenging. Indeed, additional illumination will make its application as real therapy more  
462 complex, though not impossible because PDT is already used for the treatment of lung cancer  
463 (54). Efforts should be made for the development of specific device allowing PDT for COVID  
464 patients. Nonetheless, we believe that broad-spectrum, low-toxic, non-resistance inducing  
465 molecules such as Pba can certainly prove their value to reduce environment-to-person and  
466 person-to-person transmission of microorganisms when applied as e.g a spray for  
467 decontamination of surfaces or when formulated for topical application in nose and mouth. Very  
468 recently, a study describing such topical application of synthetic SARS-CoV-2 fusion inhibitor  
469 has demonstrated that the topical treatment of upper respiratory tract infections might prove its  
470 value in reducing virus transmission, particularly in cases where many people gather (55). In  
471 contrast to the SARS-CoV-2 fusion inhibitor described by de Vries *et al.* (55), Pba is a widely  
472 commercially available natural molecule with broad-spectrum activity against many enveloped  
473 viruses. Therefore, one should explore whether it can exert similar effects upon topical  
474 administration to the nose or oral cavity. If so, Pba might help to make people less susceptible to  
475 and/or less contagious upon upper respiratory infections with enveloped viruses, many of them  
476 causing seasonal outbreaks of respiratory disease such as common colds and flu.

477 Given that 1) onset of resistance to this product is very unlikely, 2) the activity of the compound  
478 is not dependent of envelope variants, 3) coronaviruses and other enveloped viruses can cause  
479 major problems in animals and 4) there is a potential risk for virus transmission from those  
480 animal to humans, the possibility to formulate Pba in such a way that also veterinary medicine  
481 and facilities with large numbers of animals can benefit from the strong antiviral properties that  
482 this molecule might have in the environment (decontamination of air, water and surfaces) should  
483 additionally be explored.

484

485 **Materials and Methods**

486 **Chemicals**

487 Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, phosphate buffered saline (PBS),  
488 4',6-diamidino-2-phenylindole (DAPI), were purchased from Life Technologies. Goat and foetal  
489 bovine sera (FBS) were obtained from Eurobio. Pheophorbide a (Pba) >90% pure, pyroPba,  
490 chlorin e6, HPPH, N-methyl protoporphyrin IX, N-methyl mesoporphyrin IX, and Zn  
491 protoporphyrin IX were from Cayman chemicals (Merck Chemicals, Darmstadt, Germany).  
492 Remdesivir (GS-5734) was from Selleck Chemicals (Houston TX). Mowiol 4-88 was obtained  
493 from Calbiochem. Rose Bengale, trolox and other chemicals were from Sigma (St. Louis, MO).  
494 Stocks of compounds were resuspended in dimethylsulfoxide (DMSO) at 50 mM. Plant extracts  
495 were resuspended in DMSO at 25 mg/mL.

496

497 **Antibodies**

498 Mouse anti-HCV E1 mAb A4 (56) and mouse anti-YFV E mAb 2D12 (anti-E, ATCC CRL-  
499 1689) were produced *in vitro* by using a MiniPerm apparatus (Heraeus). Mouse anti-dsRNA mAb  
500 (clone J2) was obtained from Scicons. Mouse anti-SARS-CoV-2 spike protein mAb were  
501 obtained from GeneTex. Polyclonal rabbit anti-SARS-CoV-2 nucleocapsid antibodies were from  
502 Novus. Cyanine 3-conjugated goat anti-mouse IgG and HRP-labeled goat-anti rabbit IgG  
503 antibodies were from Jackson Immunoresearch.

504

505 **Cells and culture conditions**

506 Huh-7, Vero-81 (ATCC number CCL-81) and Vero-E6 were grown in DMEM with glutaMAX-I  
507 and 10% FBS in an incubator at 37°C with 5% CO<sub>2</sub>. Vero-81 cells were subcloned to obtain a  
508 better overall infection rate. The primary human bronchial epithelial cells Mucilair™ were from  
509 Epithelix (Geneva, Switzerland) and maintained in Mucilair™ culture medium (Epithelix) as  
510 recommended by the manufacturer.

511

## 512 **Plant collection and extraction**

513 The fifteen plants were collected in the Bafing region (North-West Côte d'Ivoire, Touba  
514 department). They were authenticated at the Centre National de Floristique (CNF), University of  
515 Félix Houphouët Boigny de Cocody (Abidjan), where voucher specimens were deposited in an  
516 Herbarium. *M. oppositifolius* voucher number is UCJ006172. Plants were cleaned and air-dried at  
517 constant temperature (26°C) for 1 to 2 weeks at the Nangui Abrogoua University (Abidjan). They  
518 were then powdered and stored in the dark until extractions. For each plant, 20 g of dried powder  
519 were mixed with 100 mL methanol for 24 h. After filtration, the grounds were extracted again  
520 twice in the same way. The 3 resulting filtrates were combined and dried under vacuum at 40°C.  
521 These extracts were then dissolved in DMSO for antiviral assays.

522

## 523 **Bioguided fractionation of Mo extract and Pba identification**

524 For *Mallotus oppositifolius*, three other solvents were used to extract more compounds from these  
525 plant leave: methylene chloride (MC) for the first extraction of the dried leaves, then methanol to  
526 extract the first ground, and ethanol/water (50:50) to extract the second ground. The  
527 corresponding extracts were tested against HCoV-229E-Luc. Since the MC extract was the most  
528 active, it was fractionated by chromatography (CPC), leading to 10 fractions (F1-10) that were  
529 tested again. F7 was selected for further fractionation by another chromatography (HPLC) and  
530 led to 9 partitions (7.1-7.9). Partition 7.7 (the most active against HCoV-229E) purity and  
531 identity was determined by UPLC-MS and NMR (more details in supplementary materials).

532

## 533 **Viruses**

534 The following viral strains were used: HCoV-229E strain VR-740 (ATCC), and a recombinant  
535 HCoV-229E-Luc (kind gift of Pr. V. Thiel) (57); SARS-CoV-2 (isolate SARS-CoV-

536 2/human/FRA/Lille\_Vero-81-TMPRSS2/2020, NCBI MW575140) was propagated on Vero-81-  
537 TMPRSS2 cells. MERS-CoV was recovered by transfecting the infectious clone of MERS-CoV-  
538 EMC12 (kindly provided by Dr Luis Enjuanes) in Huh-7 cells. A cell culture-adapted strain  
539 (JFH1-CSN6A4) of HCV was produced as previously described (58). A recombinant Sindbis  
540 virus (SINV) expressing HCV E1 glycoprotein was employed as previously described (59).  
541 Yellow fever virus strain 17D (YFV) was obtained from Dr Philippe Desprès (Institut Pasteur de  
542 Paris, France). Coxsackievirus B4 strain E2 (CVB4) was provided by Dr Didier Hober  
543 (Université de Lille, France).

544

#### 545 **Cell toxicity assay**

546  $6 \times 10^4$  Huh-7, Vero-E6 and Vero-81 cells were seeded in 96-well plates and incubated for 16 h at  
547 37°C 5% CO<sub>2</sub> incubator. The cells were then treated with increasing concentrations of the  
548 compound of interest. One hour after inoculation, cells were taken out of the incubator to be  
549 exposed to the white light of the biosafety cabinet (BSC) for 10 min, after which cells were  
550 further incubated in the dark at 37°C 5% CO<sub>2</sub> for 23 h. The BSC's light source lamp consists of  
551 one fluorescent tube of 36W, 3350 lumen white light. An MTS [3-(4,5-dimethylthiazol-2-yl)-5-  
552 (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]-based viability assay (Cell Titer  
553 96 Aqueous non-radioactive cell proliferation assay, Promega) was performed as recommended  
554 by the manufacturer. The absorbance of formazan at 490 nm was detected using a plate reader  
555 (ELX 808 Bio-Tek Instruments Inc). Each measure was performed in triplicate and each  
556 experiment was repeated at least 3 times.

557

#### 558 **Micilair<sup>TM</sup> toxicity assays**

#### 559 ***LDH secretion assay***



560 Mucilair™, three wells per condition, were incubated with Pba in 100 µl Mucilair™ culture  
561 medium, or 50 µl of lysis solution (Cytotoxicity LDH assay kit-WST, Dojindo) at the apical  
562 surface for 1 h. Pba or lysis solution was removed and the cells were placed in the incubator for  
563 72 h. At 24 h, 48 h and 72 h. LDH secretion was measured in the basolateral medium according  
564 to the manufacturer's instructions, by recording the absorbance of WST-formazan at 490 nm as  
565 described. The results are expressed relative to the LDH secretion values obtained in the lysed  
566 wells, for which a value of 100% toxicity was attributed.

567

#### 568 *Trans-epithelial electrical resistance*

569 Mucilair™ were incubated with Pba and lysis solution as described above. Trans-epithelial  
570 electrical resistance was measured using a volt/ohm meter and electrode (Millicell ERS2,  
571 Millipore) at 24 h, 48 h and 72 h. 200 µl of prewarmed Mucilair™ culture medium was added at  
572 the apical surface prior resistance measurement. Data are expressed relative to control untreated  
573 cells.

574

#### 575 **HCoV-229E infection inhibition assays**

##### 576 *Luciferase assay*

577 HCoV-229E-Luc was first mixed with the crude extracts or the compounds at the appropriate  
578 concentrations for 10 minutes. Huh-7 cells and Huh-7-TMPRSS2 cells were inoculated with  
579 HCoV-229E-Luc at a MOI of 0.5 in a final volume of 50 µL for 1 h at 37°C in the presence of  
580 the plant crude extracts or the different compounds. The virus was removed and replaced with  
581 culture medium containing the extracts or the different compounds for 6 h at 37°C. Cells were  
582 lysed in 20 µL of Renilla Lysis Buffer (Promega, Madison, USA) and luciferase activity was  
583 quantified in a Tristar LB 941 luminometer (Berthold Technologies, Bad Wildbad, Germany)  
584 using Renilla Luciferase Assay System (Promega) as recommended by the manufacturer.

585 This experiment was either performed under white light exposure, in which the virus and the  
586 compounds were exposed to the light of the BSC lamp. To maximize light exposure, the tubes  
587 were laid flat on the bench of the BSC. For dark condition, the light of the BSC and the room was  
588 shut down and all the tubes and plates were covered with foil paper.

#### 589 ***HCoV-229E titers***

590 Huh-7 and Huh-7-TMPRSS2 cells seeded in 24-well plates were inoculated with HCoV-229E at  
591 a MOI of 0.5 in the presence of Pba at different concentrations for 1 h at 37°C. The inoculum was  
592 removed and replaced with culture medium containing Pba and the cells were incubated at 37°C  
593 for 8 h (for TMPRSS2 condition) or 10 h (without TMPRSS2). Supernatants were collected and  
594 serial dilutions were performed and used to infect naive Huh-7 cells in 96-well plates. Six days  
595 after infection, cytopathic effect was determined in each well to calculate TCID<sub>50</sub> titers by using  
596 the Reed and Muench method.

597

#### 598 **SARS-CoV-2 and MERS-CoV infection inhibition assays**

599 Vero-E6 and Huh-7 cells seeded in 24-well plates 24 h before inoculation were inoculated with  
600 SARS-CoV-2 and MERS-CoV, respectively, at a MOI of 0.3 in the presence of Pba at different  
601 concentrations for 1 h at 37°C. Then, the inoculum was removed by 3 washings with DMEM and  
602 fresh medium containing different Pba concentrations was added for 16 h at 37°C. Cell  
603 supernatants were collected and the amount of infectious virus was determined by infectivity  
604 titration. Therefore, Vero-E6 (SARS-CoV-2) and Huh-7 (MERS-CoV), seeded in 96-well plates,  
605 were inoculated with 100 µL of 1/10 serially diluted supernatants (ranging from 10<sup>-1</sup> to 10<sup>-8</sup>).  
606 Cells were incubated with the virus dilutions for 5 days at 37°C and 5% CO<sub>2</sub>. Then, the 50%  
607 tissue culture infectious dose (TCID<sub>50</sub>) was determined by assessing the CPE in each well by  
608 light microscopy and the 50% end point was calculated according to the method of Reed and  
609 Muench.

610

611 **Time-of-addition assay**

612 To determine at which stage of the replication cycle Pba executed its effect, a time-of-addition  
613 assay was performed for which 1  $\mu$ M Pba (and 10  $\mu$ M chloroquine as a control for SARS-CoV-2)  
614 was added at different time points before (referred to as the condition ‘pre-treatment cells’),  
615 during (referred to as the condition ‘inoculation’), or after inoculation. For the latter condition,  
616 Pba and chloroquine were not added before and during inoculation, but only directly after  
617 removal of the inoculum (referred to as the condition ‘p.i. - end’), or from 1 h or 2 h after  
618 removal of the inoculum onwards (referred to as the condition ‘1 h p.i.-end’ and ‘2 h p.i.-end,’  
619 respectively) and were left in the medium for the rest of the incubation time (i.e until 6 h p.i. for  
620 HCoV-229E and 16 h p.i. for SARS-CoV-2). For this experiment, Huh-7-TMPRSS2 or Vero-81  
621 cells were inoculated with HCoV-229E-Luc or SARS-CoV-2 at a MOI of 0.5 and 0.05,  
622 respectively. One hour after inoculation, cells for all conditions were washed 3 times to remove  
623 the unbound particles. HCoV-229E-luc, luciferase activity was quantified as described ahead. For  
624 SARS-CoV-2, cells were washed once with PBS and lysed in 200  $\mu$ L of non-reducing 2x  
625 Laemmli loading buffer. Lysates were incubated at 95°C for 30 min to inactivate the virus and  
626 lysates were kept at -20°C until western blot analysis (see below). For each time point, DMSO  
627 was taken as a control, and all experiments were repeated 3 times.

628

629 **Western blot detection of the SARS-CoV-2 nucleocapsid expression**

630 Sixteen hours after inoculation, cells were washed once with PBS and lysed in 200  $\mu$ L of non-  
631 reducing 2x Laemmli loading buffer. Lysates were incubated at 95°C for 30 min to inactivate the  
632 virus and the proteins were subsequently separated on a 12% polyacrylamide gel by SDS-PAGE.  
633 Next, proteins were transferred to a nitrocellulose membrane (Amersham), and the membranes  
634 were subsequently blocked for 1 h at RT in 5% (w/v) non-fat dry milk in PBS with 0.1% (v/v)

635 Tween-20. Membranes were incubated overnight at 4°C with polyclonal rabbit anti-SARS-CoV-  
636 2 nucleocapsid antibodies in 5% (w/v) non-fat dry milk in PBS with 0.1% (v/v) Tween-20. After  
637 being washed 3 times with PBS with 0.1% (v/v) Tween-20, membranes were incubated for 1 h at  
638 RT with HRP-labeled goat-anti rabbit IgG antibodies, after which membranes were washed 3  
639 times. N proteins were visualized by enhanced chemiluminescence (Pierce™ ECL, ThermoFisher  
640 Scientific). Quantification was performed by using Image J and its gel quantification function.

641

#### 642 **Infection assay with other viruses**

643 Vero (YFV, SINV, CBV4) or Huh-7 (HCV) cells grown on glass coverslips were infected with  
644 viral stocks diluted so as to obtain 20–40% infected cells in control conditions. The cells were  
645 fixed at a time that allowed for a clear detection of infected cells vs non-infected cells, and  
646 avoided the detection of reinfection events, thus limiting the analysis to a single round of  
647 infection (30 h p.i. for HCV, 20 h p.i. for YFV, 6 h p.i. for SINV, 4 h p.i. for CVB4). The cells  
648 were fixed for 20 min with 3% PFA. They were then rinsed with PBS and processed for  
649 immunofluorescence as previously described (60) using primary mouse antibodies specific to  
650 HCV E1 (for both HCV and SINV), YFV E, or dsRNA (for CVB4), followed by a cyanine-3-  
651 conjugated goat anti-mouse IgG secondary antibody for the detection of infected cells. Nuclei  
652 were stained with DAPI. Coverslips were mounted on microscope slides in Mowiol 4-88-  
653 containing medium. Images were acquired on an Evos M5000 imaging system (Thermo Fisher  
654 Scientific) equipped with light cubes for DAPI, and RFP, and a 10× objective. For each coverslip,  
655 a series of six 8-bit images of randomly picked areas were recorded. Cells labelled with anti-virus  
656 mAbs were counted as infected cells. The total number of cells was obtained from DAPI-labelled  
657 nuclei. Infected cells and nuclei were automatically counted using macros written in ImageJ.  
658 Infections were scored as the ratio of infected over total cells. The data are presented as the  
659 percentage of infection relative to the control condition.

660

661 **Effect of Pba on pseudotyped virion entry**

662 Particles pseudotyped with either SARS-CoV-2 S (SARS-2pp), MERS-CoV S proteins  
663 (MERSpp), HCoV-229E-S (HCoV-229Epp), genotype 2a HCV envelope proteins (HCVpp), or  
664 the G envelope glycoprotein of vesicular stomatitis virus (VSV-Gpp) were produced as  
665 previously described (22, 61). Pseudotyped virions were pre-treated with Pba for 30 min at room  
666 temperature under the BSC's light or covered in foil and then used to inoculate Huh-7 cells in 96-  
667 well plates for 3 h. The inoculum was removed and cells were further incubated with culture  
668 medium for 45 h. Cells were lysed and luciferase activity was detected by using a Luciferase  
669 Assay kit (Promega) and light emission measured by using a Tristar LB 941 luminometer  
670 (Berthold Technologies).

671

672 **White light exposure kinetics**

673 HCoV-229E-Luc or SARS-CoV-2 were pre-treated with Pba at room temperature and exposed to  
674 BSC's white fluorescent light during different period of time. To maximize light exposure, tubes  
675 were laid flat under the BSC's light. Next, infection was quantified for each virus as described  
676 previously.

677

678 **Fusion assay**

679 Cells were preincubated for 30 min in the presence of 25 mM NH<sub>4</sub>Cl at 37°C to inhibit virus  
680 entry through the endosomal route, and then were transferred to ice. In the meantime, the virus  
681 was preincubated under light with Pba and 25 mM NH<sub>4</sub>Cl for 10 min and then allowed to bind to  
682 the cells at 4°C for 1 h in DMEM containing 0.2% BSA, 20 mM Hepes, and 25 mM NH<sub>4</sub>Cl.  
683 Cells were then warmed by addition of DMEM containing 3 µg/mL trypsin, 0.2% BSA, 20 mM  
684 Hepes, and 25 mM NH<sub>4</sub>Cl and were incubated for 5 min in a water bath at 37°C. The cells were

685 rinsed and further incubated for 30 min in culture medium containing 25 mM NH<sub>4</sub>Cl, and then  
686 the medium was replaced by normal culture medium. Seven hours after inoculation, luciferase  
687 activity was detected by using a Renilla Luciferase Assay Kit (Promega).

688

#### 689 **Cell-cell fusion assay by transient expression of the SARS-CoV-2 spike protein**

690 Vero-81 cells were seeded on coverslips in 24-wells 16 h before transfection. Cells were  
691 transfected with 250 ng of a pCDNA3.1(+) vector encoding for the SARS-CoV-2 spike protein  
692 using the TransIT<sup>®</sup>-LT1 Transfection Reagent (Mirus Bio). Six hours post transfection (p.t.),  
693 transfection medium was replaced by normal medium containing 1 μM Pba or DMSO. Twenty-  
694 four hours p.t., cells were fixed with 3% paraformaldehyde in PBS for 20 min at RT and syncytia  
695 were visualized by immunofluorescence, by incubating the cells with a monoclonal anti-SARS-  
696 CoV-2-spike antibody in 10% normal goat serum, followed by incubation with Cyanine-3-  
697 conjugated goat anti-mouse IgG antibodies. Nuclei were visualized with 1 μg/ml of 4',6-  
698 diamidino-2-phenylindole (DAPI), and coverslips were mounted in Mowiol<sup>®</sup> mounting medium.  
699 Pictures were obtained with an Evos M5000 imaging system (Thermo Fisher Scientific).

700

#### 701 **Attachment assay**

702 Huh-7-TMPRSS2 cells seeded in 24-well plates were inoculated with HCoV-229E at a MOI of 4  
703 on ice in the presence of 4.1 or 8.2 μM Pba under the light of the BSC. 1 h after inoculation, cells  
704 were washed 3 times with cold PBS, and lysed using LBP lysis buffer for RNA extraction  
705 following manufacturer's instructions (NucleoSpin<sup>®</sup> RNA plus extraction kit, Macherey-Nagel).  
706 Reverse transcription was then performed on 10 μL of RNA using High Capacity cDNA Reverse  
707 Transcription kit (Applied Biosystems). 3 μL of cDNA were used for real-time reverse-  
708 transcription polymerase chain reaction (qRT-PCR) assay using specific primers and probe

709 targeting the N gene (forward primer 5'-TTCCGACGTGCTCGAACTTT-3', reverse primer 5'-  
710 CCAACACGGTTGTGACAGTGA-3' and probe 5'-6FAM-TCCTGAGGTCAATGCA-3') and  
711 subjected to qPCR amplification with Taqman Master mix.

712

### 713 **Quencher Assay**

714 HCoV-229E-Luc was mixed with 10 mM Trolox or  $\text{NaN}_3$  after which 0.5 or 1  $\mu\text{M}$  Pba was  
715 added and the mixture was exposed to light for 10 min. The mixture was used to inoculate Huh-  
716 7-TMPRSS2 cells for 1h. Inoculum was replaced with DMEM and cells were kept in the dark at  
717 37°C 5%  $\text{CO}_2$  for 7 h and then lysed to quantify luciferase activity as described above.

718

### 719 **CryoEM**

720 HCoV-229E was produced by inoculating a confluent Huh-7 T75 Flask at MOI of 0.008 in  
721 DMEM supplemented with 5% FBS and put at 33°C 5%  $\text{CO}_2$  for 5 days. Supernatant was  
722 harvested and treated with DMSO or 10  $\mu\text{M}$  Pba, and further kept in the dark or exposed to light  
723 for 30 min. Then NaCl was added to a final concentration of 400 mM final to induce an osmotic  
724 shock. Viruses were fixed in 4% PFA. For cryo-EM experiments of the particles, lacey carbon  
725 formvar 300 mesh copper grids were used after a standard glow discharged procedure. Plunge  
726 freezing was realized using the EM-GP apparatus (Leica). Specimens were observed at  $-175^\circ\text{C}$   
727 using a cryo holder (626, Gatan), with a ThermoFisher FEI Tecnai F20 electron microscope  
728 operating at 200 kV under low-dose conditions. Images were acquired with an Eagle 4k x 4k  
729 camera (ThermoFisher FEI).

730

### 731 **Primary airway cell infection quantification**

732 The air interface of Mucilair<sup>TM</sup> (Epithelix) was rinsed with 100  $\mu\text{L}$  of Mucilair<sup>TM</sup> culture medium  
733 for 10 min 3 times to remove mucosal secretion. The cells were then inoculated at the apical

734 membrane with SARS-CoV-2 or MERS-CoV at a MOI of 0.2 in the presence of compounds for  
735 1 h at 37°C. Inoculum was removed and the cells were rinsed with PBS. In parallel, compounds  
736 were added in the basolateral medium. 72 h post-infection, viruses secreted at the apical  
737 membrane were collected by adding 200 µL of medium in the apical chamber. Viral titers were  
738 determined as described above. In parallel, cells were lysed with lysis buffer from the kit  
739 NucleoSpin<sup>®</sup> RNA Plus (Macherey Nagel), and total RNA extracted following manufacturer's  
740 instructions, eluted in a final volume of 60 µL of H<sub>2</sub>O, and quantified.

741 For SARS-CoV-2, one-step qPCR assay was performed using 5 µL of RNA and Takyon Low rox  
742 one-step RT probe Mastermix (Eurogentec) and specific primers and probe targeting E gene,  
743 forward primer 5'-ACAGGTACGTTAATAGTTAATAGCGT-3', reverse primer 5'-  
744 ATATTGCAGCAGTACGCACACA-3' and probe FAM-ACACTAGCCATC-  
745 CTTACTGCGCTTCG-MGB.

746 For MERS-CoV and RPLP0 reference gene, 10 µM of RNA were used for cDNA synthesis using  
747 High Capacity cDNA Reverse Transcription kit (Applied Biosystems). 3 µL of cDNA were used  
748 for real-time reverse-transcription polymerase chain reaction (qRT-PCR) assay using specific  
749 probes. For MERS-CoV, the following primers and probe targeting N gene were used, forward  
750 primer 5'-GGGTGTACCTCTTAATGCCAATTC-3', reverse primer 5'-  
751 TCTGTCCTGTCTCCGCCAAT-3' and probe 5'-FAM-ACCCCTGCGCAAATGCTGGG-  
752 MGBNFQ-3' and subjected to qPCR amplification with Taqman Master mix. For RPLP0,  
753 Taqman gene expression assay (Life Technologies) was used according to the manufacturer  
754 instruction. SARS-CoV-2 E and MERS-CoV N gene expression were quantified relative to  
755 RPLP0 using  $\Delta\Delta C_t$  method. A value of 1 was arbitrary assigned to infected cells without  
756 compound.

757

758 **Statistical analysis and IC<sub>50</sub> and CC<sub>50</sub> calculation**



759 Values were graphed and  $IC_{50}$  calculated by non-linear regression curve fitting with variable  
760 slopes constraining the top to 100% and the bottom to 0%, using GraphPad PRISM software.  
761 Kruskal Wallis nonparametric test followed by a Dunn's multicomparison post hoc test with a  
762 confidence interval of 95% was used to identify individual difference between treatments. P  
763 values  $< 0.05$  were considered as significantly different from the control.

764

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771

772

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968 **FIGURE LEGENDS**

969

970 **Figure 1. Identification of Pba as active compound in *Mallotus oppositifolius* using bioguided**  
971 **fractionation of plant extracts. (A)** Huh-7 cells were inoculated with HCoV-229E in the presence of  
972 various plant extracts at 25 µg/mL. Cells were lysed 7 h post-inoculation and luciferase activity  
973 quantified. **(B)** Huh-7 cells were inoculated with HCoV-229E in the presence of sub-extracts of Mo  
974 (methylene chloride, MC; ethanol/water (50:50), EtOH/w; water, H<sub>2</sub>O; left), fractions of Mo MC sub-  
975 extract (middle), or sub-fractions of F7 fraction (right panel) at 25 µg/mL. Cells were lysed 7 h post-  
976 inoculation and luciferase activity quantified. **(C)** Huh-7 cells were inoculated with HCoV-229E-Luc in  
977 the presence of Pba extracted from Mo (Pba F7.7) or commercial Pba (Pba com) at different  
978 concentrations. At 1 h p.i., cells were washed and fresh compounds were added to the cells for 6 h after  
979 which cells were lysed to quantify luciferase activity. Data are expressed relative to the control DMSO.  
980 Results are expressed as mean ± SEM of 3 experiments.  
981

982 **Figure 2. Pba inhibits various HCoVs.** For infection assays, cells were inoculated with HCoV-229E  
983 (Huh-7 cells), SARS-CoV-2 (Vero-E6 cells) and MERS-CoV (Huh-7 cells) in presence of various  
984 concentrations of Pba. At 1 h p.i, cells were washed and fresh compounds were added to the cells for 9 h  
985 (HCoV-229E) or 16 h (SARS-CoV-2 and MERS-CoV) and the supernatants were collected for infectivity  
986 titration. For toxicity assays, cells were incubated with Pba at different concentrations for 24 h. MTS assay  
987 was performed to monitor cell viability. For both infection and toxicity tests, cells were exposed to the  
988 light of the cabinet for 10 min, once upon inoculation, once after a 1 h incubation. Results are expressed as  
989 mean ± SEM of 3 experiments.  
990

991 **Figure 3. Pba inhibits viral entry by a direct action on the viral particle. (A)** Huh-7 and Huh-7-  
992 Tmprss2 cells were inoculated with HCoV-229E-Luc in the presence of various concentrations Pba. At  
993 1 h p.i, cells were washed and fresh compounds were added to the cells for 6 h after which the cells were  
994 lysed to quantify luciferase activity. Data are expressed relative to the control DMSO. **(B)** Pba at 2 µM  
995 was added at different time points during infection of Huh-7-Tmprss2 cells by HCoV-229E-Luc, either  
996 1 h before inoculation (pre-treatment), or 1 h during inoculation (Inoculation), or for 6 h post-inoculation  
997 (p.i.-end), after 1 h post-inoculation till the end (1 h p.i.-end), or 2 h post-inoculation till the end (2 h p.i.-  
998 end). Cells were lysed 7 h after the inoculation and luciferase activity quantified. **(C)** A similar experiment  
999 was performed in Vero-81 cells inoculated with SARS-CoV-2 in the presence of Pba at 1 µM or  
1000 chloroquine at 10 µM at different time points. Cells were lysed 16 h post-inoculation and the viral  
1001 nucleocapsid protein was detected by Western blot. The graph represents the quantification of the band  
1002 intensity corresponding to the N protein, relative to the DMSO control for each time point. **(D)** Huh-7  
1003 cells were inoculated with HCoV-229E-Luc in the presence of 0.2 or 2 µM Pba, or with HCoV-229E-Luc  
1004 previously treated with 2 µM Pba and then diluted 10 times, leading to a concentration of 0.2 µM Pba for  
1005 the inoculation period (2 µM > 0.2 µM). The amount of virus used for inoculation was kept constant in the  
1006 different conditions and all the samples were exposed to the light for 10 min. At 7 h post-inoculation, cells  
1007 were lysed and luciferase activity was quantified. \*, P<0.05; \*\*\*, P<0.005.

1008

1009

1010 **Figure 4. Pba inhibits viral entry at the fusion step.** (A) Huh-7-TMPRSS2 cells were inoculated with  
1011 HCoV-229E for 1 h at 4°C in the presence of DMSO, or 4.1 and 8.2 µM Pba. Cells were washed thrice  
1012 with ice-cold PBS, and total RNA was extracted. Bound HCoV-229E virions were detected by  
1013 quantification of HCoV-229E gRNA by qRT-PCR. Relative binding is expressed as the percentage of the  
1014 control (DMSO) for which the 100% value was arbitrarily attributed. Mean values ± SEM (error bars) of  
1015 three independent experiments are presented. n.s., not significant. (B) HCoV-229E was incubated with  
1016 Pba at different concentration and was bound to Huh-7 cells for 1 h in the absence (control) or presence  
1017 (trypsin) of NH<sub>4</sub>Cl at 4°C. In the later condition, fusion was induced by 3 µg/mL trypsin for 5 min at 37  
1018 °C in the presence of NH<sub>4</sub>Cl. Cells were lysed 7 h post-infection and luciferase activity quantified.  
1019 Infectivity is expressed as the percentage of the control (DMSO) for which the 100% value was arbitrarily  
1020 attributed. Mean values ± SEM (error bars) of three independent experiments are presented. (C) Vero-81  
1021 cells transiently expressing SARS-CoV-2 spike protein were incubated with or without Pba at 1 µM from  
1022 6 to 24 h p.i., after which syncytia were visualized by immunofluorescence. Images were acquired on an  
1023 Evos M5000 imaging system (Thermo Fisher Scientific).  
1024

1025 **Figure 5. The antiviral activity of Pba depends on light exposure and its ability to generate singlet**  
1026 **oxygen species.** HCoV-229E-Luc (A) and SARS-CoV-2 (B) were incubated with Pba at given  
1027 concentration under the light of the laminar flow cabinet. At different time points of light exposure, the  
1028 mixture was used to inoculate Huh-7 cells or Vero-81 cells, respectively. At 7 h (HCoV-229E-Luc) or 16  
1029 h (SARS-CoV-2) post-inoculation, cells were lysed and infection quantified as described previously. (C)  
1030 Pba at 0.5 or 1 µM was mixed with 10 mM Trolox or 10 mM NaN<sub>3</sub> in DMEM and HCoV-229E-Luc was  
1031 added to the mixture prior to inoculation of Huh-7 cells for 1 h at 37°C. Inoculum and compounds were  
1032 removed and replaced with culture medium for 6 h. Cells were lysed and luciferase activity quantified.  
1033 Infectivity is expressed as the percentage of infection relative to the control (DMSO) to which the 100%  
1034 value was arbitrarily attributed. Mean values ± SEM (error bars) of three independent experiments are  
1035 presented.  
1036

1037 **Figure 6. Pba renders virions resistant to osmotic shock.** HCoV-229E were incubated in the presence  
1038 or absence of Pba at 10 µM with or without 30 min light exposure, after which the particles were  
1039 subjected to normal medium conditions (100 mM NaCl) or to an osmotic shock of 400 mM NaCl for 30  
1040 sec. Virions were fixed with PFA and samples were treated for CryoEM observation. Images are  
1041 representative of 30 independent images of 2 independent experiments. Scale bar: 50 nm.  
1042

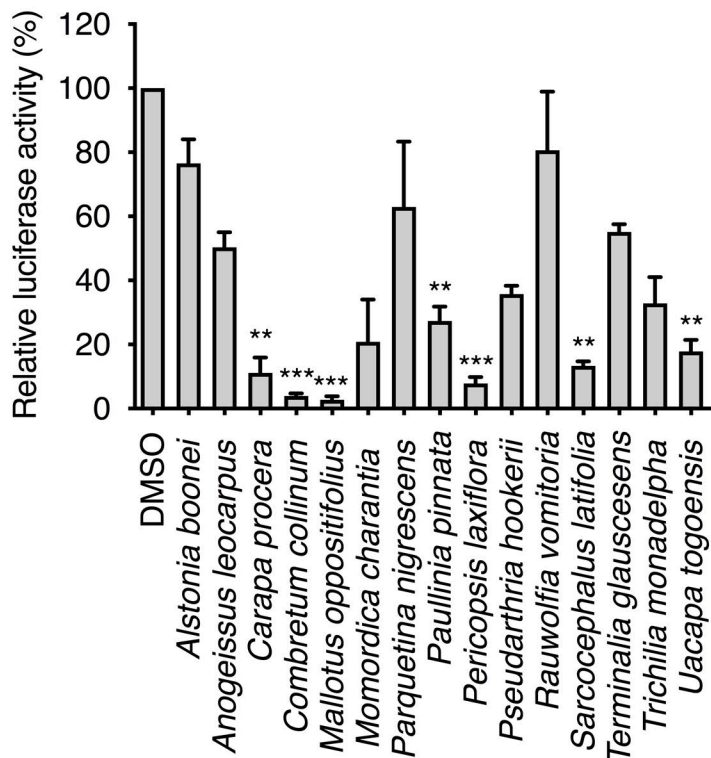
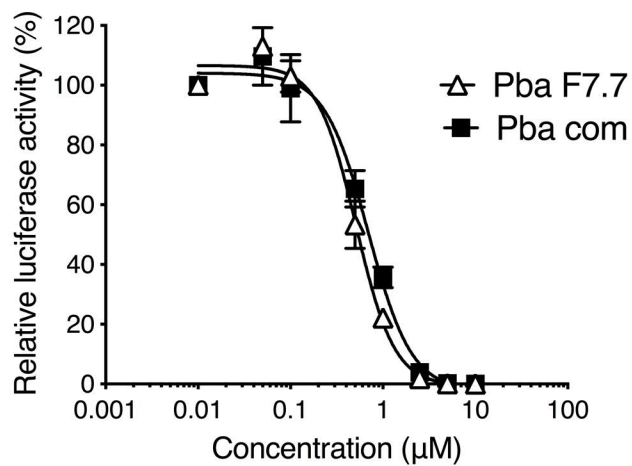
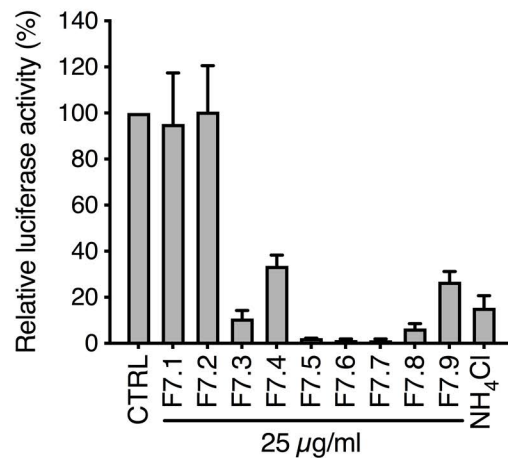
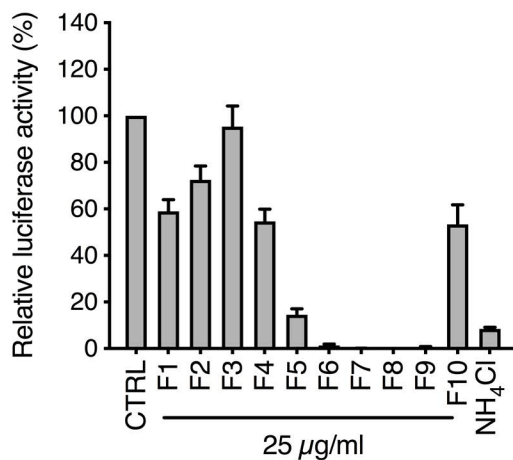
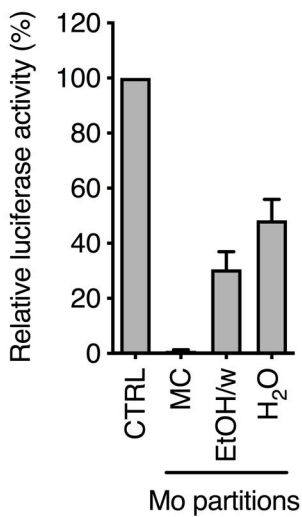
1043 **Figure 7. Pba is a broad-spectrum antiviral agent against enveloped viruses.** (A) MERSpp, HCoV-  
1044 229Epp, VSVpp, and SARS-2pp were preincubated with Pba at the indicated concentration either under  
1045 light for 30 min (Light) or without light (Dark) prior to inoculation of Huh-7 cells expressing ACE2 and  
1046 TMPRSS2 for 2 h. At 46 h post-inoculation, cells were lysed and luciferase activity was quantified.  
1047 Infectivity is expressed as the percentage relative to the control (DMSO) to which the 100% value was  
1048 arbitrarily attributed. Mean values ± SEM (error bars) of three different experiments are presented. (B).  
1049 Different viruses were incubated with Pba at 1 and 2.5 µM under light condition for 30 min prior to  
1050 inoculation. Cells were fixed at different time points depending on the virus (see Materials and Methods  
1051 section for details) and subjected to immunofluorescence labelling. Infectivity is expressed as the

1052 percentage relative to the control (DMSO). Mean values  $\pm$  SEM (error bars) of three different experiments  
1053 are presented.

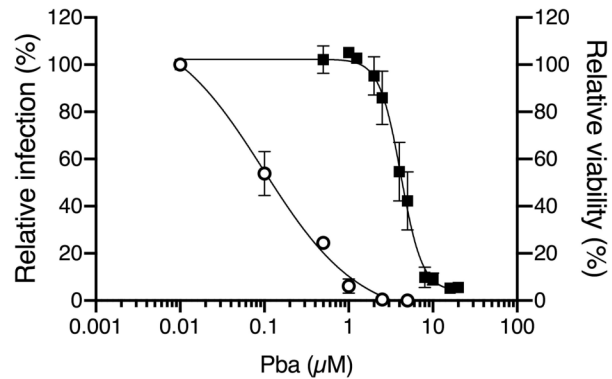
1054 **Figure 8. Antiviral efficacy of Pba in human primary bronchial epithelial cells.** Mucilair™ cells were  
1055 inoculated with SARS-CoV-2 or MERS-CoV in the presence of Pba at 0.25 or 2.5  $\mu$ M for 1 h at the apical  
1056 side. The cells were left for 10 min under the light of the cabinet before being put in the incubator.  
1057 Inoculum was removed 1 h post-inoculation. Remdesivir (Rem) at 5  $\mu$ M was added in the basolateral  
1058 medium. The cells were additionally exposed to the light for 10 min at 24 h, 48 h and 72 h post-  
1059 inoculation. 72 h post-inoculation, viruses were collected from the apical surface, and cells were lysed to  
1060 extract RNA. Viral RNA was quantified by qRT-PCR and viral titers were determined by infectivity  
1061 titrations for SARS-CoV-2 (**A**) and MERS-CoV (**B**). For RNA quantification, data are expressed relative  
1062 to the control DMSO. Results are expressed as mean  $\pm$  SEM of 3 experiments. Viral titers are  
1063 representative of three independent experiments. Toxicity of Pba was measured in Mucilair™ treated with  
1064 Pba at 0.25 and 2.5  $\mu$ M by measuring either LDH secretion (**C**) or trans-epithelial electrical resistance (**D**)  
1065 at 24 h, 48 h and 72 h incubation. As a control, cells were lysed with lysis solution as described in  
1066 materials and methods section. Results are expressed as mean  $\pm$  SEM of 2 experiments. \*,  $P < 0.05$ ; \*\*\*,  
1067  $P < 0.005$ ; n.s., not significant.

1068

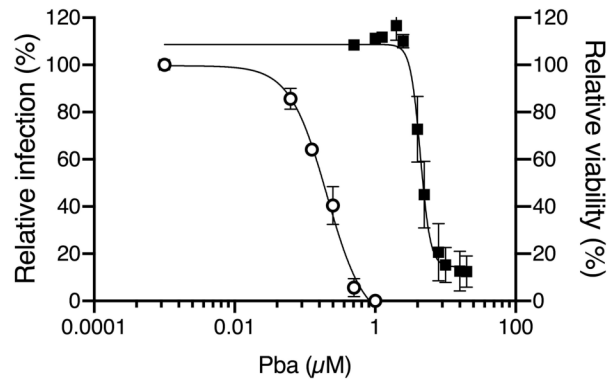


**A****C****B****Figure 1**

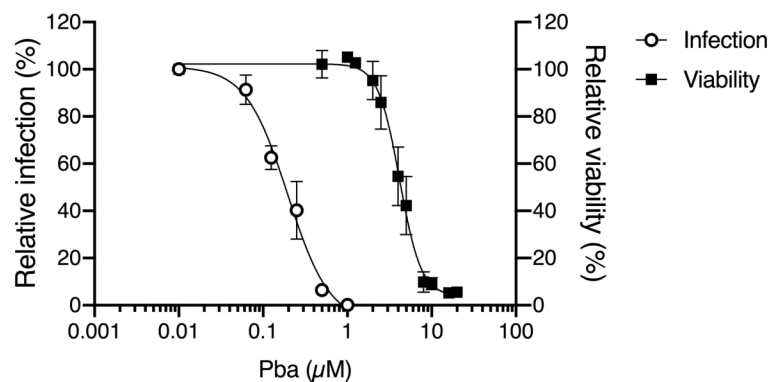
HCoV229E in Huh-7

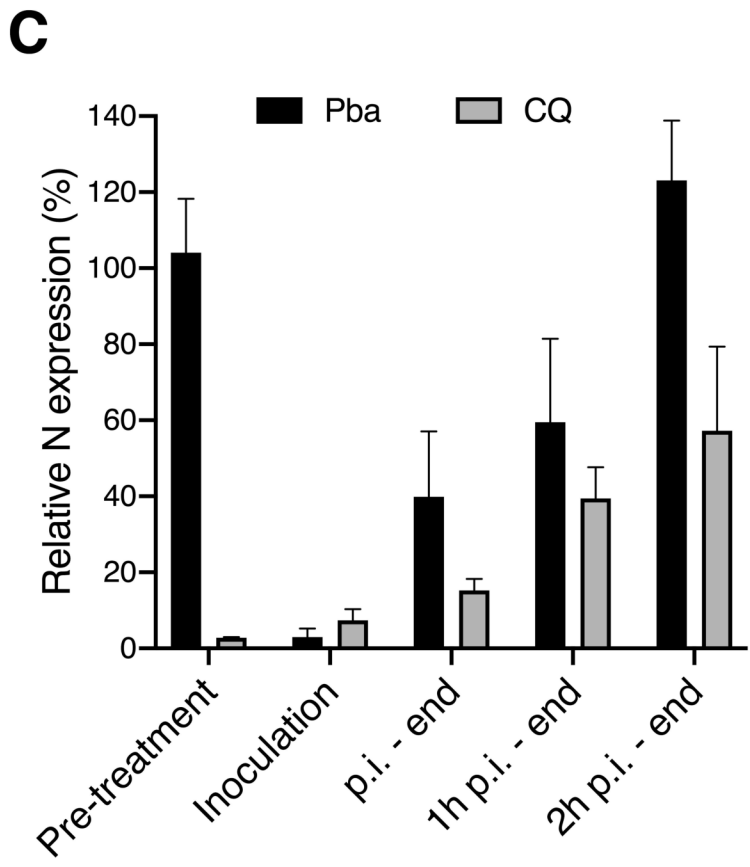
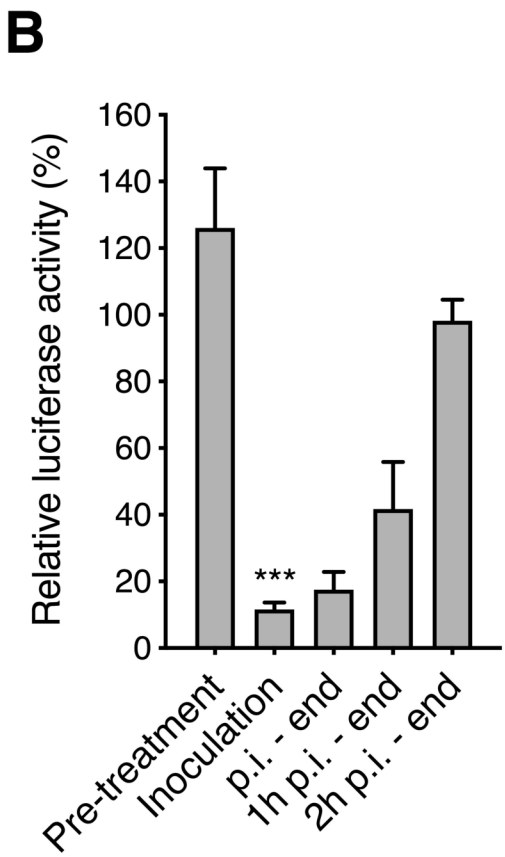
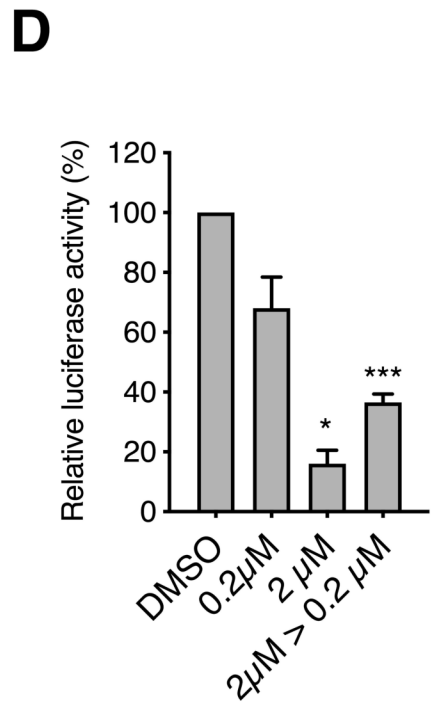
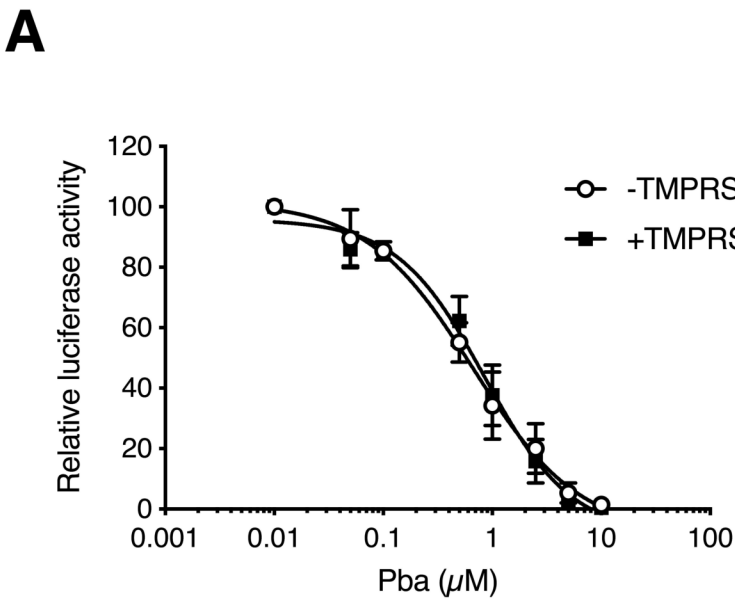


SARS-CoV-2 in VeroE6

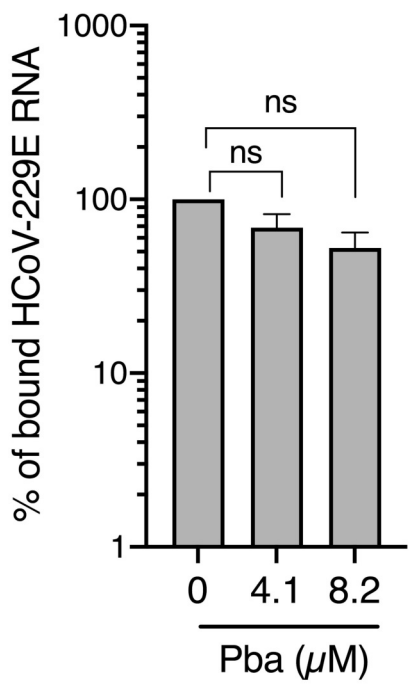
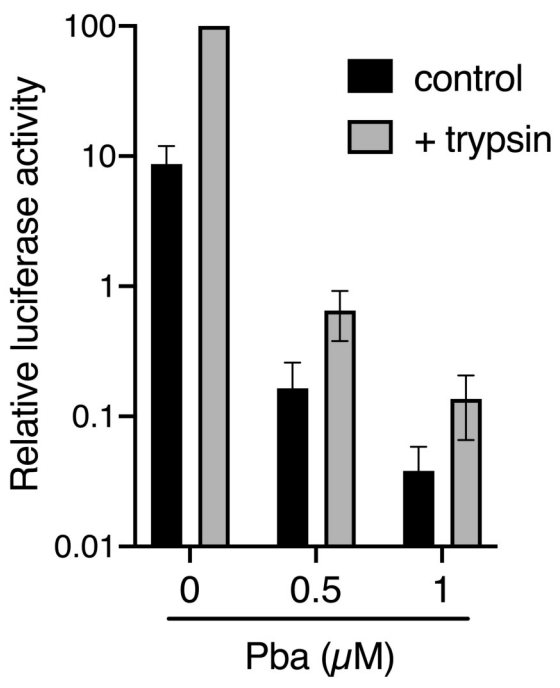
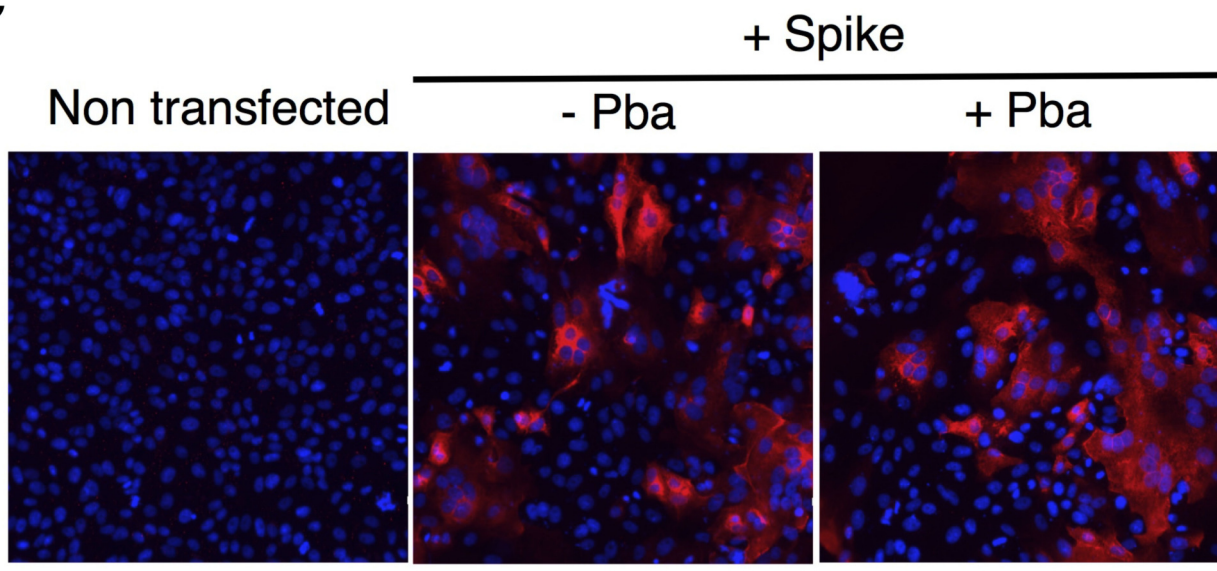


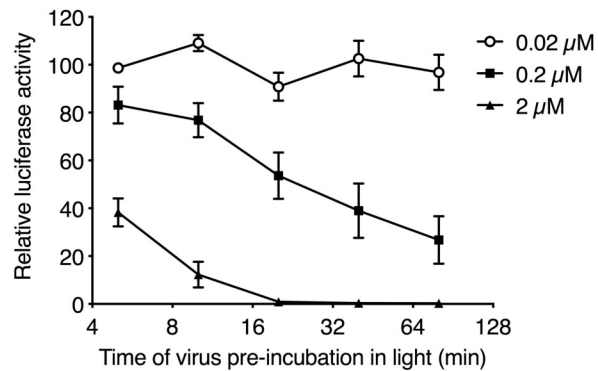
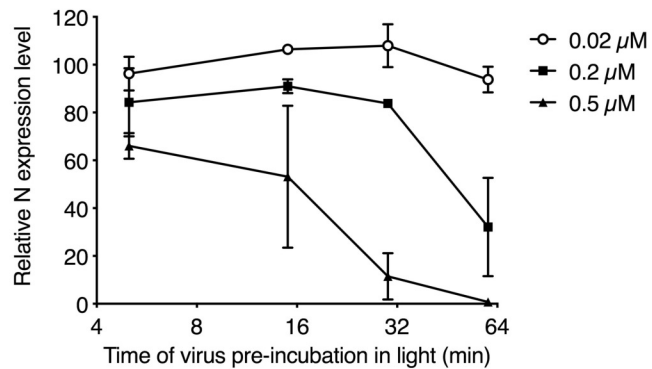
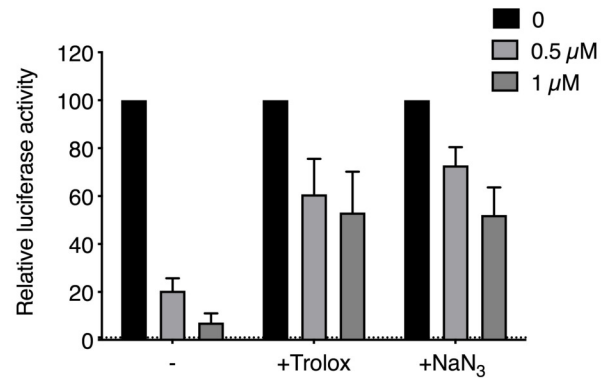
MERS-CoV in Huh-7

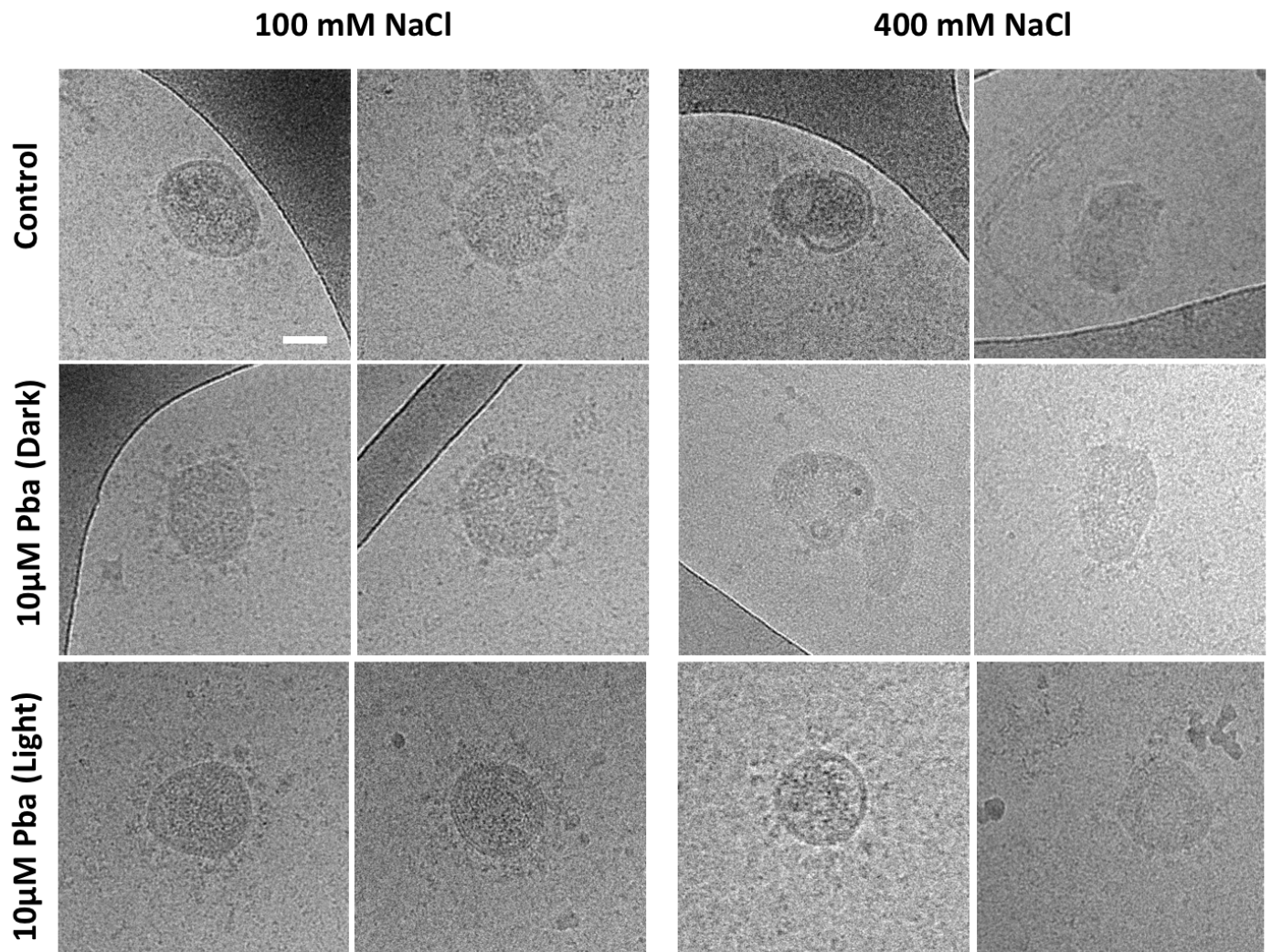
**Figure 2**



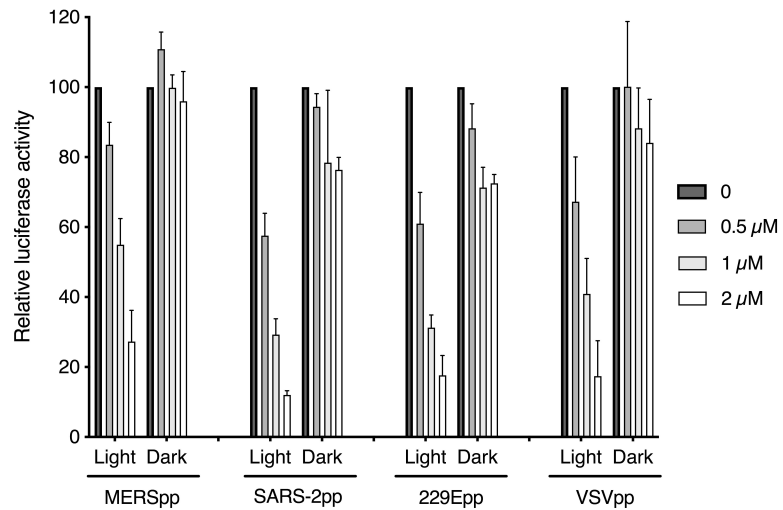
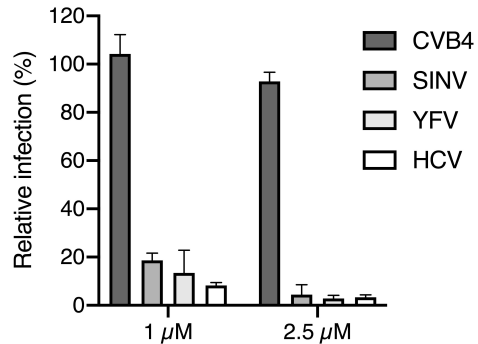
**Figure 3**

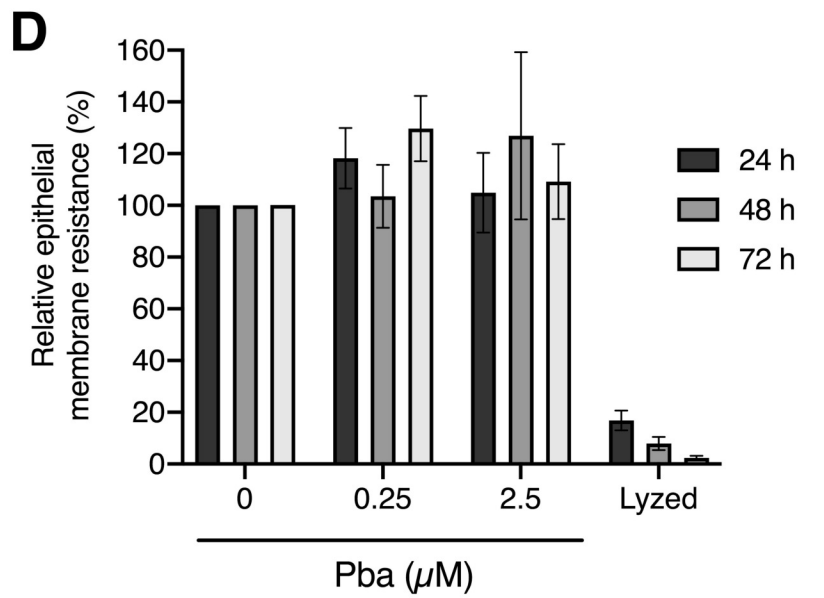
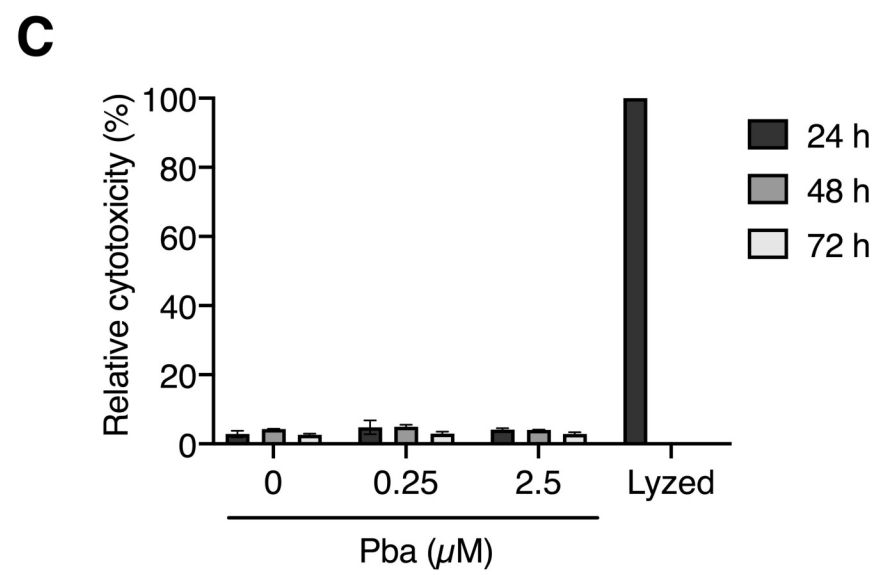
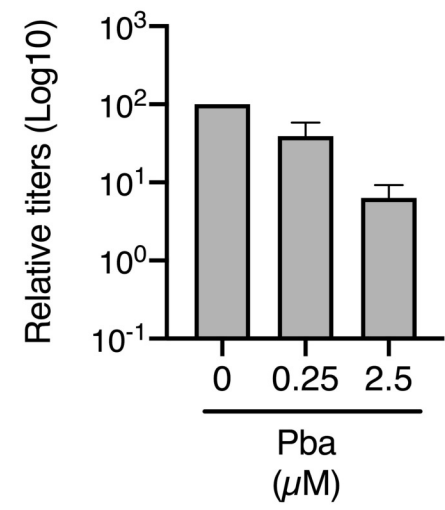
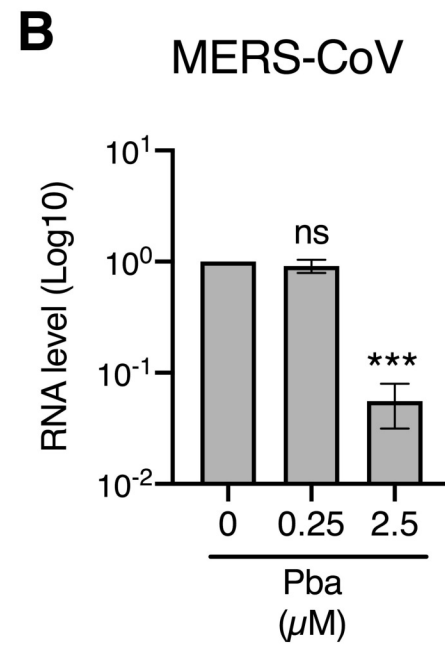
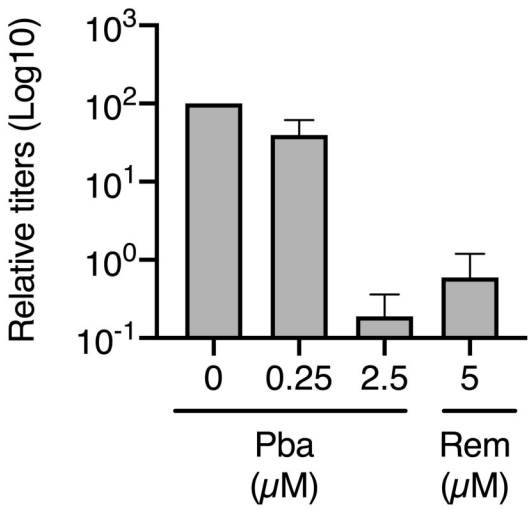
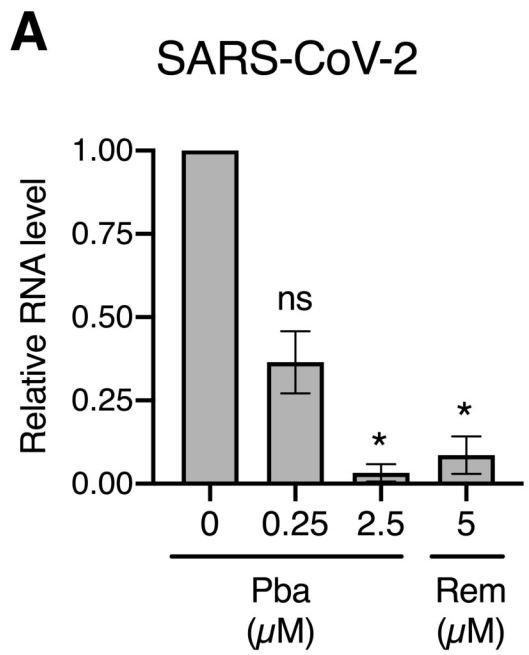
**A****B****C****Figure 4**

**A****B****C****Figure 5**



**Figure 6**

**A****B****Figure 7**



**Figure 8**