

1 **Protoporphyrin IX and verteporfin prevent SARS-CoV-2 infection *in***
2 ***vitro* and in a mouse model expressing human ACE2**

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25

1 **Abstract**

2 The SARS-CoV-2 infection is spreading rapidly worldwide. Efficacious antiviral
3 therapeutics against SARS-CoV-2 is urgently needed. Here, we discovered that
4 protoporphyrin IX (PpIX) and verteporfin, two FDA-approved drugs, completely
5 inhibited the cytopathic effect produced by SARS-CoV-2 infection at 1.25 μ M and 0.31
6 μ M respectively, and their EC₅₀ values of reduction of viral RNA were at nanomolar
7 concentrations. The selectivity indices of PpIX and verteporfin were 952.74 and 368.93,
8 respectively, suggesting broad margin of safety. Importantly, PpIX and verteporfin
9 prevented SARS-CoV-2 infection in mice adenovirally transduced with human ACE2.
10 The compounds, sharing a porphyrin ring structure, were shown to bind viral receptor
11 ACE2 and interfere with the interaction between ACE2 and the receptor-binding
12 domain of viral S protein. Our study suggests that PpIX and verteporfin are potent
13 antiviral agents against SARS-CoV-2 infection and sheds new light on developing
14 novel chemoprophylaxis and chemotherapy against SARS-CoV-2.

15

16 **Main Text**

17 The infection of SARS-CoV-2 has spread around the world since December 2019. As
18 of July 6, 2020, there are nearly 11 million confirmed cases globally, of which more
19 than five hundred thousand died ([https://www.who.int/emergencies/diseases/novel-](https://www.who.int/emergencies/diseases/novel-coronavirus-2019)
20 [coronavirus-2019](https://www.who.int/emergencies/diseases/novel-coronavirus-2019)). Although the pandemic has been contained in some countries, the
21 numbers of confirmed cases and deaths worldwide are expected to continue to rise.

22

23 SARS-CoV-2 is transmitted through respiratory droplets and close contact, which
24 causes mainly upper and lower respiratory diseases. The majority of infected healthy
25 adults and children only show mild symptoms including cough, fever, fatigue and
26 diarrhea but the elderly with various chronic diseases are at high risk of development
27 of serious diseases including pneumonia, acute respiratory distress, multiple organ
28 failure and shock. At present, the treatment of Coronavirus Disease 2019 (COVID-19)

1 is mostly supportive, including non-specific antivirals and symptom-alleviating
2 therapies. Ventilations and intensive care are required for severe cases, calling for early
3 intervention to prevent symptoms from deteriorating.

4
5 *In vitro* experiment showed that remdesivir targeting viral RNA-dependent RNA
6 polymerase (RdRp) effectively inhibited SARS-CoV-2 replication ^{1,2}. The
7 compassionate use of remdesivir for patients with severe COVID-19 indicated that
8 clinical improvement was observed in 36 of 53 patients (68%) ³. Remdesivir was
9 reported to shorten the time to recovery in adults hospitalized with COVID-19 and
10 evidence of lower respiratory tract infection in a double-blind, randomized, placebo-
11 controlled trial, though conflicting trial results have also been reported ^{4,5}. Several
12 repurposed drugs have been tested *in vitro* for inhibition of SARS-CoV-2 infection and
13 some of them were tested in clinical trial ⁶⁻⁹. Among them, chloroquine and
14 hydroxychloroquine have been shown to inhibit SARS-CoV-2 infection *in vitro*, while
15 the clinical trials of hydroxychloroquine reported controversial results ^{2,10-12}. The
16 effective concentrations (presented as the concentration for 50% of maximal effect
17 (EC50) on the reduction of viral RNA) of most previously selected drugs are in the
18 micromolar (μM) concentration range. On the other hand, neutralizing antibodies
19 against SARS-CoV-2 are also being intensively studied ¹³⁻¹⁵. In general, more
20 efficacious antiviral therapeutic agents against SARS-CoV-2 with good safety profile
21 are urgently needed.

22
23 In search of novel antivirals that can effectively inhibit SARS-CoV-2 infection, we set
24 out to screen an FDA-approved drug library of 3200 small molecules via observation
25 of viral CPE in Vero-E6 cells, followed by evaluation of the antiviral effect of candidate
26 compounds *in vitro* and in mice transduced intranasally with the recombinant
27 adenovirus 5 expressing human ACE2 (Ad5-hACE2). We discovered that
28 protoporphyrin IX (PpIX) and verteporfin displayed a potent antiviral activity and
29 prevent SARS-CoV-2 infection.

1

2 **Results**

3 **Protoporphyrin IX and verteporfin effectively inhibit SARS-CoV-2 infection in** 4 **Vero-E6 cells**

5 Two compounds, protoporphyrin IX and verteporfin, showed a complete suppression
6 of viral CPE at 1.25 μM and 0.31 μM respectively (Fig. 1B). These two compounds
7 were subject to further analysis. At 48 hours post-infection, viral RNA level in the
8 supernatant of the compound-treated cells was measured using qRT-PCR, which
9 decreased dose-dependently as the compound concentration increased. Based on the
10 RNA level-compound concentration curve, the EC₅₀ values of protoporphyrin IX,
11 verteporfin and the positive control remdesivir were calculated to be 0.23 μM , 0.03 μM ,
12 and 1.35 μM (Fig. 1A), respectively. The EC₅₀ of remdesivir was comparable to the
13 previous report ². Cell viability assay was performed, resulting in a viability-compound
14 concentration curve (Fig. 1A), from which the CC₅₀ (cytotoxicity concentration 50%)
15 values of protoporphyrin IX, verteporfin and remdesivir were determined to be 219.13
16 μM , 10.33 μM , and 303.23 μM , respectively. The selectivity indices (S.I.) for the three
17 compounds could thus be calculated as 952.74, 368.93, and 224.61, respectively. Viral
18 N protein expression in infected Vero-E6 cells was assessed by immunofluorescence.
19 The data revealed the complete inhibition of N protein expression by protoporphyrin
20 IX, verteporfin and remdesivir at 1.25 μM , 0.31 μM , and 6.25 μM , respectively (Fig.
21 1B). The results indicate that protoporphyrin IX and verteporfin strongly inhibit the
22 infection of SARS-CoV-2 at nanomolar concentrations and have a wide safety range *in*
23 *vitro*.

24

25 **Effects of treatment timing on protoporphyrin IX and verteporfin's inhibition of** 26 **SARS-CoV-2 infection**

27 We next analyzed the relationship between the antiviral effect and treatment timing of
28 protoporphyrin IX and verteporfin. As shown in Fig. 2A, Vero-E6 cells were treated
29 with protoporphyrin IX, verteporfin or the solvent DMSO before viral infection, during

1 viral entry and after viral entry. A total of 8 treatment groups were set up for each
2 compound (group I-VIII). Based on the previous results, we selected the compound
3 concentrations of 2.5 μ M and 1.25 μ M for protoporphyrin IX and verteporfin,
4 respectively. At 48 hours post infection, viral RNA level in the culture supernatant was
5 quantified with qRT-PCR. The results showed that viral RNA levels of all the
6 compound-treated groups (group I-VII of each compound in Fig. 2B, 2C) were
7 significantly lower than that of the DMSO-treated group (group VIII in Fig. 2B, 2C).
8 Importantly, pre-treatment alone resulted in the complete inhibition of SARS-CoV-2
9 infection (group IV in Fig. 2B, 2C). In addition, treatment of cells with protoporphyrin
10 IX or verteporfin after viral infection also inhibited viral RNA production, albeit to
11 different extent (group VII in Fig. 2B, 2C). The results of immunofluorescence analysis
12 on intracellular viral N protein were consistent with those of viral RNA measurement
13 (Fig. 2D). Collectively, the results indicate that protoporphyrin IX and verteporfin can
14 prevent SARS-CoV-2 infection and might suppress established SARS-CoV-2 infection
15 to some degree.

16
17 The preventive effect was further tested by the pre-treatment of cells with either
18 compound at a constant concentration and later infection with an increasing virus titer
19 (Fig. 3A). As shown in Fig. 3B, no viral N protein expression was detected in
20 protoporphyrin IX or verteporfin pre-treated cells even if the inoculated viral titer was
21 raised by 16 folds (200 PFU to 3200 PFU).

22

23 **Protoporphyrin IX and verteporfin interact with human ACE2 protein**

24 Protoporphyrin IX and verteporfin share a same structure formed by four pyrrole rings
25 (Fig. 4A) and thus likely act through a common antiviral mechanism. The above results
26 suggest that both drugs act by inhibiting an early step in viral infection. One possibility
27 was the saturation or modification of an essential cellular factor(s) required for viral
28 infection. We thus investigated firstly by molecular docking analysis whether human
29 ACE2, the viral receptor, might be the target of the compounds. The ACE2 peptidase

1 domain (PD) from the human ACE2-B⁰AT1 complex (PDB ID: 6m18) ¹⁶ was used for
2 docking with protoporphyrin IX and verteporfin (Fig. 4A). The result with the highest
3 ranking is exhibited in Fig. 4B, which represents the molecular model of protoporphyrin
4 IX or verteporfin binding to PD. Protoporphyrin IX is located in the shallow-pocket-
5 like space in the PD, with a binding energy of -5.60 kcal/mol. Similar result was
6 obtained from the docking of verteporfin with PD (with a binding energy of -5.35
7 kcal/mol). Fig. 4C provides a view of the interaction of protoporphyrin IX or
8 verteporfin with ACE2 PD residues. In the model, 25 residues of the PD interacted with
9 protoporphyrin IX, in which the benzene ring of Phe⁴⁰ interacted closely with the
10 porphyrin-ring of protoporphyrin IX, the Trp⁶⁹ formed aromatic H-bonds with the
11 porphyrin-ring, Asp³⁵⁰ and Asp³⁸² formed H-bonds with the compound. The other
12 residues involved in the interaction with protoporphyrin IX included Ser⁴³, Ser⁴⁴, Ser⁴⁷,
13 Asn⁵¹, Gly⁶⁶, Ser⁷⁰, Leu⁷³, Thr³⁴⁷, Ala³⁴⁸, Trp³⁴⁹, Leu³⁵¹, Gly³⁵², Phe³⁵⁶, His³⁷⁸, Ile³⁷⁹,
14 Tyr³⁸⁵, Phe³⁹⁰, Leu³⁹¹, Arg³⁹³, Asn³⁹⁴ and His⁴⁰¹. Similar results were observed in the
15 interaction between verteporfin and PD, except that Asn⁵¹ formed additional H-bonds
16 with the benzazole-like structure of verteporfin. Many of these PD residues are located
17 in the region that interacts with SARS-CoV-2 S protein receptor binding domain (RBD),
18 especially Phe⁴⁰, Ser⁴³, Ser⁴⁴, Trp³⁴⁹- Gly³⁵² and Phe³⁵⁶, which are very close to the key
19 residues (Tyr⁴¹, Gln⁴², Lys³⁵³ and Arg³⁵⁷) that interact with the RBD¹⁶. The results
20 suggest that protoporphyrin IX and verteporfin might interact with ACE2.

21
22 We next used BLI (Biolayer Interferometry) assay to evaluate the binding between
23 ACE2 and these two compounds. As shown in Fig. 4D, protoporphyrin IX and
24 verteporfin indeed bind to ACE2-Fc. The K_D of protoporphyrin IX and verteporfin
25 binding to ACE2-Fc were calculated to be 3.897 × 10⁻⁵ mol/L and 1.15 × 10⁻⁴ mol/L,
26 respectively. Therefore, structural simulation by molecular docking and direct drug-
27 protein binding assay support the binding of both drugs to viral receptor ACE2.

28

29 **Protoporphyrin IX and verteporfin interfere with the interaction between SARS-**

1 **CoV-2 S protein and ACE2**

2 Based on the molecular docking and the experimental data, both drugs likely interfere
3 with the interaction between ACE2 and RBD via binding ACE2, which would impair
4 viral entry. We first tested this possibility using a cell-cell fusion assay. HEK293T cells
5 that express SARS-CoV-2 S protein served as the effector cells and those co-expressing
6 human ACE2 and GFP as the target cells (Fig. 5A). The target cells were pre-treated
7 with protoporphyrin IX (2.5 μ M), verteporfin (1.25 μ M) or DMSO for 1 hour. After
8 removal of the drug, the target and effector cells were co-cultured at 37°C for 4 hours.
9 Fused cells with larger cell size than normal cells were observed in the DMSO-treated
10 group but barely in the protoporphyrin IX or verteporfin-treated group. The results
11 indicate that protoporphyrin IX and verteporfin may block interaction of ACE2 and
12 viral S protein which is required for cell-cell fusion.

13

14 To more directly demonstrate the interference of the compounds with the interaction of
15 ACE2 to RBD, we designed an ELISA assay, in which protoporphyrin IX or verteporfin
16 was added to 96-well plate pre-coated with ACE2-Fc or His-RBD. After incubation,
17 unbound drugs were washed away. His-RBD or ACE2-Fc was added to the drug-treated
18 wells pre-coated with ACE2-Fc or His-RBD. The results showed that both drugs could
19 prevent the binding of His-RBD to pre-coated ACE2-Fc, while they had no effect on
20 the binding of ACE2-Fc to pre-coated His-RBD (Fig. 5B). The data suggest that
21 protoporphyrin IX and verteporfin most likely bind to ACE2 and interfere with the
22 binding of RBD to ACE2, which is consistent with the results of the cell-cell fusion and
23 molecular docking abovementioned.

24

25 **Protoporphyrin IX and verteporfin effectively prevent SARS-CoV-2 infection in** 26 **the mouse model expressing human ACE2**

27 To investigate the inhibition of protoporphyrin IX and verteporfin of SARS-CoV-2
28 infection *in vivo*, mice were first transduced intranasally with Ad5-hACE2 which could
29 produce hACE2 in transduced HEK293T cells (Fig. S1). The mice were then infected

1 intranasally with SARS-CoV-2 (2×10^5 PFU/mouse) in a total volume of 50 μ L DMEM
2 containing protoporphyrin IX (100 μ M), verteporfin (20 μ M) or 1% DMSO (Fig. 6A).
3 Incubation of protoporphyrin IX (100 μ M) or verteporfin (20 μ M) with the virus had
4 no effect on viral infectivity (Fig. S2).

5

6 SARS-CoV-2-infected mice treated with 1% DMSO showed ruffled fur, hunching, loss
7 of appetite and difficulty in breathing beginning 2 days post infection, while SARS-
8 CoV-2-infected mice in the protoporphyrin IX and verteporfin groups were normal
9 without obvious symptoms. All the mice were euthanized at day 3 post-infection of
10 SARS-CoV-2 and lung tissues were collected. Human ACE2 expression in Ad5-hACE2
11 transduced mouse lung tissues was verified by immunochemical staining with the
12 specific antibody, which lined along the pulmonary epithelial cells in DMSO group,
13 protoporphyrin IX and verteporfin treated groups (Fig. 6C). Much fewer cells expressed
14 viral N protein in the protoporphyrin IX and verteporfin groups compared to the DMSO
15 group (Fig. 6C). Viral RNA was barely detected in most of the lung samples taken from
16 the protoporphyrin IX and verteporfin groups and the mean of viral RNA levels from
17 these groups were significantly lower than that from the DMSO group (Fig. 6B). The
18 sections of lung tissues from the DMSO group displayed a variety of lesions including
19 perivascular to interstitial inflammatory cell infiltrates, and necrotic cell debris. In
20 contrast, the sections of lung tissues from the protoporphyrin IX and verteporfin groups
21 showed no obvious histopathological change, so did those from the non-infected mice
22 (the NC group) (Fig. 6D). The heavy deposition of collagen in the thickened alveolar
23 interstitium was observed in the DMSO group with Masson-Trichrome staining, which
24 was absent in the non-infected group and barely observed in the protoporphyrin IX and
25 verteporfin groups (Fig. 6E). These results indicate that protoporphyrin IX and
26 verteporfin also effectively inhibit SARS-CoV-2 infection in mouse model.

27

28 **Discussion**

29 Protoporphyrin IX and verteporfin have been approved and used in the treatment of

1 human diseases. Protoporphyrin IX is the final intermediate in the protoporphyrin IX
2 iron complex (heme) biosynthetic pathway¹⁷. Heme is an important cofactor for oxygen
3 transfer and oxygen storage¹⁸ and is a constituent of hemoproteins which play a variety
4 of roles in cellular metabolism¹⁹. The light-activable photodynamic effect of
5 protoporphyrin IX was used for cancer diagnosis²⁰ and approved by FDA for treatment
6 of bronchial and esophageal cancers and early malignant lesions of the skin, bladder,
7 breast, stomach, and oral cavity^{21,22}. Verteporfin was approved for the treatment of age-
8 related macular degeneration²³. The potential of verteporfin for the treatment of cancers,
9 such as prostatic cancer, breast cancer, and pancreatic ductal adenocarcinoma has been
10 investigated²⁴. Verteporfin also has been reported to inhibit autophagy at an early stage
11 by suppressing autophagosome formation²⁵.

12
13 A study of clinical pharmacokinetics of verteporfin showed that in healthy volunteers
14 who were infused with verteporfin 6 to 14 mg/m² of body surface area over 1.5 to 45
15 minutes, C_{max} (peak concentration) of verteporfin was 1.24-2.74 µg/ml²⁶. The C_{max}
16 value is approximately 2.4 to 5.2-fold higher than the EC₉₀ value that was obtained in
17 this study (0.73 µM, i.e. 0.52 µg/ml). Protoporphyrin IX is the metabolite of 5-
18 aminolevulinic acid (5-ALA) in human body. After administration of 5-ALA 2 mg/kg
19 p.o., the average C_{max} of protoporphyrin IX was 27.44 µg/ml²⁷, which is about 20-
20 fold higher than the EC₉₀ value in this study (2.45 µM, i.e. 1.38 µg/ml). These data
21 indicate that the two drugs can reach a plasma concentration that is much higher than
22 the *in vitro* effective antiviral concentration. In the mouse model in this study,
23 protoporphyrin IX and verteporfin exhibited effective inhibition of SARS-CoV-2
24 infection without notable toxicity.

25
26 Both protoporphyrin IX and verteporfin have a porphyrin ring structure formed by four
27 pyrrole rings. It is most likely that they share a similar mechanism of antiviral action.
28 In the experiment when either drug was added prior to viral infection, viral RNA
29 production was inhibited even if the relevant drug was not added in the later virus

1 infection and post-infection stages (group IV in Fig. 2B, 2C). Furthermore, increasing
2 viral titer did not relieve the inhibition of the drugs added before viral infection (Fig.
3 3B). A logical hypothesis is that both drugs act by inhibiting an early step in viral
4 infection. Structural simulation by molecular docking and direct drug-protein binding
5 assay support the binding of both drugs to viral receptor ACE2. Several residues on
6 ACE2 predicted to interact with the drugs are very close to the key residues that interact
7 with the RBD of viral S protein. Based on the molecular docking and the experimental
8 data, both drugs likely interfere with the interaction between ACE2 and RBD via
9 binding ACE2, which would impair viral entry. The proposed mechanism was
10 supported by the blocking effect of both drugs on the cell-cell fusion mediated by the
11 interaction of ACE2 and viral S protein and by more direct evidence came from the
12 ELISA binding assay. To our knowledge, this is the first report on small compounds
13 that target the interaction between SARS-CoV-2 S protein and ACE2. The study
14 suggests a new venue for the development of small molecule-based entry inhibitor
15 against SARS-CoV-2. Furthermore, it may be a potential strategy for combating SARS-
16 CoV-2 infections to use the compounds inhibiting virus entry in combination with the
17 drugs acting intracellularly, such as the RdRp inhibitor remdesivir.

18
19 On the other hand, protoporphyrin IX and verteporfin were able to inhibit viral RNA
20 production to some degree when they were added after viral infection (group VII in Fig.
21 2B, 2C). It is possible that the drugs might inhibit the infection of progeny viruses and
22 hence prevent virus spreading. However, the absence of N protein expression in post-
23 infection verteporfin-treated cells suggests that there might be other antiviral
24 mechanism. Whether the drugs stimulate an antiviral innate immune response also
25 needs exploration.

26
27 In conclusion, this study has discovered protoporphyrin IX and verteporfin as potent
28 antiviral agents against SARS-CoV-2 infection in vitro and in the hACE2 mouse model.
29 The effective antiviral concentrations of these drugs are in the nanomolar concentration

1 range and the selectivity indices are greater than 200, indicating broad margin of safety.
2 Both compounds bind viral receptor ACE2, thereby disturbing the interaction between
3 ACE2 and the receptor-binding domain of viral S protein. To our knowledge, this is the
4 first report on small compounds that target the interaction between SARS-CoV-2 S
5 protein and ACE2, which sheds new light on developing novel chemoprophylaxis and
6 chemotherapy against SARS-CoV-2. The antiviral efficacy of protoporphyrin IX and
7 verteporfin *in vivo* will need clinical evaluation.

8

9 **Materials and Methods**

10 *Cell line, virus, compounds and constructs*

11 African green monkey kidney Vero-E6 cells and human embryonic kidney HEK293T
12 cells were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle medium
13 (DMEM) (Gibco, Carlsbad, USA) containing 2 mmol/L L-glutamine, 50 U/mL
14 penicillin, 100 mg/mL streptomycin, and 10% (vol/vol) fetal bovine serum (Gibco).
15 Vero-E6 cells after SARS-CoV-2 infection were maintained in DMEM containing 2
16 mmol/L L-glutamine, 50 U/mL penicillin, 100 mg/mL streptomycin, and 2% (vol/vol)
17 fetal bovine serum.

18

19 A clinical isolate of SARS-CoV-2, nCoV-SH01 (GenBank: MT121215.1)²⁸, was
20 propagated in Vero-E6 cells and the viral titer was determined as plaque forming units
21 (PFU) per milliliter (mL) by CPE (cytopathic effect) quantification. All the infection
22 experiments were performed in the biosafety level-3 (BSL-3) laboratory of Fudan
23 University.

24

25 The recombinant adenovirus 5 expressing human ACE2 (Ad5-hACE2) and control
26 adenovirus (Ad5-Ctrl) were purchased from ABM (Vancouver, Canada) or generated
27 in the laboratory. For the generation of recombinant Ad5-hACE2, hACE2 cDNA was
28 subcloned into the shuttle vector pShuttle-CMV²⁹ between KpnI and XhoI sites,
29 yielding pShuttle-CMV-hACE2. The plasmid pShuttle-CMV-hACE2 was linearized

1 with restriction enzyme PmeI, and then transformed into BJ5183-AD-1 competent cells
2 (Weidi, China), leading to the generation of pAd5-hACE2. Then, the plasmid pAd5-
3 hACE2 was linearized with restriction enzyme PacI and used to transfect HEK293 cells
4 as described previously³⁰. Adenovirus Ad5-hACE2 was rescued from pAd5-hACE2-
5 transfected cells and further amplified by several rounds of passage in HEK293 cells.
6 High-titer adenovirus was purified by CsCl gradient centrifugation and virus titer was
7 determined as described previously³¹. The resulting virus stock had a titer of 4.6×10^{12}
8 vp/mL.

9

10 Custom compound libraries containing 3200 small molecules were purchased from
11 Target Mol (MA, USA). Protoporphyrin IX (CAS No. 553-12-8), verteporfin (CAS No.
12 129497-78-5) and remdesivir (CAS No. 1809249-37-3) were purchased from
13 MedChemExpress (NJ, USA).

14

15 pCMV-GFP and pcDNA3.1-ACE2 were constructed by inserting the green fluorescent
16 protein (GFP) and human ACE2 cDNA into pcDNA3.1, respectively. pCAGGS-SARS-
17 CoV-2-S that encodes the SARS-CoV-2 Spike gene was generated by GENEWIZ
18 (Suzhou, China). Recombinant adenovirus expressing human ACE2 (Ad5-hACE2) and
19 control adenovirus (Ad5-Ctrl) were purchased from ABM (Vancouver, Canada).

20

21 ***Cell cytotoxicity assay***

22 The Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was used to assess cell viability
23 according to the manufacturer's instructions. Briefly, Vero-E6 cells were dispensed into
24 96-well plate (1.0×10^4 cells/well), cultured in medium supplemented with different
25 concentrations of the compound for 48 hours. After removal of the medium, the cells
26 were incubated with fresh serum-free medium containing 10% CCK-8 for 1 hour at 37°C
27 and then the absorbances at 450 nm were measured using a microplate reader (Bio-Rad,
28 Hercules, USA).

29

1 ***Library screening***

2 Custom compound libraries were screened via observation of CPE. Vero-E6 cells
3 cultured in 96-well plate (4.0×10^4 cells/well) were incubated with medium containing
4 SARS-CoV-2 (200 PFU/well) and each compound (10 μ M). Remdesivir (10 μ M)
5 served as positive control and DMSO as solvent control. CPE was observed under
6 microscope every 24 hours for 72 hours.

7
8 ***Evaluation of antiviral effects of the compounds***

9 Vero-E6 cells cultured in 96-well plate (4.0×10^4 cells/well) were pre-treated with the
10 compound of a tested concentration or DMSO for 1 hour. SARS-CoV-2 (200 PFU/well)
11 diluted in medium supplemented with the compound of the corresponding
12 concentration was added and allow viral infection for 1 hour at 37°C. The mixture was
13 removed and cells were washed twice with PBS, followed by culture with fresh medium
14 containing the compound of the corresponding concentration. At 48 hours post infection,
15 culture supernatant was collected for viral RNA quantification and the cells were fixed
16 in 4% paraformaldehyde for immunofluorescence analysis.

17
18 To evaluate the relationship between the timing of compound addition and the antiviral
19 efficacy, Vero-E6 cells cultured in 96-well plate (4.0×10^4 cells/well) were treated with
20 protoporphyrin IX (2.5 μ M), verteporfin (1.25 μ M) or DMSO at different timepoints
21 relative to virus infection (Fig. 2a). Briefly, four sets of cells (I-IV) were pre-treated
22 with the compound for 1 hour prior to virus infection. The medium was discarded and
23 the cells were washed twice with PBS. Two sets (I, II) were then incubated with medium
24 containing SARS-CoV-2 (200 PFU/well) and the compound for 1 hour and the other
25 two sets (III, IV) were incubated only with the virus. After the removal of the virus and
26 wash with PBS, set I and III were cultured with fresh medium containing the compound
27 while set II and IV with medium without the compound. Four more sets of cells (V-
28 VIII) were set up similarly except the initial medium contains DMSO instead of the
29 compound. At 48 hours post infection, the culture supernatant was collected for viral

1 RNA quantification and the cells for immunofluorescence analysis.

2

3 For evaluation of the prevention of viral infection by the compounds, Vero-E6 cells
4 plated in 96-well plate (4.0×10^4 cells/well) were pre-treated with protoporphyrin IX
5 ($2.5 \mu\text{M}$), verteporfin ($1.25 \mu\text{M}$) or DMSO for 1 hour. The compound was removed and
6 the cells were washed with PBS twice. Subsequently, the cells were incubated with
7 medium containing an increasing dose of SARS-CoV-2 for 1 hour. After removal of the
8 virus and wash with PBS, the cells were cultured for 48 hours for immunofluorescence
9 analysis.

10

11 For evaluation of the possible inactivation of SARS-CoV-2 by the compounds, SARS-
12 CoV-2 (2×10^5 PFU) were treated with 1% DMSO, protoporphyrin IX ($100 \mu\text{M}$),
13 verteporfin ($20 \mu\text{M}$) or 0.2% Triton X-100 for 30 minutes at room temperature. The
14 compounds were removed through centrifugal ultrafiltration (30 kDa, Millipore,
15 Darmstadt, Germany) and viral titers were measured with TCID₅₀ assay on Vero-E6
16 cells.

17

18 ***Viral RNA extraction and quantitative real time PCR (qRT-PCR)***

19 Viral RNA in tissue and cell supernatant was extracted using TRIzol reagent (Invitrogen,
20 Carlsbad, USA) following the manufacturer's instructions. After phenol/chloroform
21 extraction and isopropanol precipitation, RNA was reverse transcribed using cDNA
22 Synthesis Kit (Tiangen, Shanghai, China) according to the manufacturer's instructions.
23 Quantitative real-time PCR (qRT-PCR) was performed in a $20 \mu\text{L}$ reaction containing
24 SYBR Green (TaKaRa, Kusatsu, Japan) on MXP3000 cycler (Stratagene, La Jolla,
25 USA) with the following program: initial denaturation at 95°C for 300 seconds; 40
26 cycles of 95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 20 seconds; followed
27 by a melt curve step. The PCR primers (Genewiz) targeting the N gene (nt608-706) of
28 SARS-CoV-2 were: $5'$ -GGGGAACTTCTCCTGCTAGAAT- $3'$ / $5'$ -
29 CAGACATTTTGCTCTCAAGCTG- $3'$ (forward/reverse).

1

2 ***Immunofluorescence analysis***

3 To detect the viral nucleocapsid protein (N protein), anti-N polyclonal antibodies were
4 generated using standard immunization of BALB/c mice with recombinant N protein
5 derived from *E. coli*. Vero-E6 cells grown in 96-well plate were fixed in 4%
6 paraformaldehyde, permeabilized by 0.2% Triton X-100 (Thermo Fisher Scientific,
7 Waltham, USA), blocked with 3% BSA, and stained overnight with the anti-N antibody
8 (1:1000 dilution) at 4°C. The samples were then incubated with Alexa Fluor donkey
9 anti-mouse IgG 488-labeled secondary antibody (1:1000 dilution, Thermo Fisher
10 Scientific) for 1 hour at 37°C. The nuclei were stained with DAPI (Thermo Fisher
11 Scientific). Images were captured with fluorescence microscopy (Thermo Fisher
12 Scientific).

13

14 ***Molecular docking***

15 Cryo-electron microscopy structures of the full-length human ACE2 and a neutral
16 amino acid transporter B⁰AT1 complex with an overall resolution of 2.9 Å have been
17 reported ¹⁶. The structure files were downloaded from Protein Data Bank (PDB ID:
18 6m18). Meanwhile, the structures of the compounds, protoporphyrin IX and verteporfin,
19 were obtained from the EMBL-EBI and PubChem compound databases.

20

21 The receptor-ligand docking of the ACE2 protein with protoporphyrin IX or verteporfin
22 was performed by using AutoDock 4.2.6 software and visualized with AutoDockTools
23 1.5.6 software (<http://autodock.scripps.edu>). Firstly, the ligand and receptor coordinate
24 files were prepared respectively to include the information needed by AutoDock and
25 the PDBQT files were created. Then the three-dimension of the grid box was set in
26 AutoDockTools to create the grid parameter file. Afterwards, AutoGrid was used to
27 generate the grid maps and AutoDock was run for receptor-ligand docking. After
28 docking was completed, the results were shown in AutoDockTools, then the binding
29 energy and receptor-ligand interactions were evaluated. The docking area was

1 displayed in VMD 1.9.3 software (<http://www.ks.uiuc.edu/Research/vmd>).

2

3 ***Cell-cell fusion assay***

4 Cell-cell fusion was performed as described previously³². Briefly, target HEK293T
5 cells were transiently co-transfected with pCMV-eGFP and pcDNA3.1-ACE2 using
6 polyethyleneimine (PEI). Effector HEK293T cells were generated by transfection with
7 the envelope-expressing plasmid pCAGGS-SARS-CoV-2-S. Twenty-four hours post
8 transfection, the effector cells were pre-treated with protoporphyrin IX (2.5 μ M),
9 verteporfin (1.25 μ M) or DMSO for 1 hour. The compound was then removed and the
10 cells were washed with PBS twice. The target cells were quickly trypsinized and added
11 to adherent effector cells in a 1:1 target-to-effector cell ratio. After a 4-hour co-
12 cultivation period, five fields were randomly selected in each well and the number of
13 fused and unfused cells in each field were counted directly under an inverted
14 fluorescence microscope, based on much larger cell size of fused cells.

15

16 ***ELISA***

17 In the binding assay of viral S protein receptor binding domain (RBD), the recombinant
18 protein of the extracellular domain of human ACE2 (aa 1-740) fused to Fc (ACE2-Fc,
19 Genscript, Nanjing, China) was coated on 96-well microtiter plate (50 ng/well) at 4°C
20 overnight. The wells were blocked with 3% BSA for 1 hour at 37°C. Serial dilution
21 solutions of protoporphyrin IX, verteporfin or DMSO were added and incubated at
22 37°C for 1 hour. The free drug or DMSO was washed away with PBS. 50 ng of His-
23 tagged RBD (His-RBD, aa 319-541) (Genscript) was then added to each well and
24 incubated at 37°C for 2 hours. The wells were then washed with PBS and incubated
25 with mouse anti-His antibody (1:1000 dilution, Abmart, Berkeley Heights, USA) at
26 37°C for 1 hour, followed by incubation with Horseradish peroxidase (HRP)-
27 conjugated goat anti-mouse antibody (Abmart) at 37°C for 1 hour. Finally, TMB
28 substrate was added for color development and the absorbance at 450 nm was read on
29 a 96-well plate reader. The binding assay of ACE2 was similarly performed, except that

1 His-RBD protein (50 ng/well) was coated on 96-well microtiter plate and ACE2-Fc
2 protein was used for binding. HRP-goat anti-human Fc antibody (Abmart) was used for
3 final signal detection.

4

5 ***Transduction of HEK293T cells and Western blot analysis***

6 HEK293T cells were transduced with Ad5-hACE2 or Ad5-Ctrl at a multiplicity of
7 infection (MOI) = 100 for 4 hours at 37°C. The cells were lysed 48 hours post
8 transduction and the samples were subjected to 10% SDS-PAGE and transferred to
9 nitrocellulose membranes. The membranes were blocked with 3% bovine serum
10 albumin (BSA) in PBST (PBS containing 0.05% Tween 20, pH7.0) and incubated with
11 human ACE2 Rabbit Polyclonal antibody (1:100 dilution, Proteintech, Wuhan, China)
12 followed by HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5000 dilution,
13 Invitrogen). Immobilon Western Chemiluminescent HRP Substrate (Thermo Fisher
14 Scientific) was used for signal development.

15

16 ***Transduction and infection of mice***

17 Eight-week-old male mice (BALB/c) (SLAC Laboratory Animal, Shanghai, China)
18 were raised in pathogen-free cages in the BSL-3 laboratory of Fudan University. The
19 animal study protocol has been approved by the Animal Ethics Committee of School of
20 Basic Medical Sciences, Fudan University.

21 Mice were transduced intranasally with Ad5-hACE2 (5×10^{10} viral particles per mouse
22 in 50 μ l saline) and were randomly divided into four groups three days post transduction.
23 The mice were then infected intranasally with SARS-CoV-2 (2×10^5 PFU per mouse) in
24 a total volume of 50 μ l DMEM containing 100 μ M protoporphyrin IX (protoporphyrin
25 IX group), 20 μ M verteporfin (verteporfin group) or 1% DMSO (mock group),
26 respectively. Non-SARS-CoV-2 infected Ad5-hACE2 transduced mice were used as
27 negative control group (NC group). Mice were monitored and weighed daily. All the
28 mice were euthanized and sacrificed at day 3 post infection to collect the lungs for the
29 examinations of virus infection and histopathological changes.

1

2 ***Preparation of lung tissue samples***

3 Mouse lung tissues were fixed in 4% paraformaldehyde solution. Tissue homogenates
4 (1 g/mL) were prepared by homogenizing perfused lung tissues using an automatic
5 sample grinding instrument (Jingxin, Shanghai, China) for 1 minute in TRIzol reagent.
6 The homogenates were centrifuged at 12,000 rpm for 10 minutes at 4 °C. The
7 supernatant was collected for viral RNA extraction.

8

9 ***Histology and immunohistochemistry***

10 Mouse lungs were fixed in 4% paraformaldehyde solution. Tissue paraffin sections
11 (2~4 μm in thickness) were stained with hematoxylin and eosin (H&E) and modified
12 Masson's Trichrome. To detect hACE2 expression, the sections were first incubated in
13 blocking reagent and then with hACE2 Rabbit Polyclonal antibody (1:100 dilution,
14 Proteintech) at 4 °C overnight, followed by incubation with HRP-conjugated goat anti-
15 rabbit IgG secondary antibody (1:5000 dilution, Invitrogen). The lung sections from the
16 mouse transduced intranasally with 5×10^{10} of Ad5-hACE2 were used as negative
17 control. For viral antigen detection, the sections were sequentially incubated with
18 mouse polyclonal antibody to SARS-CoV-2 N protein (1:500 dilution) and HRP-
19 conjugated goat anti-mouse IgG secondary antibody (1:5000 dilution, Invitrogen). The
20 sections were observed under microscope (Olympus, Tokyo, Japan).

21

22 ***Biolayer interferometry (BLI) binding Assay***

23 BLI assays were carried out in 96-well black plates using an OctetRED96 device (Pall
24 ForteBio, Fremont, USA). For detecting the binding kinetics of protoporphyrin IX or
25 verteporfin with hACE2, the recombinant protein ACE2-Fc (Genscript) at 5 μg/mL
26 buffered in PBST (PBS with 0.02% Tween 20, pH 7.0) was immobilized onto activated
27 AHC biosensors (ForteBio) and incubated with 20 μM, 10 μM or 5 μM of each
28 compound in kinetics buffer (PBST). The experiment included the following steps at

1 37°C: (1) equilibration (60 seconds); (2) immobilization of ACE2-Fc onto sensors (100
2 seconds); (3) baseline in kinetics buffer (60 seconds); (4) association of the drug for
3 measurement of k_{on} (240 seconds); and (5) dissociation of the drug for measurement
4 of k_{off} (200 seconds). All the curves were fitted by a 2:1 (heterogeneous ligands)
5 binding model and mean K_D values were determined using the Data Analysis software
6 (ForteBio).

7 8 **Statistical analysis**

9 Data were analyzed using Prism 7 (GraphPad) and were presented as mean \pm SEM. The
10 dose response curves of viral RNA levels or cell viability *versus* the drug concentrations
11 were plotted and evaluated by Prism 7. Statistical significance was determined using
12 unpaired two-tailed Student's *t* test for single variables and two-way ANOVA followed
13 by Bonferroni posttests for multiple variables.

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13

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Author Contributions

Youhua Xie, Di Qu and Qing Deng drafted the manuscript. Youhua Xie, Di Qu and Qiang Deng designed the project. The majority of the experiments and data analysis were performed by Chenjian Gu, Yang Wu, Huimin Guo and Yuanfei Zhu. The other authors participated in the data analysis and manuscript revision. All the authors have approved the manuscript.

Competing interests

The authors declare no competing interests.

Figures

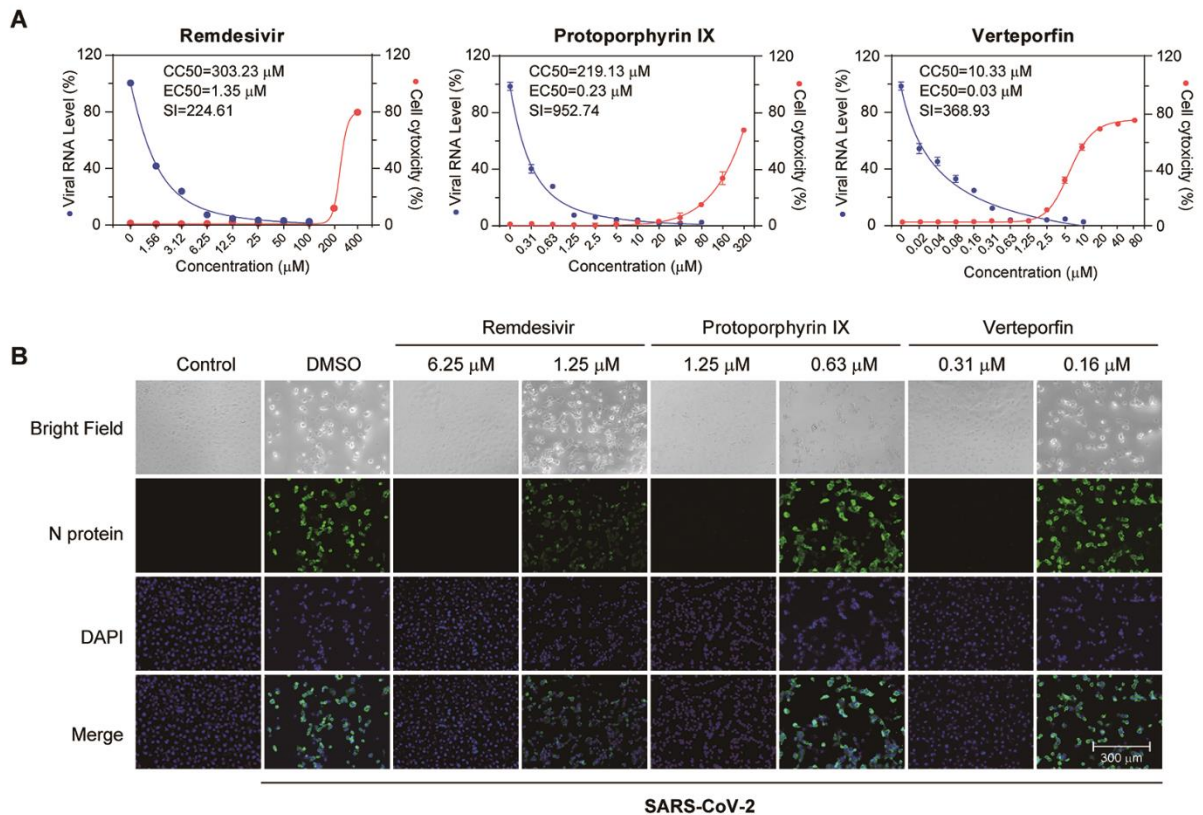


Fig. 1. Effective inhibition of SARS-CoV-2 infection by protoporphyrin IX and verteporfin

(A) Antiviral effect and cell cytotoxicity of protoporphyrin IX and verteporfin. The viral RNA production in the supernatant of infected Vero-E6 cells was quantified with qRT-PCR. The value at each compound concentration was presented relative to that at zero compound concentration that was set as 100% (blue). The percentage of reduction in viable cells at different compound concentration (red) was measured using the CCK8 assay. The value at each compound concentration was calculated using the formula, $100 - \text{Value (compound concentration)} / \text{Value (zero compound concentration)}$. EC50, concentration for 50% of maximal effect; CC50, concentration for 50% of maximal cytotoxic effect; S.I., selectivity index. Data from three independent experiments were analyzed. (B) Immunofluorescence of intracellular viral N protein. Intracellular

expression of N protein was assessed by staining of infected Vero-E6 cells with the polyclonal anti-N antibody (1:1000 dilution, green). Nuclei were stained with DAPI. CPE was shown in bright field.

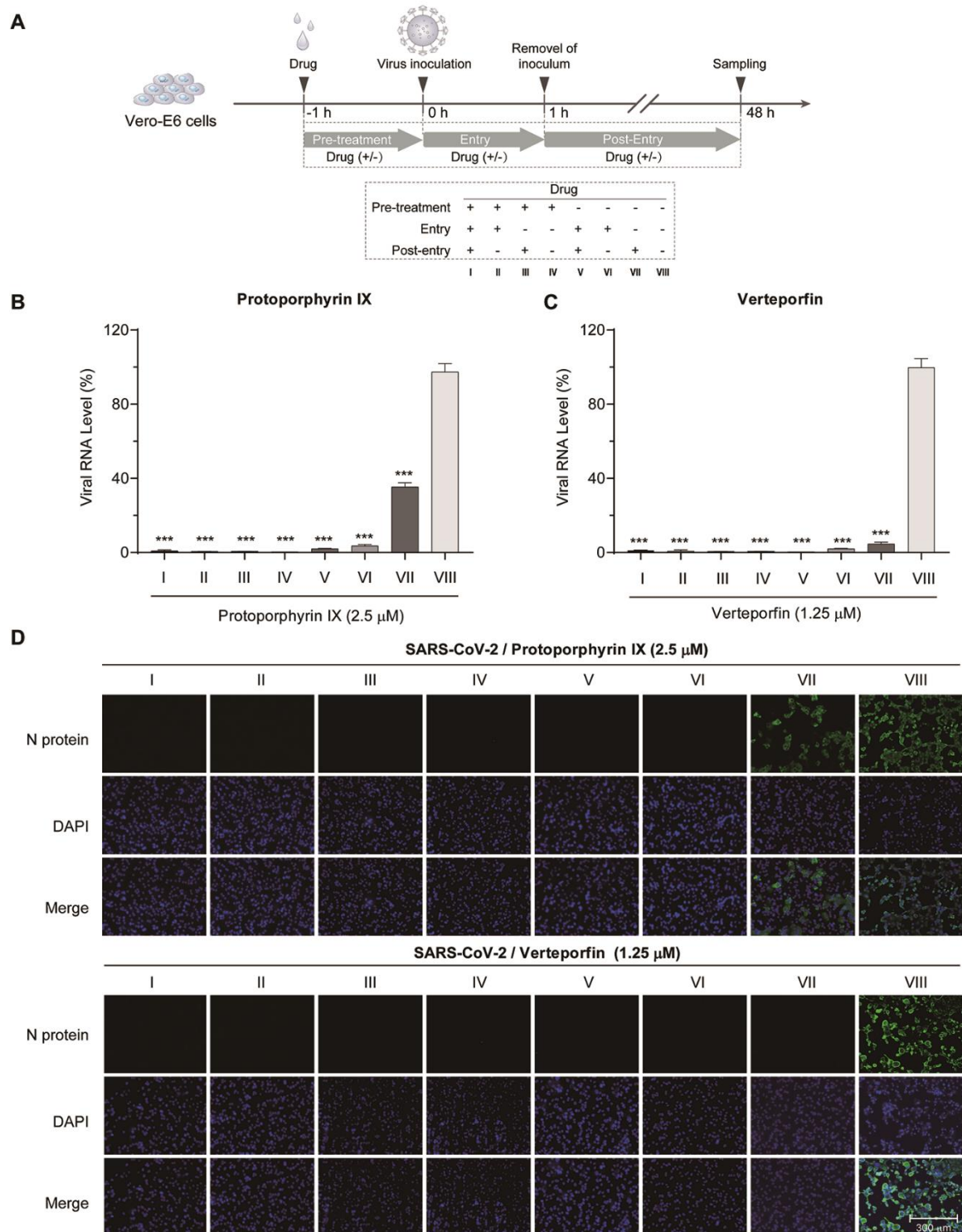


Fig. 2. Effects of treatment timing of protoporphyrin IX and verteporfin on SARS-CoV-2 infection

(A) Schematic presentation of treatment timing of protoporphyrin IX and verteporfin. Briefly, Vero-E6 cells were treated with protoporphyrin IX, verteporfin or the solvent DMSO before viral infection, during viral entry and after viral entry. A total of 8 treatment groups (I-VIII) for each compound were set up. **(B)** Antiviral effect of different treatment timing. Viral RNA level in the supernatant of infected Vero-E6 cells was quantified with qRT-PCR. The values of group I to VII were presented relative to that of group VIII which was set as 100%, respectively. Statistical significance was determined using the unpaired two-tailed Student's *t* test. *** $P < 0.001$. Data from three independent experiments were analyzed. **(C)** Immunofluorescence of intracellular viral N protein. Intracellular expression of N protein of different treatment timing was assessed by staining of infected Vero-E6 cells with the polyclonal anti-N antibody (1:1000 dilution, green). Nuclei were stained with DAPI.

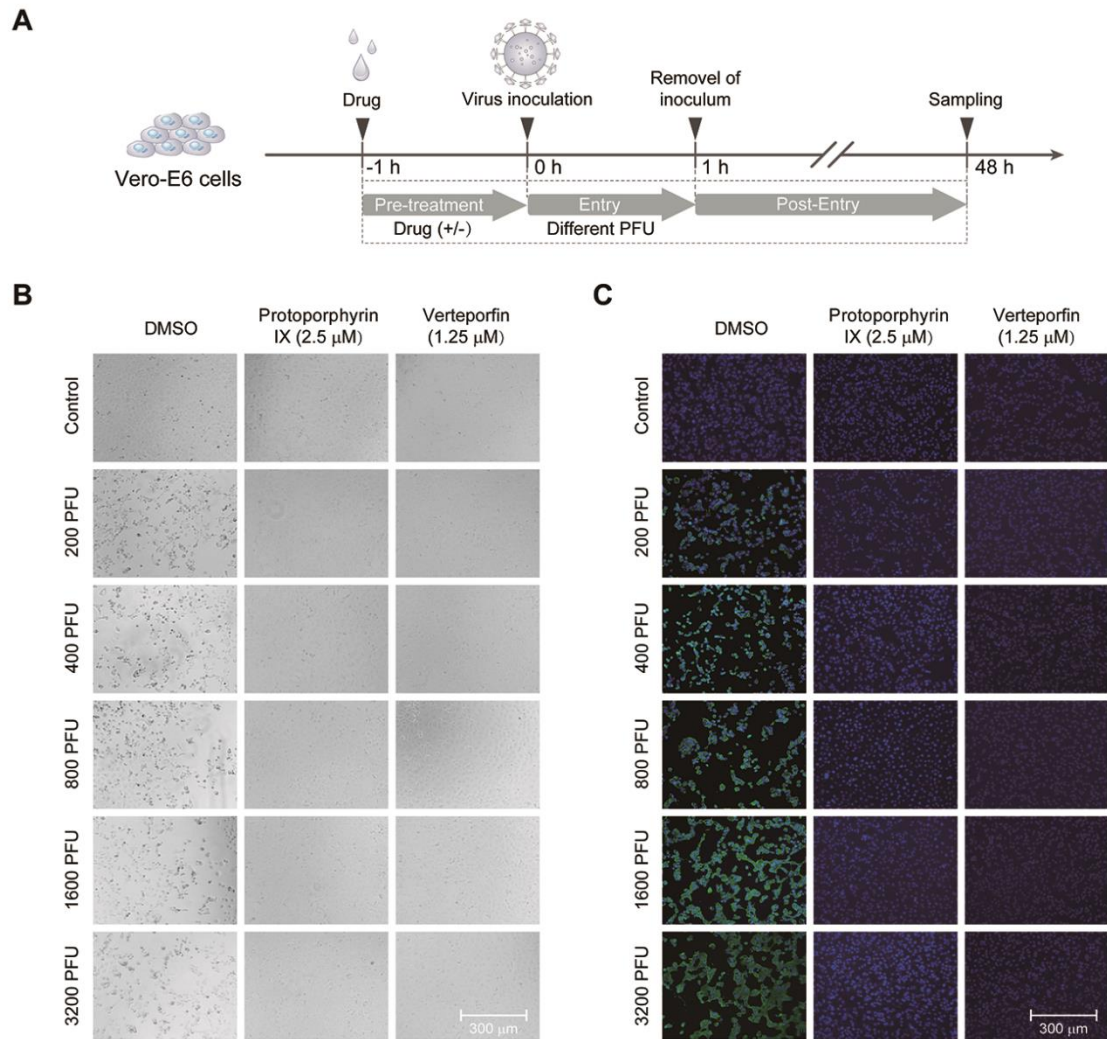


Fig. 3. Protoporphyrin IX and verteporfin prevent SARS-CoV-2 infection

(A) Schematic presentation of treatment design. Briefly, Vero-E6 cells were pre-treated with protoporphyrin IX, verteporfin or the solvent DMSO before viral infection for 1 hour, then the drugs were removed and the cells were washed and infected with an increasing titer of SARS-CoV-2. (B) CPE of the cells with the different treatment. (C) Immunofluorescence of intracellular viral N protein. Intracellular expression of N protein of different treatment was assessed by staining of infected Vero-E6 cells with the polyclonal anti-N antibody (1:1000 dilution, green). Nuclei were stained with DAPI.

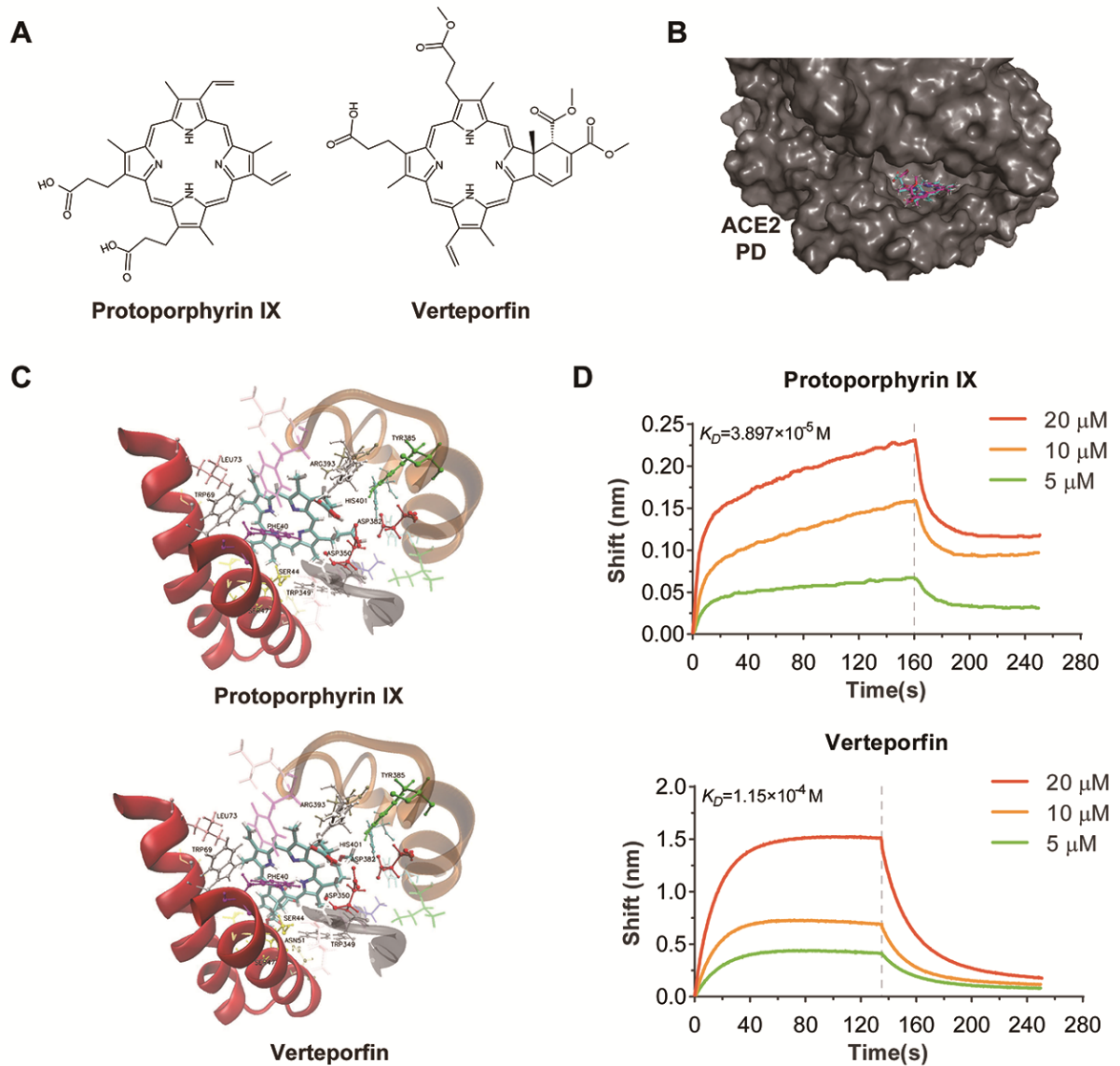


Fig. 4. Protoporphyrin IX and verteporfin bind human ACE2 protein

(A) Structures of protoporphyrin IX and verteporfin. (B) Docking of ACE2 peptidase domain (PD) with protoporphyrin IX (blue) and verteporfin (pink). The 3D structure of PD is from cryo-electron microscopy structure of the ACE2-B⁰AT1 complex (PDB ID: 6m18). The surface of PD is shown. (C) Interactions of protoporphyrin IX (upper) or verteporfin (bottom) with ACE2 residues. (D) Binding profiles of protoporphyrin IX or verteporfin to ACE2-Fc protein measured with Bi-layer Interferometry assay.

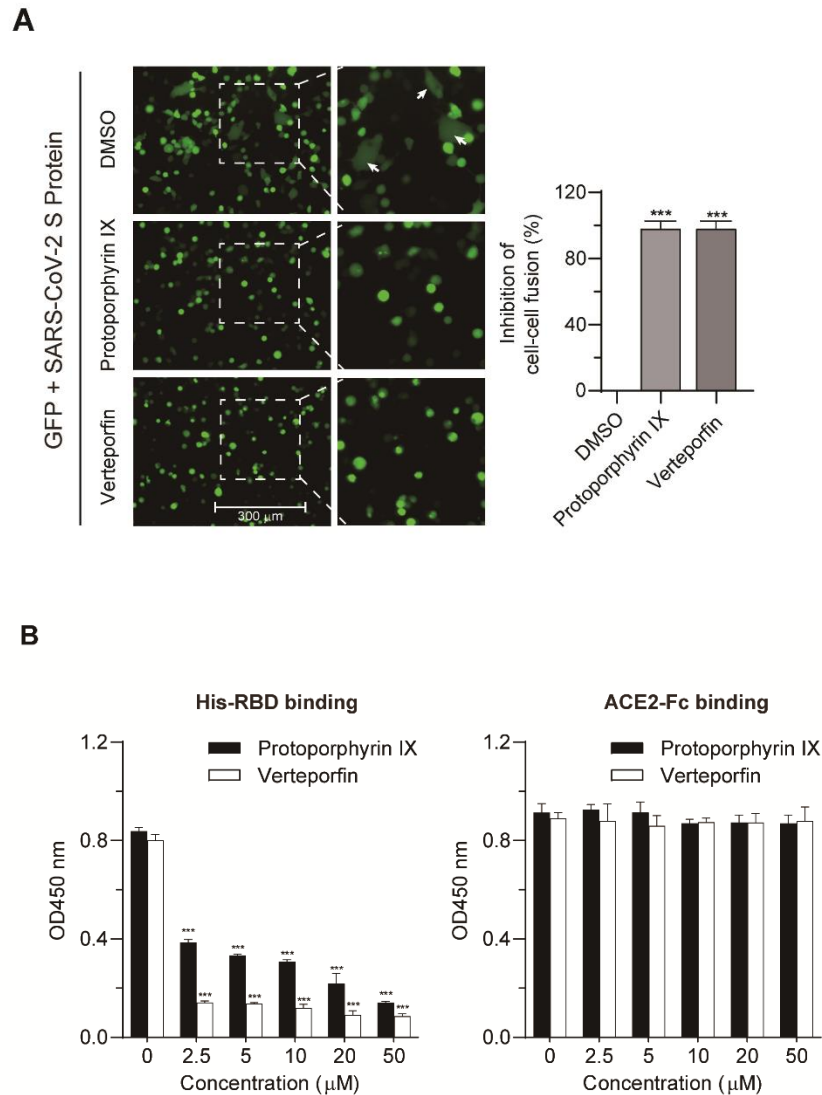


Fig. 5. Protoporphyrin IX and verteporfin interfere with the interaction between ACE2 and RBD

(A) Blocking effect on ACE2 and SARS-CoV-2 S-mediated cell-cell fusion by protoporphyrin IX and verteporfin. The inhibitory value of protoporphyrin IX or verteporfin-treated group was presented relative to that of the DMSO-treated group which was set as 100%, respectively. Statistical significance was determined using the unpaired two-tailed Student's *t* test. *** $P < 0.001$. (B) ELISA. The binding of His-RBD or ACE2-Fc to drug-treated pre-coated ACE-Fc or His-RBD was measured by absorbance at 450 nm. Statistical significance was determined using the unpaired two-tailed Student's *t* test. *** $P < 0.001$. Data from triplicate wells were analyzed.

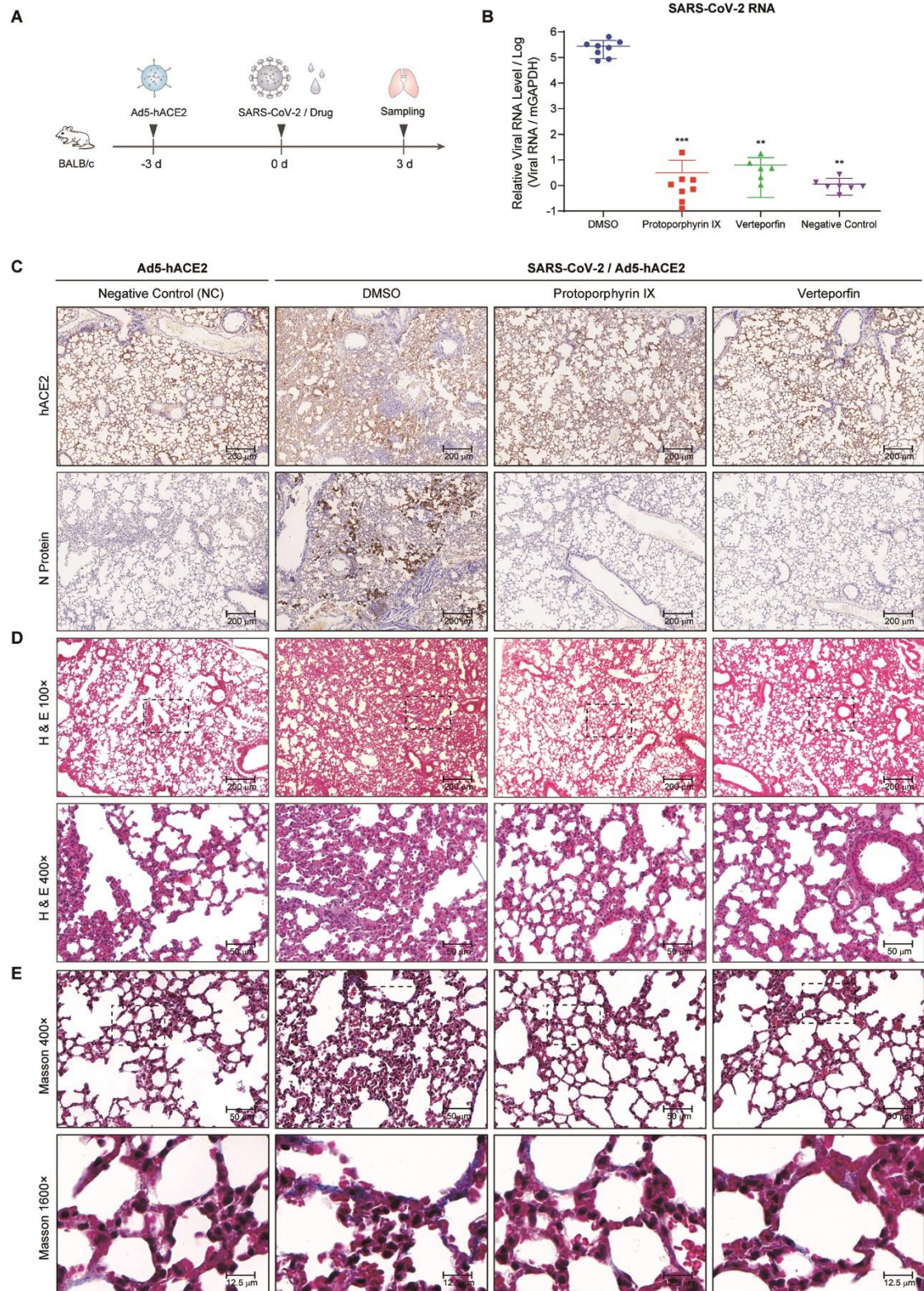


Fig. 6. Effective inhibition of SARS-CoV-2 infection by protoporphyrin IX and verteporfin in SARS-CoV-2-infected hACE2 mice

(A) Schematic representation of the experiment timeline. **(B)** Relative viral RNA levels in lung tissues from each group. Data are relative to that of the DMSO-treated group and statistical significance was calculated using unpaired two-tailed t-test. $**P < 0.01$ and $***P < 0.001$. **(C)** Immunohistochemical staining of hACE2 and viral N protein in lung tissue samples from each group. **(D)** Representative HE staining of lung tissue sections from each group. **(E)** Representative Masson's Trichrome staining of lung tissue sections from each group.

Supplementary Figure 1

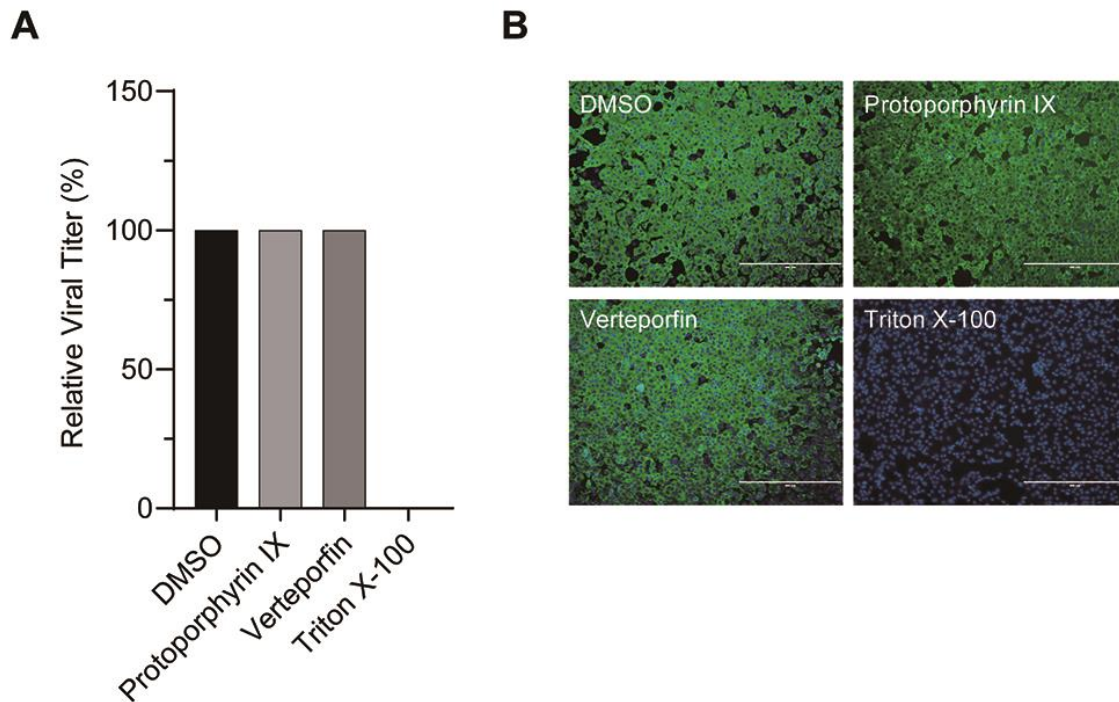


Figure S1. Incubation of protoporphyrin IX or verteporfin with SARS-CoV-2 has no effect on viral infectivity

(A) Relative viral titer measured with TCID₅₀ assay. SARS-CoV-2 (2×10^5 PFU) was treated with 1% DMSO, protoporphyrin IX (100 μ M), verteporfin (20 μ M) or 0.2% Triton X-100 for 30 minutes. The compounds were removed by centrifugal ultrafiltration and viral titers were measured with TCID₅₀ assay on Vero-E6 cells. (B) Immunofluorescence of intracellular viral N protein. Intracellular expression of N protein was assessed by staining infected Vero-E6 cells using the polyclonal anti-N antibody (1:1000 dilution, green). Nuclei were stained with DAPI.

Supplementary Figure 2

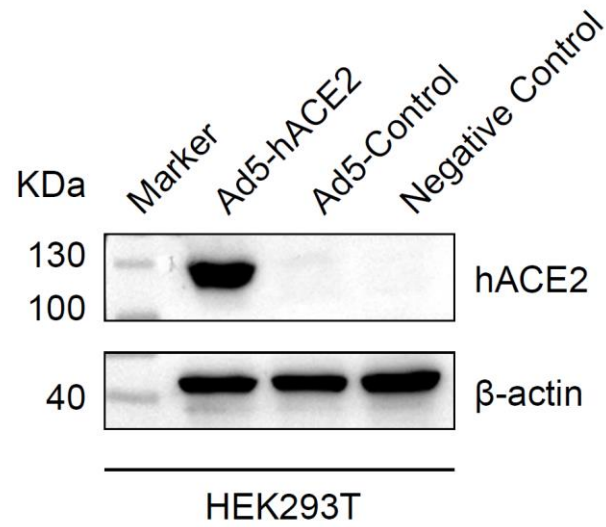


Figure S2. Expression of hACE2 in Ad5-hACE2 transduced HEK293T cells.

Western blot analysis of hACE2 protein in HEK293T cells transduced by Ad5-hACE2. HEK293T cells were transduced with Ad5-hACE2 or Ad5-Ctrl at MOI of 100 at 37 °C for 4 hours. HEK293T cells without any treatment were used as negative control.