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Protoporphyrin IX and verteporfin potently inhibit SARS-CoV-2 infection in vitro and in a mouse model expressing human ACE2

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A B S T R A C T
The SARS-CoV-2 infection is spreading rapidly worldwide. Efficacious antiviral therapeutics against SARS-CoV-2 is urgently needed. Here, we discovered that protoporphyrin IX (PpIX) and verteporfin, two Food and Drug Administration (FDA)-approved drugs, completely inhibited the cytopathic effect produced by SARS-CoV-2 infection at 1.25 μmol/L and 0.31 μmol/L, respectively, and their EC50 values of reduction of viral RNA were at nanomolar concentrations. The selectivity indices of PpIX and verteporfin were 952.74 and 368.93, respectively, suggesting a broad margin of safety. Importantly, PpIX and verteporfin prevented SARS-CoV-2 infection in mice adenovirally transduced with human angiotensin-converting enzyme 2 (ACE2). The compounds, sharing a porphyrin ring structure, were shown to bind viral receptor ACE2 and interfere with the interaction between ACE2 and the receptor-binding domain of viral S protein. Our study suggests that PpIX and verteporfin are potent antiviral agents against SARS-CoV-2 infection and sheds new light on developing novel chemoprophylaxis and chemotherapy against SARS-CoV-2.

1. Introduction
SARS-CoV-2 is transmitted through respiratory droplets and close contact, which causes mainly upper and lower respiratory diseases. The majority of infected healthy adults and children only show mild symptoms including cough, fever, fatigue, and diarrhea but the elderly with various chronic diseases are at high risk of development of serious diseases including pneumonia, acute respiratory distress, multiple organ failure, and shock. At present, treatment of coronavirus disease 2019 (COVID-19) is mostly supportive, including non-specific antivirals and symptom-alleviating therapies [1]. Ventilations and intensive care are required for severe cases, calling for early intervention to prevent symptoms from deteriorating [1,2].

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

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

2. Materials and methods

2.1. Cell line, virus, compounds, and constructs

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

A clinical isolate of SARS-CoV-2, nCoV-SH01 (GenBank: MT121215.1) [18], was propagated in Vero E6 cells and the viral titer was determined as plaque forming units (PFU) per milliliter (mL) by CPE quantification. All the infection experiments were performed in the biosafety level-3 (BSL-3) laboratory of Fudan University.

The recombinant adenovirus 5 expressing human ACE2 (Ad5-hACE2) and control adenovirus (Ad5-Ctrl) were purchased from ABM (Vancouver, Canada) or generated in the laboratory. For the generation of recombinant Ad5-hACE2, hACE2 cDNA was subcloned into the shuttle vector pShuttle-CMV [19] between KpnI and XhoI sites, yielding pShuttle-CMV-hACE2. The plasmid pShuttle-CMV-hACE2 was linearized with restriction enzyme Pmel, and then transformed into BJ5183-AD-1 competent cells (Weidi, Shanghai, China), leading to the generation of pAd5-hACE2. Then, the plasmid pAd5-hACE2 was linearized with restriction enzyme PciI and used to transfect HEK293 cells as described previously [20]. Adenovirus Ad5-hACE2 was rescued from pAd5-hACE2-transfected cells and further amplified by several rounds of passage in HEK293 cells. High-titer adenovirus was purified by CsCl gradient centrifugation and virus titer was determined as described previously [21]. The resulting virus stock had a titer of 4.6 × 1012 viral particles per mL (VP/mL).

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

The pCMV-GFP and pcDNA3.1-ACE2 were constructed by inserting the green fluorescent protein (GFP) and human ACE2 cDNA into pcDNA3.1, respectively. pCAGGS-SARS-CoV-2-S expressing the SARS-CoV-2 spike protein was generated by Gene-wiz (Suzhou, China).

2.2. Cell cytotoxicity assay

The Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was used to assess cell viability according to the manufacturer’s instructions. Briefly, Vero E6 cells were dispensed into 96-well plates (1.0 × 104 cells/well), cultured in a medium supplemented with different concentrations of the compound for 48 h. After removal of the medium, the cells were incubated with fresh serum-free medium containing 10% CCK-8 for 1 h at 37 °C and then the absorbances at 450 nm were measured using a microplate reader (Bio-Rad, Hercules, USA).

2.3. Library screening

Custom compound libraries were screened via observation of CPE. Vero E6 cells cultured in 96-well plates (4.0 × 104 cells/well) were pre-treated with the compound of a tested concentration or DMSO for 1 h. SARS-CoV-2 (200 PFU/well) were diluted in medium supplemented with the compound of the corresponding concentration and then added to allow viral infection for 1 h at 37 °C. The mixture was removed and cells were washed twice with phosphate buffered saline (PBS), followed by culture with fresh medium containing the compound of the corresponding concentration. At 48 h post-infection, culture supernatant was collected and the cells were fixed in 4% paraformaldehyde for immunofluorescence analysis.

To evaluate the relationship between the timing of compound addition and the antiviral efficacy, Vero E6 cells cultured in 96-well plate (4.0 × 104 cells/well) were treated with protoporphyrin IX (2.5 μmol/L), verteporfin (12.5 μmol/L) or DMSO at different timepoints relative to virus infection (Fig. 2a). Briefly, four sets of cells (I-IV) were pre-treated with the compound for 1 h prior to virus infection. The medium was discarded and the cells were washed twice with PBS. Two sets (I, II) were then incubated with a medium containing SARS-CoV-2 (200 PFU/well) and the compound for 1 h and the other two sets (III, IV) were incubated only with the virus. After the removal of the virus and wash with PBS, set I and III were cultured with fresh medium containing the compound while set II and IV with medium without the compound. Four more sets of cells (V–VIII) were set up similarly except the initial medium contains DMSO instead of the compound. At 48 h post infection, the culture supernatant was collected for viral RNA quantification and the cells for immunofluorescence analysis.

For evaluation of the prevention of viral infection by the compounds, Vero E6 cells plated in 96-well plates (4.0 × 104 cells/well) were pre-treated with protoporphyrin IX (2.5 μmol/L), verteporfin (12.5 μmol/L) or DMSO for 1 h. The compound was removed and the cells were washed twice with PBS. Subsequently, the cells were incubated with a medium containing an increasing dose of SARS-CoV-2 for 1 h. After removal of the virus and wash with PBS, the cells were cultured for 48 h for immunofluorescence analysis.

For evaluation of the possible inactivation of SARS-CoV-2 by the compounds, SARS-CoV-2 (2 × 106 PFU) were pre-treated with 1% DMSO, protoporphyrin IX (100 μmol/L), verteporfin (20 μmol/L) or 0.2% Triton X-100 for 30 min at room temperature. The compounds were removed through centrifugal ultrafiltration (30 kD, Millipore, Darmstadt, Germany) and viral titers were measured with 50% tissue culture infectious dose (TCID50) assay on Vero E6 cells.
2.5. Viral RNA extraction and quantitative real time PCR (qRT-PCR)

Viral RNA in tissue and cell supernatant was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) and Total RNA Purification Kit (Sangon, Shanghai, China) following the manufacturer’s instructions. Extracted RNA was reverse transcribed using cDNA Synthesis Kit (Tiangen, Shanghai, China) according to the manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) was performed in a 20 μl reaction containing SYBR Green (TaKaRa, Kusatsu, Japan) on MXP3000 cyclers (Stratagene, La Jolla, USA) with the following program: initial denaturation at 95 °C for 300 s; 40 cycles of 95 °C for 15 s, 55 °C for 20 s, and 72 °C for 20 s; followed by a melt curve step. The PCR primers (Genewiz) targeting the N gene (nt608-706) of SARS-CoV-2 were: 5′-GGGGACCTTCTCTGCTA GAA-T3′/5′-CACACATTGTTCCTCAAGCT-3′ (forward/reverse), primers targeting human ACE2 (hACE2) were 5′-TGGGTCTCTCAGTG CTCTCAGA-3′/5′-GACCTCAGATCTCCAGTT-3′ (forward/reverse), primers targeting murine GAPDH (mGAPDH) were 5′-AGGTCGGTGT GAAAGGATTGG-3′/5′-GGGGTCTGTTGAGTCGAACA-3′ (forward/reverse).

2.6. Immunofluorescence analysis

To detect the viral nucleocapsid protein (N protein), anti-N polyclonal antibodies were generated using standard immunization of BALB/c mice with recombinant N protein derived from E. coli. Vero E6 cells grown in 96-well plates were fixed in 4% paraformaldehyde, permeabilized by 0.2% Triton X-100 (Thermo Fisher Scientific, Waltham, USA), blocked with 3% bovine serum albumin (BSA), and stained overnight with the anti-N antibody (1:1000 dilution) at 4 °C. The samples were then incubated with Alexa Fluor donkey anti-mouse IgG 488-labeled secondary antibody (1:1000 dilution, Thermo Fisher Scientific) for 1 h at 37 °C. The nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific). Images were captured with fluorescence microscopy (Thermo Fisher Scientific).

2.7. Molecular docking

Cryo-electron microscopy structures of the full-length human ACE2 and a neutral amino acid transporter B₀AT1 complex with an overall resolution of 2.9 Å have been reported [22]. The structure files were downloaded from Protein Data Bank (PDB ID: 6 m18). Meanwhile, the structures of the compounds, protoporphyrin IX and verteporfin, were obtained from the EMBL-EBI and PubChem compound databases.

The receptor-ligand docking of the ACE2 protein with protoporphyrin IX or verteporfin was performed by using AutoDock 4.2.6 software and visualized with AutoDockTools 1.5.6 software (http://autodock.scripps.edu). Firstly, the ligand and receptor coordinate files were prepared respectively to include the information needed by AutoDock and the PDBQT files were created. Then, the three-dimension of the grid box was set in AutoDockTools to create the grid parameter file. Afterward, AutoGrid was used to generate the grid maps and AutoDock was run for receptor-ligand docking. After docking was completed, the results were shown in AutoDockTools, then the binding energy and receptor-ligand interactions were evaluated. The docking area was displayed using VMD 1.9.3 software (http://www.ks.uiuc.edu/Research/vmd).
followed by HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5000 dilution, Invitrogen). Immobilon Western Chemiluminescent HRP Substrate (Thermo Fisher Scientific) was used for signal development.

2.12. Transduction and infection of mice

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

Mice were transduced intranasally with Ad5-hACE2 (5 × 10^10 viral particles per mouse in 50 μL saline) and were randomly divided into four groups three days post-transduction. The mice were then infected intranasally with SARS-CoV-2 (2 × 10^6 PFU per mouse) in a total volume of 50 μL DMEM containing 100 μmol/L protoporphyrin IX (protoporphyrin IX group), 20 μmol/L verteporfin (verteporfin group) or 1% DMSO (mock group), respectively. Non-SARS-CoV-2 infected Ad5-hACE2 transduced mice were used as the negative control group (NC group). Mice were monitored and weighed daily. All the mice were euthanized and sacrificed at day 3 post infection to collect the lungs for the examinations of virus infection and histopathological changes.

2.13. Preparation of lung tissue samples

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

2.14. Histology and immunohistochemistry

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

2.15. Biolayer interferometry (BLI) binding assay

BLI assays were carried out in 96-well black plates using an Octet RED96 device (Pall ForteBio, Fremont, USA). For detecting the binding kinetics of protoporphyrin IX or verteporfin with hACE2, the recombinant protein ACE2-Fc [Genscript] at 5 μg/mL buffered in PBST (PBS with 0.02% Tween 20, pH 7.0) was immobilized onto activated AHC biosensors (ForteBio, Fremont, USA) and incubated with 20 μmol/L, 10 μmol/L or 5 μmol/L of each compound in kinetics buffer (PBST). The experiment included the following steps at 37 °C: (1) equilibration (60 s); (2) immobilization of ACE2-Fc onto sensors (100 s); (3) baseline in kinetics buffer (60 s); (4) association of the drug for measurement of k_on (240 s); and (5) dissociation of the drug for measurement of k_off (200 s). All the curves were fitted by a 2:1 (heterogeneous ligands) binding model and mean K_d values were determined using the Data Analysis software (ForteBio).

2.16. Statistical analysis

Data were analyzed using GraphPad Prism (Version 7.0, San Diego, USA) and were presented as mean ± standard error of the mean (SEM). The dose response curves of viral RNA levels or cell viability vs. the drug concentrations were plotted and evaluated by Prism 7. Statistical significance was determined using unpaired two-tailed Student’s t-test for single variables and two-way ANOVA followed by Bonferroni posttests for multiple variables.

3. Results

3.1. Protoporphyrin IX and verteporfin effectively inhibit SARS-CoV-2 infection in Vero E6 cells

A compound library of 3200 small molecules was screened via observation of viral CPE in Vero E6 cells for novel antivirals that can effectively inhibit SARS-CoV-2 infection. Vero E6 cells cultured in 96-well plate were pre-treated with the compound (10 μmol/L) for 1 h. After the removal of the compound, SARS-CoV-2 (200 PFU/well) with the compound (10 μmol/L) was added. Simultaneously, remdesivir (10 μmol/L) served as positive control and DMSO as solvent control. At 48 h post-infection, structural and morphological changes induced by SARS-CoV-2 were observed. Two compounds, protoporphyrin IX and verteporfin, showed a complete suppression of viral CPE. These two compounds were subjected to further analysis.

As shown in Fig. 1a, protoporphyrin IX and verteporfin, displayed a complete suppression of viral CPE at 1.25 μmol/L and 0.31 μmol/L, respectively. Viral N protein expression in infected Vero E6 cells was assessed by immunofluorescence. The data revealed the complete inhibition of N protein expression by protoporphyrin IX, verteporfin and remdesivir at 1.25 μmol/L, 0.31 μmol/L, and 6.25 μmol/L, respectively (Fig. 1a). At 48 h post-infection, viral yield in the supernatant of the compound-treated cells was measured using qRT-PCR, which decreased dose-dependently as the compound concentration increased. Based on the RNA level-compound concentration curve, the EC50 values of protoporphyrin IX, verteporfin and remdesivir were calculated to be 0.23 μmol/L, 0.03 μmol/L, and 1.35 μmol/L (Fig. 1b), respectively. The EC50 of remdesivir was comparable to the previous report[4]. Cell viability assay was performed, resulting in a viability-compound concentration curve (Fig. 1b), from which the CC50 (cytotoxicity concentration 50%) values of protoporphyrin IX, verteporfin and remdesivir were determined to be 219.13 μmol/L, 10.33 μmol/L, and 303.23 μmol/L, respectively. The selectivity indices (S.I.) for the three compounds could thus be calculated as 952.74, 368.93, and 224.61, respectively. The results indicate that protoporphyrin IX and verteporfin strongly inhibit the infection of SARS-CoV-2 at nanomolar concentrations and have a wide safety range in vitro.

3.2. Effects of treatment timing on protoporphyrin IX and verteporfin’s inhibition of SARS-CoV-2 infection

We next analyzed the relationship between the antiviral effect and treatment timing of protoporphyrin IX and verteporfin. As shown in Fig. 2a, 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 were set up for each compound (group I–VIII). Based on the previous results, we selected the compound concentrations of 2.5 μmol/L,
and 1.25 μmol/L for protoporphyrin IX and verteporfin, respectively. At 48 h post infection, viral RNA level in the culture supernatant was quantified with qRT-PCR. The results showed that viral RNA levels of all the compound-treated groups (group I–VII of each compound in Fig. 2b, c) were significantly lower than that of the DMSO-treated group (group VIII in Fig. 2b, c). Importantly, pre-treatment alone resulted in the complete inhibition of SARS-CoV-2 infection (group IV in Fig. 2b, c). In addition, treatment of cells with protoporphyrin IX or verteporfin after viral infection showed a 64.6% or 95.4% reduction of viral RNA production, respectively (group VII in Fig. 2b, c). The results of immunofluorescence analysis on intracellular viral N protein were consistent with those of viral RNA measurement (Fig. 2d). Collectively, the results indicate that protoporphyrin IX and verteporfin can prevent SARS-CoV-2 infection and also suppress established SARS-CoV-2 infection to some degree.

The preventive effect was further tested by the pre-treatment of cells with either compound at a constant concentration and later infection with an increasing virus titer (Fig. 3a). As shown in Fig. 3b, c, no CPE or viral N protein expression was detected in protoporphyrin IX or verteporfin pre-treated cells even if the inoculated viral titer was raised by 16 folds (200 PFU to 3200 PFU). Pre-treatment of the virus with protoporphyrin IX (100 μmol/L) or verteporfin (20 μmol/L) did not affect viral infectivity (Fig. S1 online).
PD interacted with protoporphyrin IX, in which Phe\textsuperscript{40} interacted closely with the porphyrin-ring of protoporphyrin IX, the Trp\textsuperscript{69} formed aromatic H-bonds with the porphyrin-ring, Asp\textsuperscript{350} and Asp\textsuperscript{382} formed H-bonds with the compound. Similar results were observed in the interaction between verteporfin and PD, except that Asn\textsuperscript{51} formed additional H-bonds with the benzazole-like structure of verteporfin. Many of these PD residues are located in the region that interacts with SARS-CoV-2 S protein receptor binding domain (RBD), especially Phe\textsuperscript{40}, Ser\textsuperscript{43}, Ser\textsuperscript{44}, Trp\textsuperscript{349}, Gly\textsuperscript{352}, and Phe\textsuperscript{356}, which are very close to the key residues (Tyr\textsuperscript{41}, Gln\textsuperscript{42}, Lys\textsuperscript{353}, and Arg\textsuperscript{357}) that interact with the RBD \[22\]. As a negative control, docking DMSO with the region in Fig. 2.
PD failed, indicating that DMSO did not bind to PD. The results suggest that protoporphyrin IX and verteporfin might interact with ACE2.

We next used BLI assay to evaluate the binding between ACE2 and these two compounds. As shown in Fig. 4d, protoporphyrin IX and verteporfin indeed bind to ACE2-Fc. The KD of protoporphyrin IX and verteporfin binding to ACE2-Fc was calculated to be $3.897 \times 10^{-6}$ and $1.15 \times 10^{-4}$ mol/L, respectively. Therefore, structural simulation by molecular docking and direct drug-protein binding assay support the binding of both drugs to viral receptor ACE2.

3.4. Protoporphyrin IX and verteporfin interfere with the interaction between SARS-CoV-2 S protein and ACE2

Based on the molecular docking and the experimental data, both drugs likely interfere with the interaction between ACE2 and RBD via binding ACE2, which would impair viral entry. We first tested this possibility using a cell–cell fusion assay. HEK293T cells that express SARS-CoV-2 S protein served as the effector cells and those co-expressing human ACE2 and GFP as the target cells (Fig. 5a). The target cells were pre-treated with protoporphyrin IX (2.5 μmol/L), verteporfin (1.25 μmol/L) or DMSO for 1 h. After removal of the drug, the target and effector cells were co-cultured at 37 °C for 4 h. Fused cells with larger cell size than normal cells were observed in the DMSO-treated group but not in the protoporphyrin IX or verteporfin-treated group. The results indicate that protoporphyrin IX and verteporfin may block the interaction of ACE2 and viral S protein which is required for cell–cell fusion.

To confirm whether protoporphyrin IX and verteporfin inhibit SARS-CoV-2 infection by interfering with ACE2, SARS-CoV-2 spike (SARS-CoV-2-S)-pseudotyped virus based on replication-defective human immunodeficiency virus type-1 (HIV-1) was generated. Also, vesicular stomatitis virus G (VSV-G)-pseudotyped lentivirus was used as the negative control. HEK293T cells overexpressing hACE2 (HEK293T-hACE2) were pre-treated with protoporphyrin IX (2.5 μmol/L), verteporfin (1.25 μmol/L) or DMSO for 1 h, and then infected with these two types of pseudotyped viruses for 12 h. After 48 h, cells were lysed and detected NanoLuc luciferase activities. The results showed that protoporphyrin IX and verteporfin blocked the infection of SARS-CoV-2 pseudotyped HIV-1 virions, but not VSV-G (Fig. 5b).

To more directly demonstrate the interference of the compounds with the interaction of ACE2 to RBD, we designed an ELISA assay, in which protoporphyrin IX or verteporfin was added to the 96-well plate pre-coated with ACE2-Fc or His-RBD. After incubation, unbound drugs were washed away. His-RBD or ACE2-Fc was added to the drug-treated wells pre-coated with ACE2-Fc or His-RBD. The results showed that both drugs could prevent the binding of His-RBD to pre-coated ACE2-Fc, while they did not affect the binding of ACE2-Fc to pre-coated His-RBD (Fig. 5c). The data suggest that protoporphyrin IX and verteporfin most likely bind to ACE2 and interfere with the binding of RBD to ACE2, which is consistent with the results of the cell–cell fusion and molecular docking abovementioned.
3.5. Protoporphyrin IX and verteporfin effectively prevent SARS-CoV-2 infection in the mouse model expressing human ACE2

To investigate the effect of protoporphyrin IX and verteporfin on SARS-CoV-2 infection in vivo, a mouse model for SARS-CoV-2 infection was established. The BALB/c mice were first transduced intranasally with Ad5-hACE2 (5 × 10¹⁰ viral particles per mouse in 50 μL saline), then infected intranasally with SARS-CoV-2 (2 × 10⁵ PFU/mouse) in a total volume of 50 μL DMEM containing protoporphyrin IX (100 μmol/L), verteporfin (20 μmol/L) or 1% DMSO (Fig. 6a). Ad5-hACE2 inducing expression of hACE2 was confirmed in transduced HEK293T cells and mice by Western blot and qRT-PCR respectively (Fig. S2a, b online).

SARS-CoV-2-infected mice treated with 1% DMSO showed ruffled fur, hunching, loss of appetite, and difficulty in breathing beginning 2 days post-infection, while SARS-CoV-2-infected mice in the protoporphyrin IX and verteporfin groups were normal without obvious symptoms. All the mice were euthanized at day 3 post-infection of SARS-CoV-2 and lung tissues were collected. Human ACE2 expression in Ad5-hACE2 transduced mouse lung tissues was verified by immunochemical staining with the specific antibody, which lined along with the pulmonary epithelial cells in DMSO group, protoporphyrin IX and verteporfin treated groups (Fig. 6c). Much fewer cells expressed viral N protein in the protoporphyrin IX and verteporfin groups compared to the DMSO group (Fig. 6c). Viral RNA levels in the lung samples taken from the protoporphyrin IX and verteporfin groups were significantly lower than that from the DMSO group (4 log reduction) and were close to that from the negative control (Fig. 6b). The sections of lung tissues from the DMSO group displayed a variety of lesions including perivascular to interstitial inflammatory cell infiltrates and necrotic cell debris. In contrast, the sections of lung tissues from the protoporphyrin IX and verteporfin groups showed no obvious histopathological change, neither did those from the non-infected mice (the NC group) (Fig. 6d). These results indicate that protoporphyrin IX and verteporfin also effectively inhibit SARS-CoV-2 infection in the mouse model.

4. Discussion and conclusion

Protoporphyrin IX and verteporfin have been approved and used in the treatment of human diseases. Protoporphyrin IX is the final intermediate in the protoporphyrin IX iron complex (heme) biosynthetic pathway [24]. Heme is an important cofactor for oxygen transfer and oxygen storage [25] and is a constituent of hemoproteins which play a variety of roles in cellular metabolism [26]. The light-activable photodynamic effect of protoporphyrin IX was used for cancer diagnosis [27] and approved by FDA for the treatment of bronchial and esophageal cancers and early malignant lesions of the skin, bladder, breast, stomach, and oral cavity [28,29]. Verteporfin was approved for the treatment of age-related macular degeneration [30]. The potential of verteporfin for the treatment of cancers, such as prostatic cancer, breast cancer, and pancreatic ductal adenocarcinoma has been investigated [31]. Verteporfin also has been reported to inhibit autophagy at an early stage by suppressing autophagosome formation [32].

A study of the clinical pharmacokinetics of verteporfin showed that in healthy volunteers who were infused with verteporfin 6 to 14 mg/m² of body surface area over 1.5 to 45 min, Cmax (peak concentration) of verteporfin was 1.24–2.74 μg/mL [33]. The Cmax value is approximately 2.4 to 5.2-fold higher than the EC90 value.
that was obtained in this study (0.73 μmol/L, i.e., 0.52 μg/mL). Protoporphyrin IX is the metabolite of 5-aminolevulinic acid (5-ALA) in human body. After administration of 5-ALA 2 mg/kg p.o., the average Cmax of protoporphyrin IX was 27.44 μg/mL [34], which is about 20-fold higher than the EC90 value in this study (2.45 μmol/L, i.e., 1.38 μg/mL). These data indicate that the two drugs can reach a plasma concentration that is much higher than the in vitro effective antiviral concentration. In the mouse model in this study, protoporphyrin IX and verteporfin exhibited effective inhibition of SARS-CoV-2 infection without notable toxicity.

Both protoporphyrin IX and verteporfin have a porphyrin ring structure formed by four pyrrole rings. It is most likely that they share a similar mechanism of antiviral action. In the experiment when either drug was added prior to viral infection, viral RNA production was inhibited even if the relevant drug was not added in the later virus infection and post-infection stages (group IV in Fig. 2b, c). Furthermore, increasing viral titer did not relieve the inhibition of the drugs added before viral infection (Fig. 2b, c). A logical hypothesis is that both drugs act by inhibiting an early step in viral infection. Structural simulation by molecular docking and direct drug-protein binding assay support the binding of both drugs to viral receptor ACE2. Several residues on ACE2 predicted to interact with the drugs are very close to the key residues that interact with the RBD of viral S protein. Based on the molecular docking and the experimental data, both drugs likely interfere with the interaction between ACE2 and RBD via binding ACE2, which would impair viral entry. The proposed mechanism was supported by the blocking effect of both drugs on the cell-cell fusion mediated by the interaction of ACE2 and viral S protein and by more direct evidence that came from the ELISA binding assay. To our knowledge, this is the first report on small compounds that target the interaction between SARS-CoV-2 S protein and ACE2. The study suggests a new venue for the development of small molecule-based entry inhibitor against SARS-CoV-2. Furthermore, it may be a potential strategy for combating SARS-CoV-2 infections to use the compounds inhibiting virus entry in combination with the drugs acting intracellularly, such as the RdRp inhibitor remdesivir.

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

In conclusion, this study has discovered protoporphyrin IX and verteporfin as potent antiviral agents against SARS-CoV-2 infection in vitro and in the hACE2 mouse model. The effective antiviral concentrations of these drugs are in the nanomolar concentration range and the selectivity indices are greater than 200, indicating a broad margin of safety. Both compounds bind viral receptor ACE2, thereby disturbing the interaction between ACE2 and the receptor-binding domain of viral S protein. To our knowledge, this is the first report on small compounds that target the interaction between SARS-CoV-2 S protein and ACE2, which sheds new light on the molecular mechanisms of viral entry and suggests potential therapeutic strategies.
on developing novel chemoprophylaxis and chemotherapy against SARS-CoV-2. The antiviral efficacy of protoporphyrin IX and verteporfin *in vivo* will need clinical evaluation.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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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.

**Appendix A. Supplementary materials**

Supplementary materials to this article can be found online at [https://doi.org/10.1016/j.scib.2020.12.005](https://doi.org/10.1016/j.scib.2020.12.005).

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