Chloroquine and hydroxychloroquine as ACE2 blockers to inhibit viropexis of COVID-19 Spike pseudotype virus

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Abstract

The pandemic coronavirus COVID-19 affected global health from the end of 2019 to 2020 and may challenge global health in the future. There have been reports of Chloroquine (CQ) and Hydroxychloroquine (HCQ) used in clinical treatment. In our study, we used CCK-8 stain, flow cytometry and immunofluorescent stain to evaluated the toxicity and autophagy of CQ and HCQ respectively on ACE2 high expressed HEK293T cells ($ACE2^{hi}$ cells). We further analysied the binding character of CQ and HCQ to ACE2 by molecular docking, surface plasmon resonance (SPR) assays and molecule docking, COVID-19 spike pseudotype virus was also taken to investigate the suppression viropexis effect of CQ and HCQ. Results showed that HCQ is slightly more toxic to $ACE2^{hi}$ cells than CQ, both CQ and HCQ could bind to ACE2, and they also exhibit equivalent suppression effect for the entrance of COVID-19 Spike pseudotype virus into $ACE2^{hi}$ cells. Our finding provides a theoretical and experimental basis for the clinical treatment of CQ and HCQ for COVID-19.

Key words: Chloroquine, Hydroxychloroquine, COVID-19, ACE2

Introduction

Chloroquine (CQ) and hydroxychloroquine (HCQ) are effective antimalarial drugs [1]. The only difference between their chemical structure is that HCQ is formed when a methyl group on CQ is replaced with a hydroxymethyl group, thereby resulting in HCQ absorbed in the human gastrointestinal tract faster and distributed in the body broader [2, 3]. Since 2004, reports on anti-viruses of CQ and HCQ have gradually increased. For example, CQ can inhibit the replication of SARS and HIV viruses *in vitro*, and it also has significant inhibitory effects on Borna, avian leukemia, Zika, etc. [4-6]. Therefore, CQ and HCQ have been considered as broad-spectrum antiviral drugs.

Since the outbreak of the COVID-19 in 2020, there have been reports of CQ and HCQ used in clinical treatment. For example, clinical trial reports issued by 10 hospitals in China indicate that CQ may shorten the duration of the disease [7, 8]. A small non-random clinical trial in France showed that HCQ combined with azithromycin has a significant therapeutic effect [8], and it has been reported that CQ can effectively inhibit the deterioration of new coronary pneumonia, improve lung imaging performance, promote viral reversion and shorten the time of disease onset [9]. However, it has also been reported that the combination of HCQ and azithromycin in 11 patients with severe COVID-19 has not achieved a positive clinical effect [10]. Another observational study showed that HCQ had no effect on the intubation or composite endpoint of death [9]. There is still a lack of randomized controlled trials of HCQ in the treatment of COVID-19 patients.

At present, it is generally believed that the COVID-19 virus enters the host cell by binding to ACE2 on the plasma membrane of the cells, causing infection [11-13]. Therefore, blocking or antagonizing the ACE2 signaling pathway of susceptible cells should be beneficial to prevent COVID-19 virus infection [14]. Abdelli I *et al*. conducted a molecular docking experiment between CQ and ACE2 and found that CQ binds to ACE2 with low binding energy and forming a stable complex system [15]. Studies have shown that ACE2 is a type I membrane-bound glycoprotein composed of 805 amino acids, mainly distributed in vascular endothelial cells, alveolar and renal tubular epithelial cells, and profoundly expresses in tissues such as heart, kidney, retina, and

gastrointestinal tissue [16]. Flow cytometry and immunoprecipitation studies have shown that during alveolar epithelial cell infection with SARS virus, CQ and HCQ can prevent the binding of viral S protein to ACE2 by disrupting ACE2 terminal glycosylation [17, 18]. Virus infection experiments *in vitro* confirmed that CQ could reduce the infection of cells by COVID-19 virus, and play a role in both the entry stage and the post-entry stage of viral infection. At the same time, HCQ can effectively reduce the COVID-19 virus copy number [19].

Recently, there have also been reports of clinical adverse reactions of HCQ and CQ. A clinical trial of 90 COVID-19 patients in the United States showed that COVID-19 infected patients receiving HCQ treatment had a higher risk of prolonged QTc, suggesting a risk of cardiotoxicity [20]. At the same time, in a clinical trial of 197 COVID-19 patients in China, CQ showed a significant therapeutic effect without severe adverse reactions [21]. The above evidence suggests that the adverse effects of CQ treatment of COVID-19 may be lower than that of HCQ. The curative effect and mechanism of the anti-COVID-19 virus of CQ and HCQ are still controversial.

In this study, we have found that CQ and HCQ can antagonize ACE2 receptor and inhibit the entry of COVID-19 Spike pseudotype virus into ACE2 expressed HEK293T cells ($ACE2^{hi}$ cells).

Results and discussion

The effect of CQ and HCQ on $ACE2^{hi}$ cells viability

The expression of ACE2 protein in human lung and bronchial-related cells was higher than that of HEK293T cells. The expression of ACE2 protein in $ACE2^{hi}$ cells was significantly higher than that of other cells, indicating that $ACE2^{hi}$ cells were successfully constructed. It has been reported that AT2 cells expressed the highest ACE2 receptors in lung and bronchial cells [22]. We also confirmed the high expression of ACE2 protein in AT2 cells, and found that EOL-1 cells express ACE2 protein for the first time (Figure 1A).

As it shows in Figure 1B, CQ and HCQ had no significant effect on the activity of $ACE2^{hi}$ cells when the concentration was less than 50 μM, and the survival rate of $ACE2^{hi}$ cells could be reduced in a dose-dependent manner when the concentration was above 50 μM. The inhibition of HCQ on the activity of $ACE2^{hi}$ cells was more significant than that of CQ. It can be concluded that the toxicity of HCQ was higher than CQ on $ACE2^{hi}$ cells at different time points at the same concentrations (Figure 1C). When the concentration was 20 μM, the statistical difference appeared at 6 h. Ca^{2+} is an essential second messenger in several cell pathways, as shown in Figure 1D, whether CQ or HCQ rarely affect Ca^{2+} influx change in $ACE2^{hi}$ cells. Figure 1E shows that within 24 h, the concentrations of both drugs had no significant effect on apoptosis.

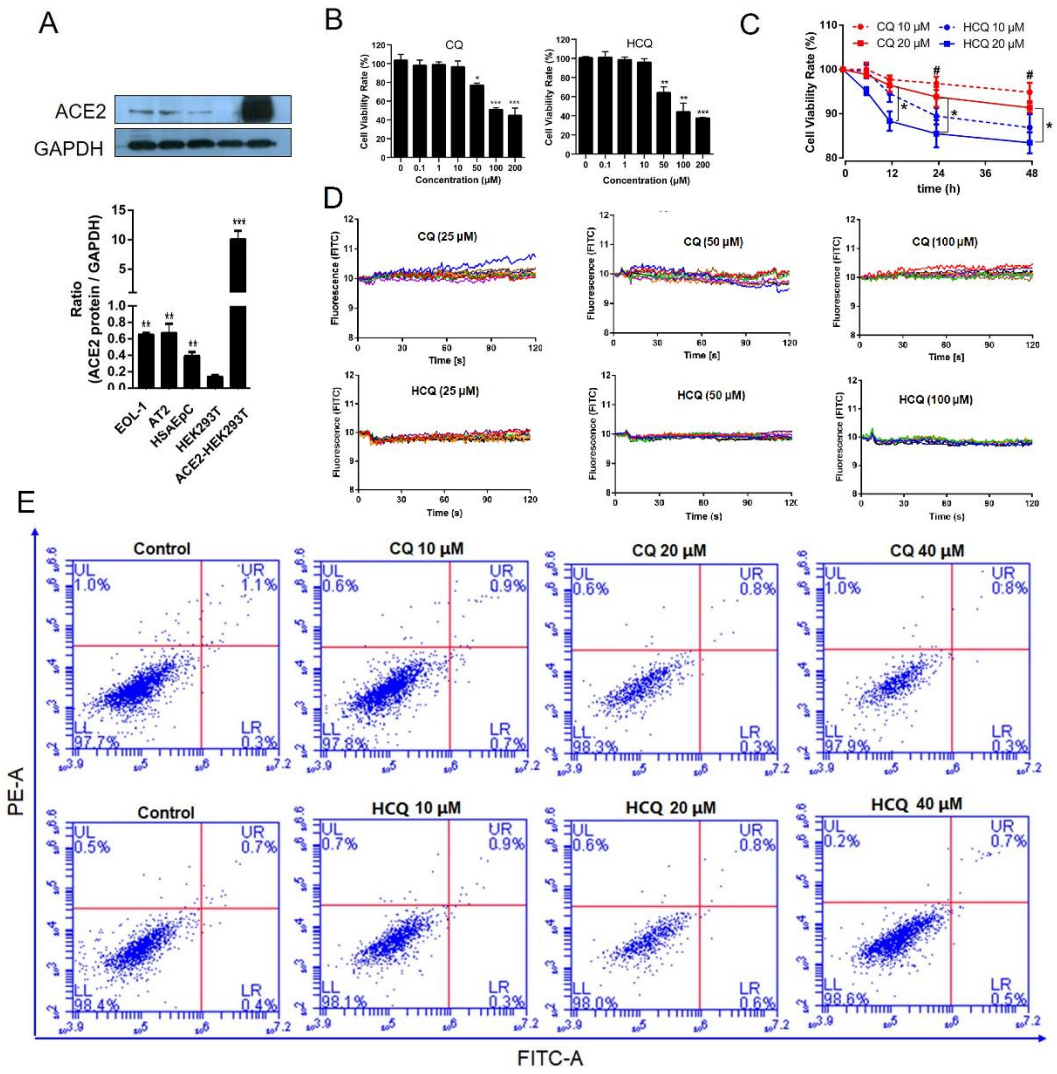

Figure 1. The effect of cell viability on ACE2 cell by CQ and HCQ. A. Western blotting analysis of the expression levels of ACE2 protein in EOL-1 cells, AT2 cells, HSAEpC cells, and ACE2^{hi} cells. B. Viability of ACE2 cells treated with CQ or HCQ for 24 h. C. The toxicity of HCQ and CQ on ACE2 cells at different time points. D. Calcium (Ca^{2+}) flux change in ACE2^{hi} cells. E. The apoptosis of ACE2^{hi} cells treated with CQ or HCQ for 24 h. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with HEK293T, or concentration was 0, or HCQ 20 μM, $^{\#}p < 0.05$ compared with HCQ 10 μM at corresponding time points)

CQ and HCQ induces LC3-mediated autophagy in ACE2 cells

Autophagosome is a spherical structure and as an essential marker for the autophagy cells, and LC3 is known as a stable association with the membrane of autophagosomes. LC3 includes two forms (LC3-I and LC3-II), LC3-I is found in the cytoplasm, while LC3-II is membrane-bound and converted from LC3-I to initiate formation and lengthening of the autophagosome. Therefore, to investigate the effects of CQ and HCQ induced autophagy on ACE2^{hi} cells, the FITC-LC3, TRITC-Phalloidin and DAPI staining were used. Activating lysosomal (Green) and Filamentous actin (F-actin, Red) was detected after stimulation with 10, 20, 40 μM of CQ and HCQ in ACE2 cells (Figure 2A).

We further pretreated ACE2^{hi} cells with CQ and HCQ, and measured the expression of ACE2^{hi} cells autophagy proteins LC3-I and LC3-II by Western blotting. We found that the expression level of LC3 and LC3-II increased in CQ and HCQ-treated ACE2^{hi} cells (Figure 2B). The protein level of the LC3-II/LC3-I ratio was significantly increased compared to the control group (Figure 2B). All of these results suggested that CQ and HCQ could induce LC3-mediated autophagy in ACE2^{hi} cells.

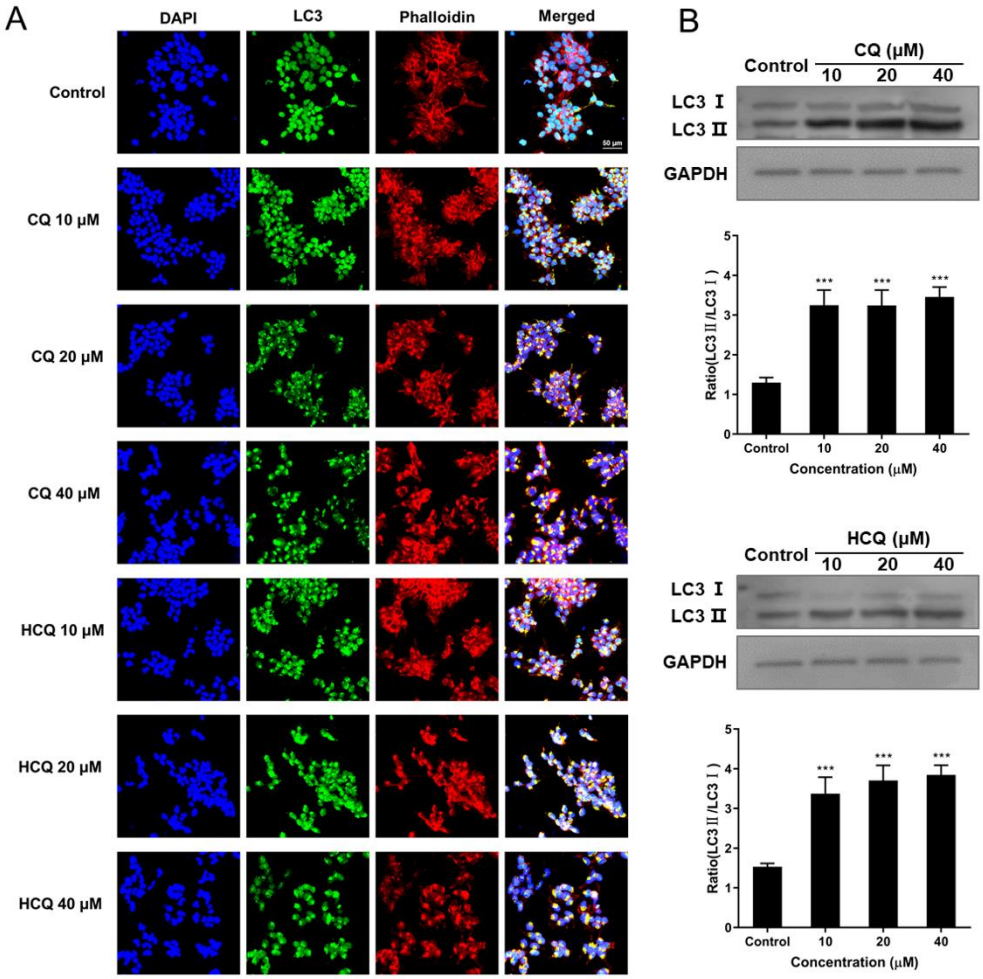

Figure 2. Effects of CQ and HCQ on the LC3 levels of ACE2^{hi} cells. ACE^{hi} cells were treated with different doses of CQ or HCQ for 24 h. (A) Effects of CQ and HCQ on the fluorescent staining of FITC-LC3 and TRITC-Phalloidin in ACE^{hi} cells. (B) The representative blots of autophagy proteins and the changes of LC3-II/LC3-I ratio. Data are presented as mean ± S.D. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with control.

The binding character of CQ and HCQ with ACE2

Since it is believed that the COVID-19 virus enters the host cell by binding to the ACE2 receptor, we focused on whether CQ or HCQ could bind with ACE2. The virtual molecular docking test was performed to investigate the binding character of CQ and HCQ with ACE2. Figure 3A shows that both CQ and HCQ can bind to R393 and D350 (both in green) of ACE2 with their quinoline and imino group. Besides, due to the replacement of a methyl group by a hydroxymethyl group, HCQ can form two additional hydrogen bonds with D350 and S44 (in red).We further used SPR to confirm the binding between CQ or HCQ and ACE2. The binding constant K_D of these two compounds and ACE2 protein were (3.63±0.40)e^{-7} M and (1.02±0.38)e^{-8} M respectively (Figure 3B).

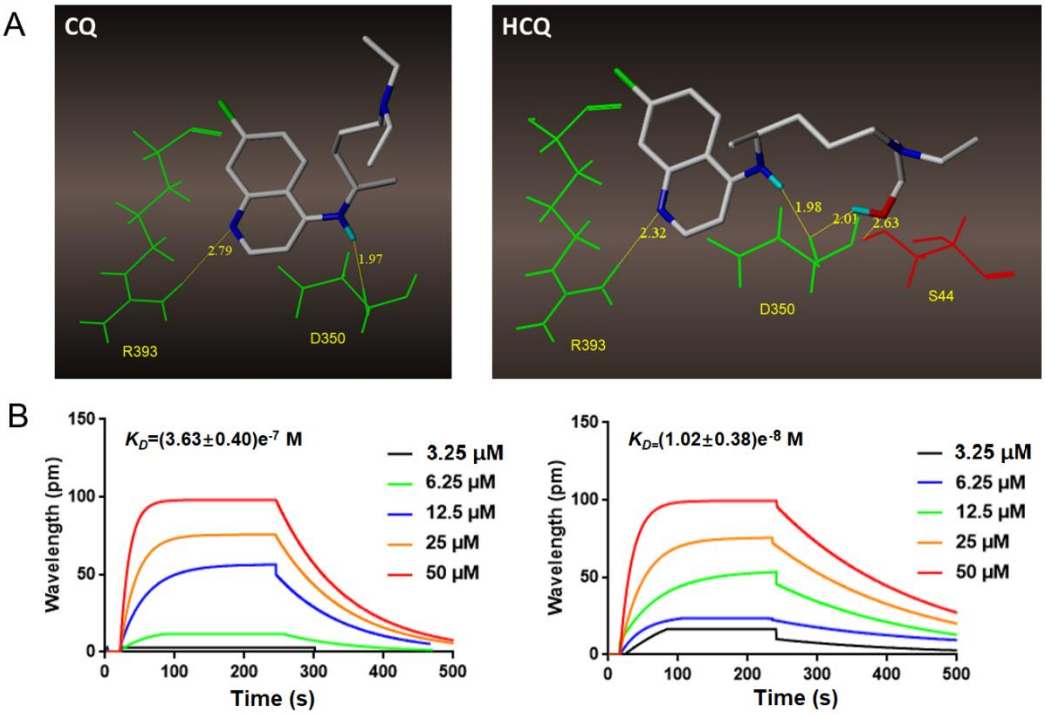

Figure 3. The binding character of CQ and HCQ with ACE2. A. SPR analysis of CQ or HCQ and ACE2. B. The molecular docking of CQ and HCQ with ACE2.

CQ and HCQ suppressed the entrance of COVID-19 Spike pseudotype virus into ACE^{hi} cells

ACE^{hi} cells infected only by COVID-19 Spike pseudotype virus were considered as control, and luciferase luminescence value of control was defined as 1. Under the treatment of 0.625 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM, and 20 μM CQ, the COVID-19 Spike pseudotype virus entrance ratio were reduced to 0.86±0.11, 0.69±0.13, 0.62±0.19, 0.56±0.13, 0.44±0.18, and 0.23±0.10, and treated by the same dosage of HCQ, the ratios were 0.77±0.07, 0.58±0.12, 0.53±0.09, 0.44±0.08, 0.35±0.05, and 0.29±0.05 (Figure 4). The ability of the COVID-19 Spike pseudotype virus to enter the ACE2 cells were both reduced significantly.

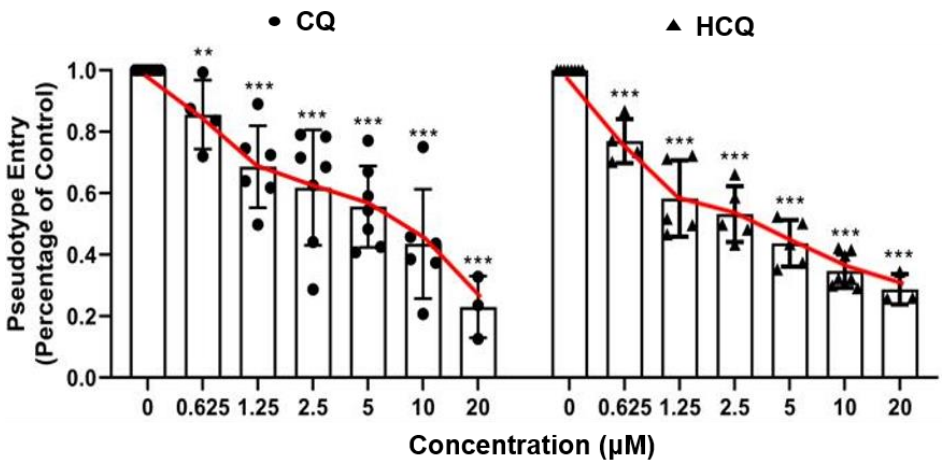

Figure 4. The effect of CQ and HCQ on the entrance of COVID-19 Spike pseudotype virus into ACE2 cells. Data are presented as mean ± S.D. *p<0.05, **p<0.01, ***p<0.001 compared with group 0.

Discussion

COVID-19 is globally prevalent in 2020, and there are currently no specific drugs against the virus. ACE2 is the target receptor of COVID-19 virus, chloroquine and hydroxychloroquine have shown certain efficacy in clinical use. This study confirmed that both CQ and HCQ can interact with ACE2 and inhibit the entry of pseudoviruses.

CQ has been reported to be an endocytosis blocking agent, and can inhibit ZIKA virus infection in different cell models[23], similarly, COVID-19 was driven by endocytosis after binding to ACE2, and was blocked by CQ and HCQ. For this experiment, we demonstrated that CQ reduced the number of COVID-19 Spike pseudotype virus into ACE2 cells without cytotoxic effects. Akram et al. reported that CQ was toxic towards hepatocytes, which explained its hepatotoxicity during case reports[24], and we showed that CQ and HCQ had cytotoxic effects on $ACE2^{hi}$ cells at certain concentrations. CQ and HCQ were reported to be autophagic flux inhibitors, and Mario et al. demonstrated the mechanism of this process was to impair autophagosome fusion with lysosomesrather[25]. Therefore, we speculated that the increased amounts of LC3-II was due to the inhibition of autophagosome fusion with lysosomes.

CQ and HCQ possess structural difference, and the change of hydroxymethyl group in HCQ makes it form additional hydrogen bonds with ACE2 according to the molecule docking result. These different modes may finally reveal different bioactivity and affinity of CQ and HCQ on ACE2. Based on the above results, we further analyzed the binding strength of CQ and HCQ to the ACE2 protein, and found that both CQ and HCQ have good binding to the ACE2 protein. Further experiments confirmed that both CQ and HCQ have abilities to suppress the entrance of COVID-19 Spike pseudotype virus into $ACE2^{hi}$ cells. The COVID-19 virus uses ACE2 for cellular entry. Thus CQ and HCQ could be good inhibitors to block COVID-19 infection of human cells expression ACE2.But the difference in the inhibitory effect of these two drugs on the COVID-19 virus needs further study.

Our study revealed that CQ and HCQ as ACE2 blockers to inhibit COVID-19 pseudovirus enter into cells, which provide new sights on COVID treatment and further control.

Methods

Drugs and reagents

Dulbecco's Modification of Eagle's Medium (DMEM) high glucose (Cat. No. SH30022.01) and fetal bovine serum(FBS) (Cat. No. 16140071) was obtained from HyClone (Logan, UT, USA). Penicillin–streptomycin solution was purchased from Xi'an Hat Biotechnology Co., Ltd (Xi'an, Shaanxi, China). Protease inhibitor and phosphatase inhibitor cocktails were purchased from Roche Diagnostic (Mannheim, Germany). 5×loading buffer was purchased from Thermo Fisher Scientific,lnc (MA,USA), SDS-PAGE was procured from Pioneer Biotech Co.,Ltd (Xi’an, China). Polyvinylidene fluoride membranes were procured from Hangzhou Microna Membrane Technology Co., Ltd (Hangzhou, China). Tween-20 was procured from Shaanxi Pioneer Biotech Co., Ltd (Xi’an, Shaanxi, China). Enhanced chemiluminescence (ECL) kit was purchased from Proteintech Group,Lnc (Rosemont, USA). Annexin V-FITC/PI Apoptosis Detection Kit (Cat. No. A005-3) and Cell Counting Kit was purchased from 7Sea Pharmatech Co., Ltd (Shanghai, China).

Cell lines

HEK293T cells, HSAEpC cells (human airway epithelial cells), AT2 cells (alveolar type II epithelial cells) were kept in DMEM high glucose medium containing 10% FBS and 1% penicillin-streptomycin; EOL-1 cells (human eosinophilic Leukemia cells) were kept in 1640 medium containing 10% FBS and 1% penicillin-streptomycin; ACE2 high expressing HEK293T cells ($ACE2^{hi}$ cells) were kept in DMEM high glucose medium containing 10% FBS, 1% penicillin-streptomycin,4μg/mL puromycin and cultured at 37°C in a 5% CO_2 incubator.

Cytotoxicity assay

Cell viability was determined using Cell Counting Kit assays (7 Sea, China). ACE2 high expression cells were seeded into 96-well plates at a density of 5×10^3 cells per well and then treated with different concentrations of CQ or HCQ (0, 0.1, 1, 10, 50, 100, 200, 300and 400 μM) for 24 h. Next, 10 μL of Cell Counting Kit solution was added to each well followed by the incubation for 2 h. Further, the relative cell viability was assessed by the detection of the absorbance at 450 nm using a microplate reader (Bio-Rad, Carlsbad, CA, USA). The survival rate of $ACE2^{hi}$ cells was calculated as the following formula:

$[(OD_{Treated} - OD_{Blank}) / (OD_{Control} - OD_{Blank})] \times 100\%$. The effects of HCQ and CQ at low concentrations (10, 20 μM) on $ACE2^{hi}$ cell viability at different time points (6, 12, 24, 48 h) were also investigated.

Apoptosis assay

$ACE2^{hi}$ cells were planted in a six-well plate And treated different concentrations of CQ and HCQ (0, 10, 20, 40 M). After incubation for 24 hours, the cell culture medium was moved to the appropriate centrifuge tube, and the cells were washed with PBS. The cells were digested by trypsin, and the collected cell culture medium was added. Then gently blown cells evenly and transferred to the centrifuge tube, and centrifuged at 500 g for 5 minutes. After discarding the supernatant, the cells were resuspended with 1 mL PBS and counted (the number of cells was no less than 10^5). The cells were centrifuged at 500 g for 5 minutes, resuspended with 400 μL of 1 × Binding Buffer, and centrifuged again. Five μL Annexin V-FITC was added into the cells, incubated at room temperature in the dark for 15 minutes. Next, 10 μL PI was added into the cells, incubate in an ice bath for 5 minutes. Detection should be performed within 30 minutes. The excitation wavelength of the flow cytometer (Accuri C6 Plus, BD Biosciences, Beijing, China) was 488 nm, and the emission wavelength was 530 nm to detect FITC, while PI was detected at 575 nm. Normal cells had low fluorescence intensity. However, apoptotic cells had strong green fluorescence, and necrotic cells had double staining with green and red fluorescence.

Western blotting

Total proteins from different cells were extracted in ice-cold condition by using RIPA lysis buffer containing 10% protease inhibitor and a phosphatase inhibitor cocktail. After 30 min, insoluble protein lysate was removed by centrifuging the samples at 12000 g for 10 min at 4°C. The protein concentration was determined by using a BCA Protein Quantification kit according to the manufacturer's instructions. The protein in the cell lysates was denatured by boiling the samples for 5 min with a 5 × loading sample buffer. Furthermore, equal amounts of protein were separated on a 10% gel using SDS-PAGE. The separated proteins were transferred onto polyvinylidene fluoride

membranes and blocked by constant stirring with 5% non-fat milk in Tris-buffered saline containing Tween-20. The membranes were then incubated overnight at 4°C with the following primary antibodies: anti-ACE2 (1:500, EPR4435, Abcam), anti-LC3 (1:1000, #2775, Cell Signaling Technology [CST]) and anti-GAPDH (1:2000, a#2118, CST). The membranes were then washed thrice with TBST at every 10 min interval and then incubated with secondary antibodies (at a dilution of 1:20,000 in TBST) for 1 h at 37°C. The membranes were again washed three times with TBST for 10 min and developed using an enhanced chemi-luminescence (ECL) kit. A Lane 1 DTM transilluminator (Beijing Creation Science Co., Ltd., Beijing, China) was used to capture the images of the developed blots, and Image-Pro Plus 5.1 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to quantify the protein levels.

The Immunofluorescence assays

The cell slides were prepared by using 24 mm×24 mm coverslips, which were sterilized by 75% ethanol and coated by polylysine. 2×10^3 ACE2 cells were seeded on the coverslip and cultured in a six-well plate. The plate was incubated overnight at 37 °C with 5 % CO_2. After incubation, the cells were treated with 10 μM, 20 μM or 40 μM CQ and HCQ prepared in serum-free DMEM for 24 h, respectively. Then the cells were fixed with 4 % paraformaldehyde for 10 minutes at room temperature. After washing with PBS, the cells were incubated with 0.5 % Triton X-100 for 5 min. Then the cells were incubated with 5 % BSA solution for 1 h at room temperature after 3 times washing with PBS. The cells were then continuously incubated with LC3 primary antibody at 37°C for 3 h, and the fluorescent secondary antibody at room temperature for 2 h. After the incubation of LC3 antibody, the cells were treated with TRITC-Phalloidin to stain for 30 min at room temperature. At last, the cells were mounted by 50 μL of DAPI-containing anti-fluorescence quenching reagent. All the cells were observed by a laser confocal fluorescence microscope.

Docking Studies

Molecular docking studies were carried out with SYBYL-X 2.0 version. The small molecules and the X-ray crystal structure of protein (PDB code: 6M0J) were imported. Water molecules were removed and hydrogen was added. Tripos force field and Pullman charge were applied to minimize. CQ and HCQ were depicted by Sybyl/Sketch module (Tripos Inc.), optimized by Powell's method with Tripos force field with convergence criterion at 0.005 kcal/(Å mol), and assigned with Gasteiger–Hückel method.

Surface plasmon resonance assay

For assessment of surface plasmon resonance (SPR), ACE2 protein with a 6-his tag (30 μg/mL) was fixed on a Carboxyl sensor chip (Nicoya, Canada) by capture-coupling, then CQ or HCQ at 6.25, 12.5, 25, 50 and 100 μM was injected sequentially into the chamber in PBS running buffer. The interaction of ACE2 with the fixed small molecules was detected by using Open SPRTM (Nicoya Lifesciences, Waterloo, Canada) at 25°C. The binding time and disassociation time were both 250 s, the flow rate was 20 μL/s, and the chip was regenerated with hydrochloric acid (pH 2.0). A one-to-one diffusion-corrected model was fitted to the wavelength shifts corresponding to the varied drug concentration. The data were retrieved and analyzed by using TraceDrawer.

The detection of COVID-19 Spike pseudotype virus Entry into ACE2 cells

5×10^4 of $ACE2^{hi}$ cells in 50 μL DMEM per well were seeded into white 96-well plates. The cells were cultured in a 37 °C incubator containing 5% CO_2 for 2 h. 25 μL medium was aspirated carefully from 96 wells, 25 μL medium containing corresponding dose of the medicine was added and incubated for 2 h. Then 5 μL of COVID-19 Spike pseudotype virus was added (Sino Biological, PSC001), and incubated in the 37 °C incubator containing 5% CO_2 for 4 h followed with adding 100 μL of complemented DMEM per well. After 6-8 h of further infection, the culture medium containing the virus was sucked away and replaced by 200 μL of fresh DMEM, and incubated continuously at 37°C for 48 h, the culture medium was aspirated and 20 μL of cell lysate was add from the Luciferase Assay System (Promega, E1500) to each well, then 100 μL of luminescence solution was added to wells before the luciferase luminescence detection, chemiluminescence was detected by a microplate reader under 560 nm, the exposure time was 1 s.

Conflicts of Interest

The authors declare no competing financial interest.

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