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# Antiviral drugs suppress infection of 2019-nCoV spike pseudotyped virus by interacting with ACE2 protein

Jue Wang | Yongjing Zhang | Shiling Hu | Haoyun Bai | Zhuoyin Xue | Yanhong Liu | Weina Ma [ORCID]

School of Pharmacy, Health Science Center, Xi'an Jiaotong University, Xi'an, Shaanxi, China

**Correspondence**
Weina Ma, School of Pharmacy, Health Science Center, Xi'an Jiaotong University, Xi'an Shaanxi 710061, China.
Email: maweina2015@xjtu.edu.cn

## Abstract

The outbreak of coronavirus disease 2019 (COVID-19) has induced a large number of deaths worldwide. Angiotensin-converting enzyme 2 (ACE2) is the entry receptor for the 2019 novel coronavirus (2019-nCoV) to infect the host cells. Therefore, ACE2 may be an important target for the prevention and treatment of COVID-19. The aim of this study was to investigate the inhibition effect of valaciclovir hydrochloride (VACV), zidovudine (ZDV), saquinavir (SQV), and efavirenz (EFV) on 2019-nCoV infection. The results of molecule docking and surface plasmon resonance showed that VACV, ZDV, SQV, and EFV could bind to ACE2 protein, with the $K_D$ value of $(4.33 \pm 0.09)\ e^{-8}$, $(6.29 \pm 1.12)\ e^{-6}$, $(2.37 \pm 0.59)\ e^{-5}$, and $(4.85 \pm 1.57)\ e^{-5}$ M, respectively. But only ZDV and EFV prevent the 2019-nCoV spike pseudotyped virus to enter ACE2-HEK293T cells with an $EC_{50}$ value of $4.30 \pm 1.46$ and $3.92 \pm 1.36\ \mu M$, respectively. ZDV and EFV also have a synergistic effect on preventing entry of virus into cells. In conclusion, ZDV and EFV suppress 2019-nCoV infection of ACE2-HEK293T cells by interacting with ACE2.

**KEYWORDS**
2019-nCoV, ACE2, efavirenz, zidovudine

## 1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19) broke out in Hubei, China at the end of December 2019 and has spread immediately all over the world.[1] COVID-19 was caused by the 2019 novel coronavirus (2019-nCoV), a novel coronavirus that infected more than 70 million persons and caused 1.74 million deaths by the end of December 2020.[2] The pathogenesis and transmission mechanisms of COVID-19 have not been fully understood till now with the result that COVID-19 patients are empirically administered symptomatic treatments.[3] At present, a great deal of research is focused on the effective prevention and treatment against COVID-19. The current potential drugs under development for COVID-19 include antiviral drugs, convalescent plasma, and angiotensin-converting enzyme 2

(ACE2) blockers.[3,4] ACE2 is widely expressed in the lungs, the primary target organs of 2019-nCoV, and especially highly expressed in human lung epithelial cells. 2019-nCoV enters the host cells by using its spike (S) protein binding with ACE2.[5] Furthermore, ACE2 is associated with organ injury in COVID-19, such as acute cardiac injury and digestive system injury.[6] Therefore, ACE2 is suggested to be a potential target for the prevention and treatment of COVID-19.[7]

Currently, selecting potential antiviral drugs against 2019-nCoV from drugs already in the market may be an effective option to combat COVID-19.[8] Some antiviral drugs have shown effective ability to inhibit 2019-nCoV in vitro, such as remdesivir, lopinavir/ritonavir, and chloroquine (CQ).[9] Remdesivir has also been proven to have antiviral activity against 2019-nCoV in the United States.[10] In the present study, we aim to investigate the preventive effect of four

antiretroviral drugs on COVID-19 infection, including zidovudine (ZDV, a nucleoside analog),[11] efavirenz (EFV, a nonnucleoside reverse transcriptase inhibitor),[12] saquinavir (SQV, a protease inhibitor),[13] and valaciclovir hydrochloride (VACV, an antiherpes simplex virus drug).[14] Despite the different mechanisms of which these drugs originally acted, our research mainly focused on whether they could have an affinity for ACE2. Of these four drugs, only ZDV has been reported to have high binding activities with ACE2 and 2019-nCoV S protein.[8] However, there are no studies on its antiviral effect against 2019-nCoV.

In this study, we found that VACV, ZDV, SQV, and EFV have good binding activities with ACE2. ZDV and EFV suppress 2019-nCoV S pseudotyped virus infection of ACE2-HEK293T cells by binding with the allosteric site of ACE2.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials and reagents

VACV, ZDV, SQV, and EFV (purity ≥ 98%) were from Yuanye Bio-Technology Co., Ltd. CQ (purity ≥ 98%) was from Energy Chemical. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Thermo Fisher Scientific Inc. Annexin V-FITC/PI Apoptosis Detection Kit and puromycin dihydrochloride were from Beyotime Biotechnology. Cell Counting Kit assays were from Abbkine Scientific Co., Ltd. The 2019-nCoV spike pseudotyped virus was obtained from Sino Biological Inc. Luciferase Assay System was procured from Promega Corporation.

### 2.2 | Cell culture

ACE2-HEK293T cells were constructed by Genomeditech and were cultured in DMEM medium supplemented with 10% FBS, 1% penicillin–streptomycin, and 4 μg/ml puromycin in 5% $CO_2$ at 37°C.

### 2.3 | Cell viability analysis

The cytotoxic effect of these four drugs was detected as previously described. In brief, ACE2-HEK293T cells ($5 \times 10^3$ cells/well) were seeded in 96-well plates and treated with VACV, ZDV, SQV, and EFV (1.56–400 μM). After culturing for 12, 24, or 48 h, the cells were incubated with Cell Counting Kit solution for 2 h. The absorbance at 450 nm by a microplate reader (Bio Tek Instruments Inc.).

### 2.4 | Apoptosis detection

According to the instructions, ACE2-HEK293T cells were seeded in six-well plates and treated with VACV, ZDV, SQV, and EFV (0, 5, 10, and 20 μM) for 24 h. The cells were collected and washed twice with phosphate buffer saline (2000 rpm, 5 min). Then the cells were suspended with a binding buffer solution (500 μl) and incubated with the buffer solution added with Annexin V-fluorescein isothiocyanate (FITC; 1 μl). Later, propidium iodide (PI, 5 μl) was added to the cells andincubated for 5 min in a dark place at room temperature. Apoptosis was detected by flow cytometry (Ex = 488 nm, Em = 530 nm). Annexin V-FITC fluorescence signal was green and the PI fluorescence signal was red.

### 2.5 | Molecular docking studies

To study the receptor–analyte interactions, molecular docking methods were conducted using the Sybyl-X program package (New Tripos International). The docking model of ACE2 protein was obtained from the PDB protein data bank (PDB code: 6M0J). The molecular structures of VACV, ZDV, SQV, and EFV were from the Sybyl/Sketch module (Tripos Inc.).

### 2.6 | Surface plasmon resonance (SPR) assay

Measurements were performed using Open SPRTM (Nicoya Lifesciences). Nitrilotriacetic acid (NTA) sensor chip was loaded into the open surface plasmon resonance (SPR) instrument and activated with $NiCl_2$ solution (40 mM). His-tagged ACE2 protein were diluted in 2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (dissolve 10 mM HEPES, 150 mM NaCl, 0.005%–0.05% Tween 20 in deionized $H_2O$ at pH 7.4) Prepared 200 μL solution of His-tagged ACE2 protein in HEPES buffer, and loaded it into the injection port and fixed it on the NTA sensor chip. The pump speed is 20 μl/min, VACV, ZDV, SQV, and EFV at 5, 10, 20, and 40 μM was injected in order. The binding time and disassociation time were both 250 s, and the response graph of analyte-protein interactions was measured. The data were analyzed with TraceDrawer software.

### 2.7 | Detection of 2019-nCoV spike pseudotyped virus infection ACE2-HEK293T cells

ACE2-HEK293T cells ($5 \times 10^4$ cells/well) were seeded in Opaque 96-well plates. After culturing overnight, cells were pretreated with DMEM medium containing VACV, ZDV, SQV, and EFV for 2 h. Then 2019-nCoV spike pseudotyped virus (5 μl) was added to each plate and incubated for 10 h. Later, the supernatant was removed and cells were cultured in a new DMEM medium at 37°C for 48 h. After that, cells were lysed with 0.1% Triton X-100 (20 μl) and exposed to luciferin substrate (100 μl). Luminescence value was measured using flex Station 3 (Molecular Devices LLC.), with an exposure time of 1000 ms.

## 2.8 | Statistical analysis

Data are presented as mean ± standard deviation. $t$-Test was used for comparisons between two groups and analysis of variance (ANOVA) followed by Turkey's post hoc test was used for comparisons in multiple groups. Significant differences were considered when $p < 0.05$.

## 3 | RESULTS

### 3.1 | Binding characteristics of VACV, ZDV, SQV, and EFV with ACE2

The molecular structures of VACV, ZDV, SQV, and EFV were obtained from Chemical BOOK and shown in Figure 1A. We used two methods to study the interaction between VACV, ZDV, SQV, and EFV with ACE2. A molecular docking method showed that VACV, ZDV, SQV, and EFV could interact well with ACE2, but the binding sites are different. VACV forms three hydrogen bonds with Tyr[83] and Thr[27] on ACE2; ZDV forms two hydrogen bonds with Asn[33] and Phe[390] on ACE2; SQV forms four hydrogen bonds with Gln[24], Phe[28], and Gln[76] on ACE2; and EFV forms a hydrogen bond with Phe[390] on ACE2 (Figure 1B). Furthermore, SPR analysis also confirmed that VACV, ZDV, SQV, and EFV can bind to ACE2, with the $K_D$ value calculated by TraceDrawer™ being $(4.33 \pm 0.09)$ e$^{-8}$, $(6.29 \pm 1.12)$ e$^{-6}$, $(2.37 \pm 0.59)$ e$^{-5}$, and $(4.85 \pm 1.57)$ e$^{-5}$ M, respectively (Figure 1C).

### 3.2 | Effect of VACV, ZDV, SQV, and EFV on ACE2-HEK293T cells viability

VACV and ZDV were nontoxic to ACE-HEK cells at 400 μM, while the inhibition effect of SQV and EFV on cell growth reached 20% at 25 μM (Figure 2A). Then we determined the toxic effect of these four drugs at different time points. It could be concluded that 20 μM VACV, ZDV, and SQV have no effect on the viability of ACE2-HEK293T cells by 48 h, while EFV has no obvious toxicity under the concentration of 10 μM when incubated by 24 h (Figure 2B). Figure 2C shows the effect of these four drugs on ACE-HEK cells apoptosis as determined by flow cytometry. A total of 20 μM EFV induced necrosis of 57% cells by 24 h, while 20 μM VACV, ZDV, SQV and 10 μM EFV had no obvious influence on apoptosis of ACE2-HEK293T cells by 24 h (Figure 2c). To sum up, 20 μM VACV, ZDV, SQV and 10 μM EFV were used for the following experiments.

### 3.3 | ZDV and EFV suppressed 2019-nCoV S pseudotyped virus infection of ACE2-HEK293T cells

ACE2-HEK293T cells pretreated with a vehicle and incubated with 2019-nCoV S pseudotyped virus were used as the control group, and the luminescence value of the control group was regarded as 1.

We measured the inhibitory effect of VACV, ZDV, SQV, and EFV on 2019-nCoV S pseudotyped virus infection of ACE2-HEK293T cells, and CQ (20 μM) was served as a positive control. Under the treatment of VACV, ZDV, SQV (20 μM), and EFV (10 μM), the invasion rate of virus were $1.12 \pm 0.11$, $0.37 \pm 0.05$, $1.18 \pm 0.14$, and $0.61 \pm 0.06$, respectively (Figure 3A).

ZDV and EFV could suppress the infection of the 2019-nCoV S pseudotyped virus in a dose-dependent manner. The EC$_{50}$ of ZDV and EFV is $4.30 \pm 1.46$ and $3.92 \pm 1.36$ μM, respectively (Figure 3B,C). Furthermore, ZDV in combination with EFV led to an improvement in inhibition of the infection of the 2019-nCoV S pseudotyped virus, which is better than that of a single drug (Figure 3D,E). The combination index (CI) was then calculated to assess a potential synergy between ZDV and EFV. All of the CI values listed in Figure 3F were <1, indicating the synergy between ZDV and EFV.

## 4 | DISCUSSION

As COVID-19 continues to spread around the world, it is a very efficient and fast way to identify approved or investigated drugs for COVID-19 treatment. 2019-nCoV shows a high affinity to ACE2, making ACE2 receptor a potential prevention and treatment target for COVID-19. This study demonstrated that ZDV and EFV, alone or in combination, suppress 2019-nCoV S pseudotyped virus infection of ACE2-HEK293T cells by combined with ACE2.

ZDV, SQV, and EFV remain the most commonly used anti-HIV drugs in third-world countries.[15] VACV is the first-line treatment for HSV.[16] The sequence of 2019-nCoV is similar to severe acute respiratory syndrome coronavirus and Middle East respiratory syndrome coronavirus (MERS-CoV).[17] Recent studies suggest that ZDV and VACV did not inhibit the MERS-CoV RdRp activity,[18] but ZDV showed molecular binding activity with the 2019-nCoV S protein/human ACE2.[8] SQV was identified as an effective inhibitor for 3CL$^{PRO}$ main proteinase by docking models[19] and showed interaction with the ACE2 receptor by molecular docking (in silico).[20] EFV has been predicted to have a good inhibition effect on the 2019-nCoV 3C-like proteinase.[21] However, the prediction has not been validated for efficacy and safety. There is no report describing the inhibition effect on 2019-nCoV infection of these four drugs in vitro till now.

The result of molecular docking studies showed that VACV is the best chemical compound binding with ACE2, followed by ZDV, SQV, and EFV. The SPR assay supported the opinion that VACV, ZDV, SQV, and EFV interact with ACE2, the result also showed that VACV had the best affinity to ACE2, with $K_D$ of $(4.33 \pm 0.09)$ e$^{-8}$ M, followed by ZDV, EFV, and SQV. It has already been reported that amino acids Tyr[41], Gln[42], Lys[353], Arg[357], Asp[30], His[34], Gln[24], and Met[82] are active sites of ACE2 that interact with RBD of 2019-nCoV.[22] Molecular docking results showed that none of these four drugs combined with the active sites of ACE2. However, both ZDV and EFV bind to Phe[390] on ACE2, which has been reported to be the allosteric site of ACE2.[23] VACV and SQV may bind with inactive
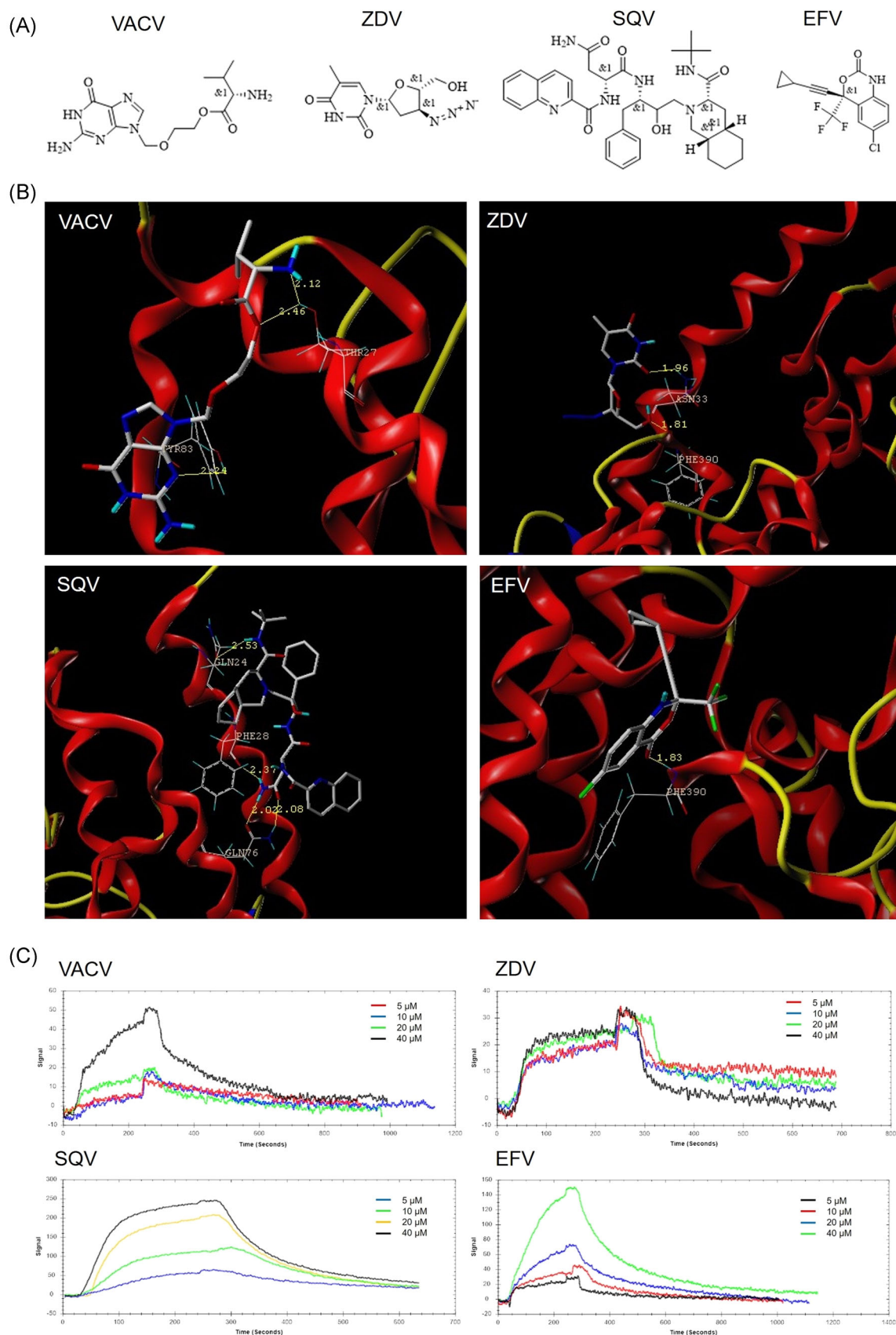
FIGURE 1 Binding character of VACV, ZDV, SQV, and EFV with ACE2. (A) Structural formulas of VACV, ZDV, SQV, and EFV. (B) SPR analysis of VACV, ZDV, SQV, and EFV with ACE2. (C) Schematic diagram of the binding between VACV, ZDV, SQV, EFV, and ACE2 protein determined via a molecular docking assay. ACE2, angiotensin-converting enzyme 2; EFV, efavirenz; SPR, surface plasmon resonance; SQV, saquinavir; VACV, valaciclovir hydrochloride; ZDV, zidovudine
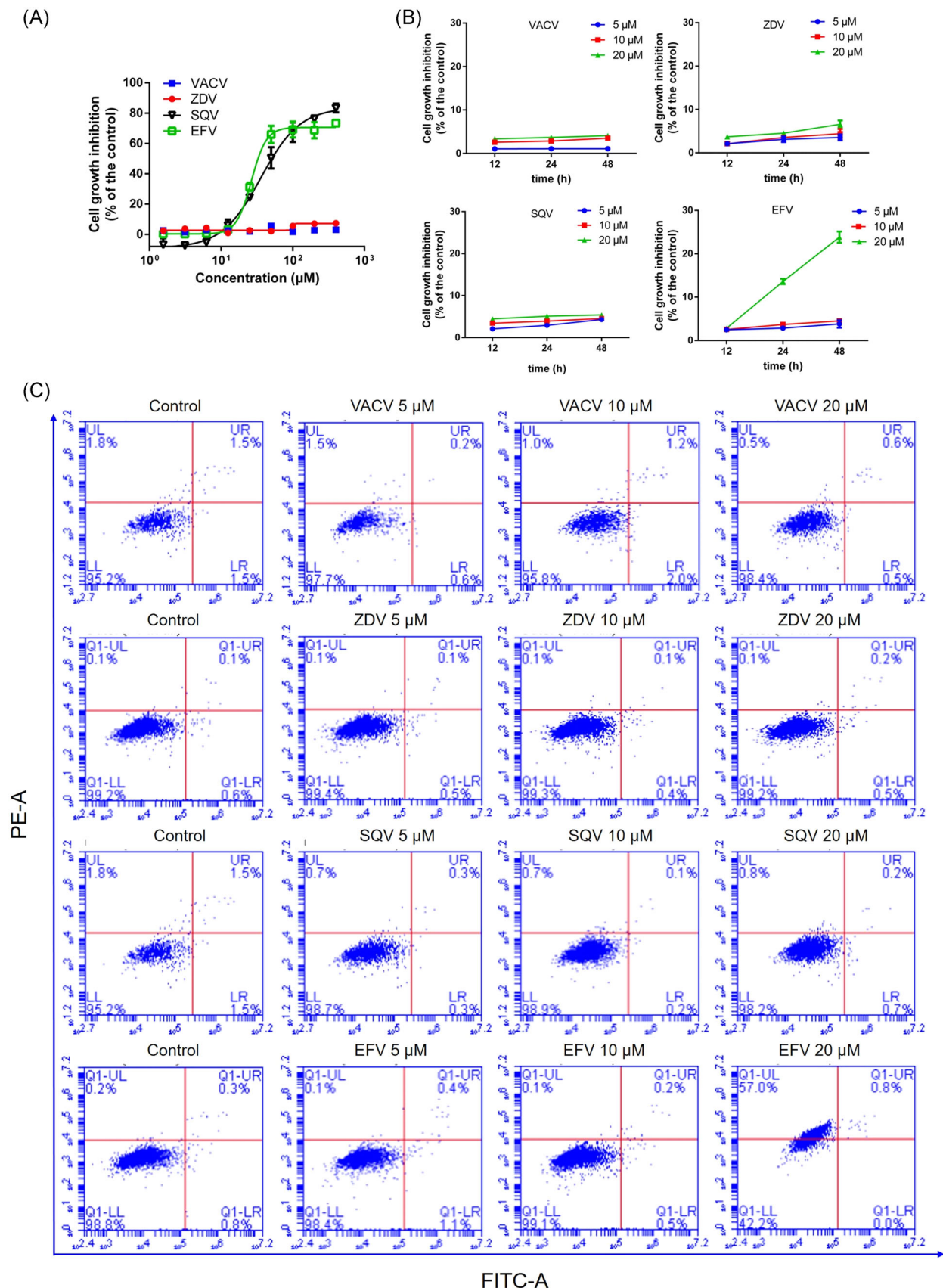
**FIGURE 2** Effect of VACV, ZDV, SQV, and EFV on the viability of ACE2-HEK293T cells. (A) Viability of ACE2-HEK293T cells treated with VACV, ZDV, SQV, and EFV for 24 h. (B) The toxicity of VACV, ZDV, SQV, and EFV on ACE2-HEK293T cells at different time points. (C) The apoptosis of ACE2-HEK293T cells treated with VACV, ZDV, SQV, and EFV for 24 h. The experiments were repeated three times. ACE2, angiotensin-converting enzyme 2; EFV, efavirenz; SQV, saquinavir; VACV, valaciclovir hydrochloride; ZDV, zidovudine
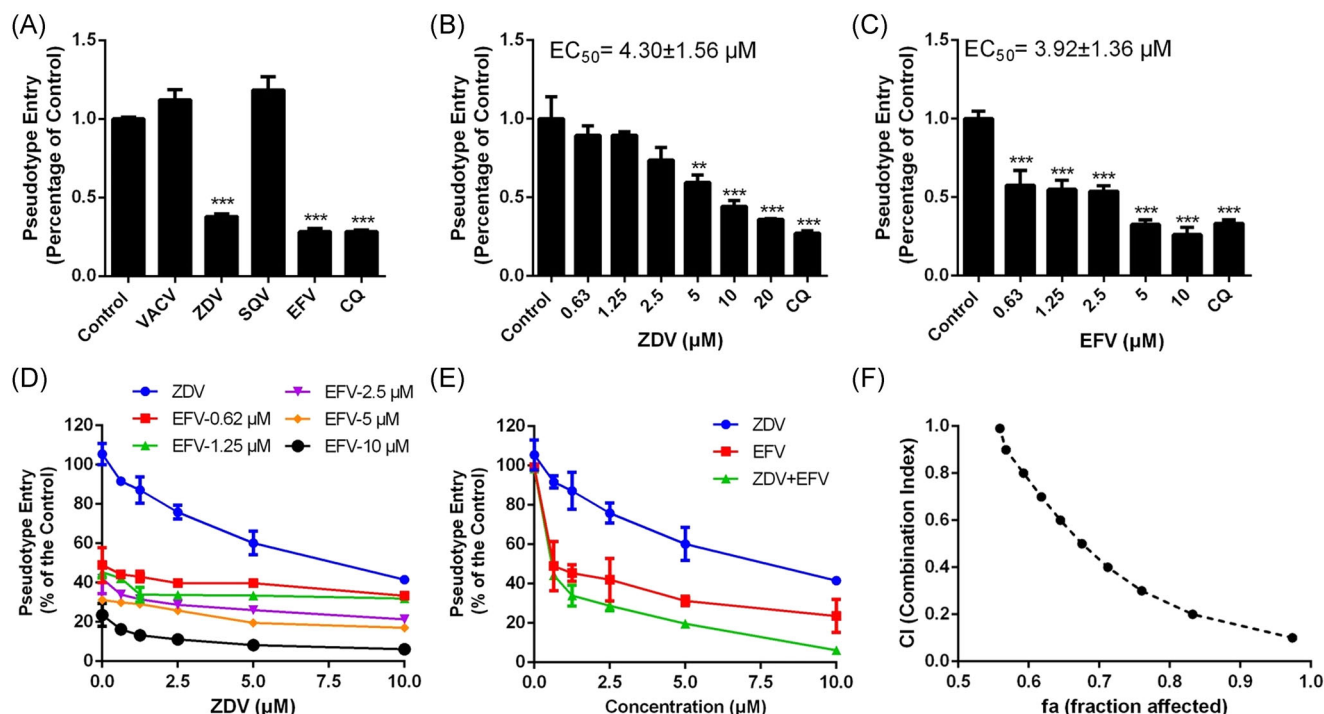
**FIGURE 3** Effect of VACV, ZDV, SQV, and EFV on the entrance of 2019-nCoV spike pseudotyped virus into ACE2-HEK293T cells. (A) Effect of VACV, ZDV, SQV, and EFV on the entrance of 2019-nCoV spike pseudotyped virus into ACE2-HEK293T cells. (B) Effect of ZDV on the entrance of 2019-nCoV spike pseudotyped virus into ACE2-HEK293T cells. (C) Effect of EFV on the entrance of 2019-nCoV spike pseudotyped virus into ACE2-HEK293T cells. (D) dose–response study of different doses of ZDV and EFV on the entrance of 2019-nCoV spike pseudotyped virus into ACE2-HEK293T cells. (E) Dose–response study of a fixed-ratio combination of ZDV (0, 0.65, 1.25, 2.5, 5, and 10 μM) and EFV (0, 0.65, 1.25, 2.5, 5, and 10 μM) on the entrance of 2019-nCoV spike pseudotyped virus into ACE2-HEK293T cells. (F) fa–CI plot in which fa and CI indicate fraction affected and combination index, respectively. CI < 1, CI = 1, and CI > 1 denote synergistic, additive, and antagonistic interaction, respectively. The experiments were repeated three times. Data are presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with Group 0. 2019-nCoV, 2019 novel coronavirus; ACE2, angiotensin-converting enzyme 2; EFV, efavirenz; SPR, surface plasmon resonance; SQV, saquinavir; VACV, valaciclovir hydrochloride; ZDV, zidovudine

sites on ACE2 and showed no inhibition effect on 2019-nCoV pseudovirus infection.

2019-nCoV spike pseudovirus assay was performed to assess the antivirus effect of ACV, ZDV, SQV, and EFV in vitro. This kind of pseudovirus expresses the 2019-nCoV Spike protein on the surface and carries the luciferase reporter gene, which retains its ability to bind to host cell surface receptors for viral infection and makes luciferase express in the cells.[24] Our results illustrated that both ZDV and EFV prevent 2019-nCoV S pseudotyped virus from infecting ACE2-HEK293T cells, with EC$_{50}$ of 4.30 ± 1.46 and 3.92 ± 1.36 μM, respectively. EFV along or in combination with ZDV suppressed infection of the 2019-nCoV S pseudotyped virus in a dose-dependent manner and showed a better inhibitory effect than the same concentration of CQ. Since CQ been proved to be an ACE2 block,[24] the combination of EFV and ZDV may be a promising treatment for the infected population. We have checked these four drugs in a repository for clinical trials, ClinicalTrials.gov by the U.S. National Library of Medicine (https://clinicaltrials.gov/), which includes 388,959 research studies in all 50 states and in 219 countries. The clinical evidence on their efficacy for the prevention of 2019-nCoV infection is lacking.

Our study first indicated that ZDV and EFV could suppress 2019-nCoV pseudovirus infection of the cells through ACE2, suggesting that ZDV and EFV could be a potential drug candidate for preventing infection of 2019-nCoV, which need novel coronavirus research, clinical trials, and epidemiological data for efficacy and safety test.

## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## ORCID

Weina Ma ![ORCID] http://orcid.org/0000-0002-6077-904X

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