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Original Article

Brequinar inhibits enterovirus replication by targeting biosynthesis pathway of pyrimidines

Han Fu[2*], Zhe Zhang[2*], Ying Dai[2*], Shunmin Liu[3], Enqing Fu[1]

[1]Department of Pulmonary Disease and Critical Care Medicine, TangDu Hospital, The Air Force Military Medical University, Xi'an, Shaanxi, China; [2]Department of Clinical Diagnosis, TangDu Hospital, The Air Force Medical University, Xi'an, Shaanxi, China; [3]Department of Respiratory Occupational Diseases, 521 Hospital of Norinco Group, Xi'an, Shaanxi, China. [*]Equal contributors.

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Abstract: Infection of human enteroviruses could cause diverse diseases ranging from mild respiratory symptoms to neurological complications, and even death. Currently, no-FDA approved antiviral drug is available for clinical treatment of human enteroviruses infection. Brequinar is an immunosuppressive drug currently being used for the prevention of organ graft rejection. The drug repurposing studies show that Brequinar exhibits potent antiviral activity against diverse viruses, including flaviviruses, alphavirus, rhabdovirus, and influenza viruses. The antiviral effect of Brequinar on human enterovirus infection has not been investigated yet. Here, the in vitro study shows that Brequinar potently inhibited EV71, EV70, and CVB3 replication at 50% inhibitory concentration (IC50) of 82.40 nM, 29.26 nM, and 35.14 nM, respectively. The antiviral activity of Brequinar was reversed by supplement exogenous pyrimidines, indicating that the antiviral effect of Brequinar against enterovirus relies on the inhibition of dihydroorotate dehydrogenase (DHODH) activity, which is responsible for the de novo biosynthesis of pyrimidines. These data extend the antiviral spectrum of Brequinar and indicate that Brequinar could serve as a promising antiviral drug to treat EV71 and other enterovirus infections.

Keywords: Enterovirus, Brequinar, pyrimidine biosynthesis, DHODH

Introduction

Human enteroviruses, including poliovirus (PV), coxsackieviruses (CVs), echovirus, enterovirus D68, D70, and A71, are non-enveloped, single-stranded RNA viruses of the *Picornaviridae* family and are the pathogens of human diseases ranging from mild to severe in outcomes, including encephalomyelitis, myocarditis, encephalitis, dilated cardiomyopathy, acute flaccid paralysis, pleurodynia, and even death [1]. Human enterovirus infection is considered as a persistent global public health threat and has caused large outbreaks in recent years. Specifically, infection of enterovirus 71 (EV71) and coxsackie A16 virus (CVA16) can result in herpetic angina and hand-foot-and-mouth disease (HFMD) [2]. Young children are particularly susceptible to the severe form of EV71-associated neurological complications. EV71 is considered to be the most severe neurotoxic enterovirus and severe EV71 disease has become a major public health problem in China [3].

The inactivated EV71 vaccine derived from pandemic genogroup C4 in China has been approved and provides specific protection against HFMD and severe EV71 disease [4]. However, the coverage rate of EV71 vaccine is relatively low across the country and the immune boost by the EV71 vaccine could not provide cross-protection against all kinds of EV71 genogroup [5]. Currently, owing to the lack of effective drugs for the treatment of EV71 infection, supportive therapy is still the main method to treat severe cases. Therefore, it is urgent to find novel antiviral drugs to treat EV71 and other enteroviruses infections [6]. However, it is a long and complicated process to develop new anti-EV71 drugs, which requires multiple levels of clinical trials, including effectiveness and safety testing. The drug repurpos-

Table 1. List of primers used for RT-qPCR

Primer	Sequence (5'-3')
qhActin-F	AGCGAGCATCCCCCAAAGTT
qhActin-R	GGGCACGAAGGCTCATCATT
qEV71-F	GCAGCCCAAAAGAACTTCAC
qEV71-R	ATTTCAGCAGCTTGGAGTGC
qCVB3-F	GATTTTGTGCTTTGTGTCAGC
qCVB3-R	GTATCTGCTGGTACAACCTGTG
qEV70-F	GAGGGATTCACCAGACATTG
qEV70-R	CTCTGCAGTACCATGCATA

ing approaches provide an alternative choice to develop antiviral drugs as the pharmacology and toxicology of the repurposed candidate drugs have been tested, which would reduce the time and resources required to advance a candidate antiviral drug into the clinic [7, 8].

Brequinar is a commercial immunosuppressive drug effective against allograft and xenograft rejection after transplantation [9, 10], with the inhibition of the proliferation of T-cells, tumor growth, and production of antibodies [11]. Brequinar was shown to inhibit the activity of dihydroorotate dehydrogenase (DHODH) and to inhibit the de novo biosynthesis pathway of pyrimidines sequentially [12]. DHODH is the fourth enzyme and rate-limiting enzyme to convert dihydroorotate to orotate at the inner membrane of mitochondria [13]. Cellular nucleosides including pyrimidines and purines play a very important role in the biosynthesis of viral RNA and DNA. The viral replication efficiency heavily relies on the supply of nucleoside pool. Suppression of pyrimidine biosynthesis by targeting DHODH enzyme is promising to inhibit virus infections [14]. Brequinar has recently been found to have antiviral activity against a broad spectrum of viruses including flaviviruses (Yellow fever virus, Dengue virus, West Nile virus, Zika virus, and Powassan virus), Ebola virus, Western equine encephalitis virus, Vesicular stomatitis virus, and Influenza A and B viruses [15-23].

Here we investigated the antiviral activity of Brequinar on the replication of enteroviruses. The results show that Brequinar potently inhibited EV71 replication. Brequinar specifically targeted enterovirus RNA synthesis since the antiviral effect was reversed by the supplement of exogenous uridine. Additionally, Brequinar inhibited coxsackievirus B3 (CVB3) and EV70 with promising efficiency. Taken together, our results expand the antiviral spectrum of Brequinar and provide insight into the possibility of developing Brequinar as an effective anti-enterovirus therapeutic.

Materials and methods

Cells, viruses, antibodies, and regents

RD cells (American Type Culture Collection (ATCC), Manassas, VA, United States; CCL-136), were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, United States) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/mL streptomycin and 500 U/mL penicillin at 37°C. EV71 (strain 87-2008 Xi'an Shaanxi) was obtained from the Xi'an Centre for Disease Control. Rabbit Enterovirus 71 VP1 antibody was purchased from GeneTex (GTX132338, Hsinchu, China). β-actin monoclonal antibody (mAb) was purchased from ProteinTech (66009-1-Ig, Wuhan, China). Goat anti-Mouse IgG H&L (IRDye® 680RD) preadsorbed (ab216776), Goat anti-Rabbit IgG H&L (IRDye® 800CW) preadsorbed (ab216773), and Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (ab150081) were purchased from Abcam (Shanghai, China). Brequinar (HY-108325, purity 99.57%) and Uridine (HY-B1449, purity 99.99%) were purchased from MedChemExpress LLC (Shanghai, China).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay

RD cells were seeded in 12-well plates with ~80% confluence. The next day, the cells were treated as designed. Total RNA was extracted using the TRIzol reagent (Invitrogen) and was subjected to reverse transcription using Hifair® III 1st Strand cDNA Synthesis Kit (Yeasen, Shanghai, China). Next, the cDNAs were subjected to RT-qPCR performed using Hieff® qPCR SYBR® Green Master Mix (Yeasen). The data represent absolute numbers of mRNA copies normalized to β-actin. Relative changes in expression were determined by using the comparative threshold cycle (CT) method. The sequences of RT-qPCR primers are listed in **Table 1**.

Immunofluorescence assay (IFA)

RD cells were seeded in 48-well plates with ~70% confluence. The next day, the cells were infected with EV71 (MOI=0.5) for 1 h at 37°C

and then treated with serially diluted Brequinar for 24 h. The next day, the cells were fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.25% Triton X-100. The cells were then incubated with Rabbit Enterovirus 71 VP1 antibody overnight at 4°C and with Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) for 1 h at room temperature. Nuclei were stained with 4=,6-diamidino-2-phenylindole (DAPI) (Roche) for 10 min at room temperature. The stained cells were imaged with a BX60 fluorescence microscope (Olympus, Tokyo, Japan).

Western blot analysis

RD cells were seeded in 6-well plates with ~80% confluence. The next day, the plate was treated as designed. Cells were lysed in Pierce RIPA buffer, and the protein concentration in the lysates was determined using the BCA assay. Totally 40 μg aliquots of each cell lysate were boiled and resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to 0.45 um polyvinylidene fluoride membranes. The membranes were blocked for 1 h and incubated with Rabbit EV71 VP1 antibody, followed by incubation with Goat anti-Rabbit IgG H&L (IRDye® 800CW) preadsorbed. The β-actin serves as the loading control. The membranes were visualized with the Odyssey infrared imaging system (Li-Cor Biosciences).

Cytotoxicity assay

To investigate the cytotoxicity of Brequinar on RD cells, the Cell Counting Kit-8 (CCK8) assay (Yeasen, Shanghai, China) was performed according to the manufacturer's instructions. Briefly, RD cells were seeded in 96-well culture plates at a density of 2×10^4 cells/well. The medium was removed next day, and the plate was washed three times with DMEM, and then the medium was replaced with medium containing serially diluted Brequinar ranging from 0.001 μM to 100 μM. As a control, 100 μM DMSO was added to the cells. Cytotoxicity was determined after 72 h of treatment. The medium was replaced with 100 μL of DMEM and 10 μL of CCK8 solution and incubated at 37°C for 4 h in dark. The plate was shaken for 1 min and measured at 450 nm using a BioTek Synergy HT microplate reader. Cell viability was calculated as previously [24].

Antiviral assays

Pre-treatment: RD cells in 12-well plates were treated with serially diluted Brequinar for 1 h at 37°C. After incubation, Brequinar was removed and cells were washed with PBS. Pre-treated cells were infected with EV71 (MOI=0.5) at 37°C for 1 h. The inoculum was removed, cells were washed with PBS and cultured with 10% FBS supplemented DMEM for an additional 22 h. EV71 RNA copy number was determined using RT-qPCR.

Attachment: Serially diluted Brequinar was pre-mixed with EV71 (MOI=0.5) and incubated with pre-chilled RD cells at 4°C for 1 h to allow virus attachment. The inoculum was removed after attachment, and cells were washed with PBS and cultured with 10% FBS supplemented DMEM for an additional 23 h. EV71 RNA copy number was determined using RT-qPCR.

Entry: To assess the effect of Brequinar on the entry step of EV71, the pre-chilled RD cells were incubated with EV71 (MOI=0.5) at 4°C for 1 h to allow virus attachment. The unattached virus was removed and the cells were washed with PBS. The cells were incubated with serially diluted Brequinar for 1 h at 37°C to allow virus entry. The medium was removed and cells were then washed with serum-free media and cultured with 10% FBS supplemented DMEM for an additional 22 h. EV71 RNA copy number was determined using RT-qPCR.

Replication: The pre-chilled RD cells were incubated with EV71 (MOI=0.5) at 4°C for 1 h to allow virus attachment. The inoculum was removed and washed with PBS after 1 h, and cells were cultured with 10% FBS supplemented DMEM for 1 h at 37°C. The medium was then replaced with serially diluted Brequinar. Total RNA was extracted 22 hours later and EV71 RNA copy number was determined using RT-qPCR.

Post-infection: The RD cells were infected with EV71 (MOI=0.5) for 1 h at 37°C. The inoculum was removed and washed with PBS after 1 h, and cells were cultured with 10% FBS supplemented DMEM for 6 h at 37°C. The medium was then replaced with serially diluted Brequinar. Total RNA was extracted 17 hours later and EV71 RNA copy number was determined using RT-qPCR.

Uridine reversal assay

In uridine reversal experiments, RD cells in plates were infected with EV71 (MOI=0.5) for 1 h and then treated with 1 μM Brequinar and uridine (50, 100, and 200 μM) for an additional 24 h. The relative levels of viral RNA and EV71 VP1 were detected by RT-qPCR and western blot, respectively.

Statistical analysis

All experiments were reproductive and repeated at least three times. The data are presented as the means ± SDs unless stated otherwise. Statistical significance for comparison of two means was determined by the two-tailed unpaired t-test in GraphPad Prism 7 software (San Diego, CA, US). $P < 0.05$ was considered statistically significant.

Results

Brequinar potentially inhibits EV71 infection in vitro

Brequinar has been reported to inhibit various viruses with different efficacies. To determine whether Brequinar has a potential anti-EV71 effect, RD cells were treated with various concentrations of Brequinar for 24 h. The results show that Brequinar potently inhibited EV71 replication in a dose-dependent manner as measured in both RNA and protein levels (**Figure 1A** and **1D**), although low concentrations (0.01~0.1 μM) of Brequinar slightly enhanced EV71 replication. The IC50 value of Brequinar against EV71 on RD cells is 82.40 nM (**Figure 1C**), while cell viability was not affected under 10 μM (**Figure 1B**), indicating that the selective index of Brequinar on EV71 is better than 121.36. Consistently, indirect fluorescence microscopy analysis further confirmed that Brequinar could inhibit EV71 replication (**Figure 1E**), and the EV71 titer was also significantly decreased by Brequinar treatment in a dose-dependent manner (**Figure 1F**). Taken together, these results suggest that Brequinar potently inhibits EV71 replication without affecting cell viability.

Brequinar inhibits EV71 replication after virus entry

To verify whether Brequinar inhibits EV71 infection after the entry stage, RD cells were treated by Brequinar and EV71 with different strategies (**Figure 2A**). As depicted in **Figure 2B**, pretreatment with Brequinar at 0.01 μM to 10 μM did not inhibit EV71 infection, while the inhibition effect of 100 μM Brequinar on virus replication may be due to the insufficient removal of Brequinar or the intracellular level of Brequinar. Additionally, the low concentration (0.01~10 μM) of Brequinar did not affect EV71 infection at the virus attachment stage (**Figure 2C**), and 100 μM of Brequinar significantly inhibited EV71 replication. Regarding the virus entry stage (**Figure 2D**), Brequinar (1~100 μM) could affect EV71 entry into the cells. As expected, Brequinar dramatically inhibited EV71 replication after virus entry (**Figure 2E**) and exhibited an inhibitory effect even at 6 h post-infection (**Figure 2F**). Brequinar has an ideal IC50 value against EV71 (**Figure 1C**), the low level of intracellular Brequinar may interfere with the results when cells were treated with 100 μM Brequinar. Combined, these data illustrate that Brequinar potently inhibits EV71 replication post virus entry.

Exogenous supplement of pyrimidines reversed the anti-EV71 activity of Brequinar

DHODH is the rate-limiting enzyme in the *de novo* biosynthesis pathway of pyrimidines. A previous study has shown that the antiviral activity of Brequinar is dependent on the inhibition of DHODH activity and the depletion of intracellular pyrimidine pools. To determine whether the biosynthesis pathway of pyrimidines was involved in the inhibition of EV71 infection, uridine was added to EV71-infected cells when treated with 1 μM Brequinar. The results show that viral protein and RNA expression are significantly inhibited when treated with Brequinar and partially restored with the addition of uridine (25, 50, and 200 μM) for 24 h (**Figure 3A** and **3B**). The 200 μM uridine alone slightly enhances EV71 RNA replication with no significance (**Figure 3B**).

Brequinar exhibits broad-spectrum inhibition of enterovirus replication

The enteroviruses apply a similar strategy for viral replication and heavily rely on the host supply of nucleoside biosynthesis. Therefore, Brequinar targeting pyrimidines biosynthesis may exhibit a broad antiviral spectrum against other enteroviruses. Here we tested the antivi-

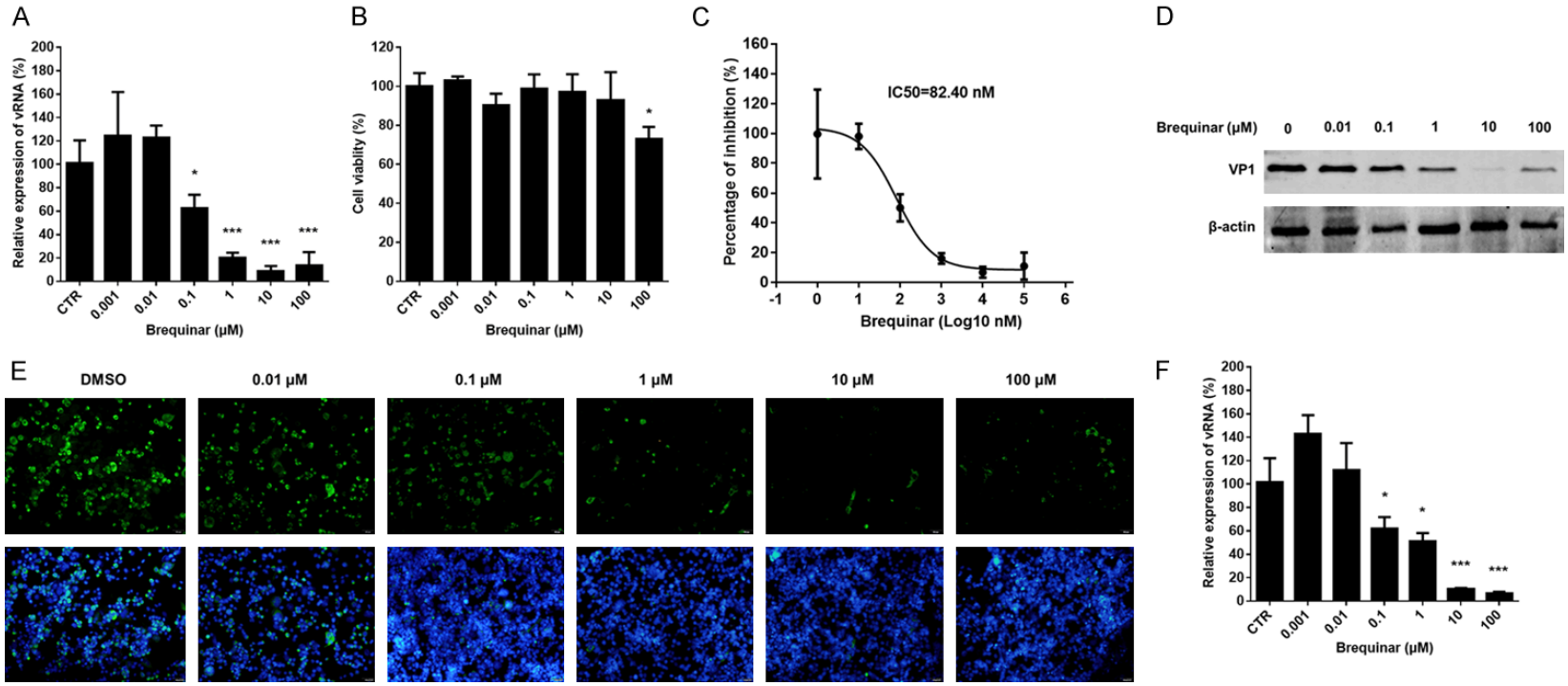

Figure 1. Brequinar potently inhibits EV71 infection without significant cytotoxicity. RD cells were infected with EV71 (MOI=0.5) for 1 h and were incubated with the indicated concentrations of Brequinar for an additional 24 h: (A) The viral RNA copy number was quantified by RT-qPCR, the β-actin was used as housekeeping gene; (D) The EV71 VP1 protein expression in cells was determined by western blot, the β-actin was determined as loading control; (E) The EV71 infected cells were visualized by indirect fluorescence microscopy, nuclei were labeled with DAPI (blue). (B) Cell viability after cultured with indicated concentrations of Brequinar was assessed by CCK8 assay at 48 h later (mean ± SD, n=6). (C) The IC50 of Brequinar against EV71 on RD cells was calculated with GraphPad. (F) The culture supernatant of each sample in (A) was collected and infected RD cells for 24 h. The viral RNA copy number indicating the virus yield in (A) was quantified by RT-qPCR (mean ± SD, n=3). *P < 0.05, **P < 0.01, ***P < 0.001.

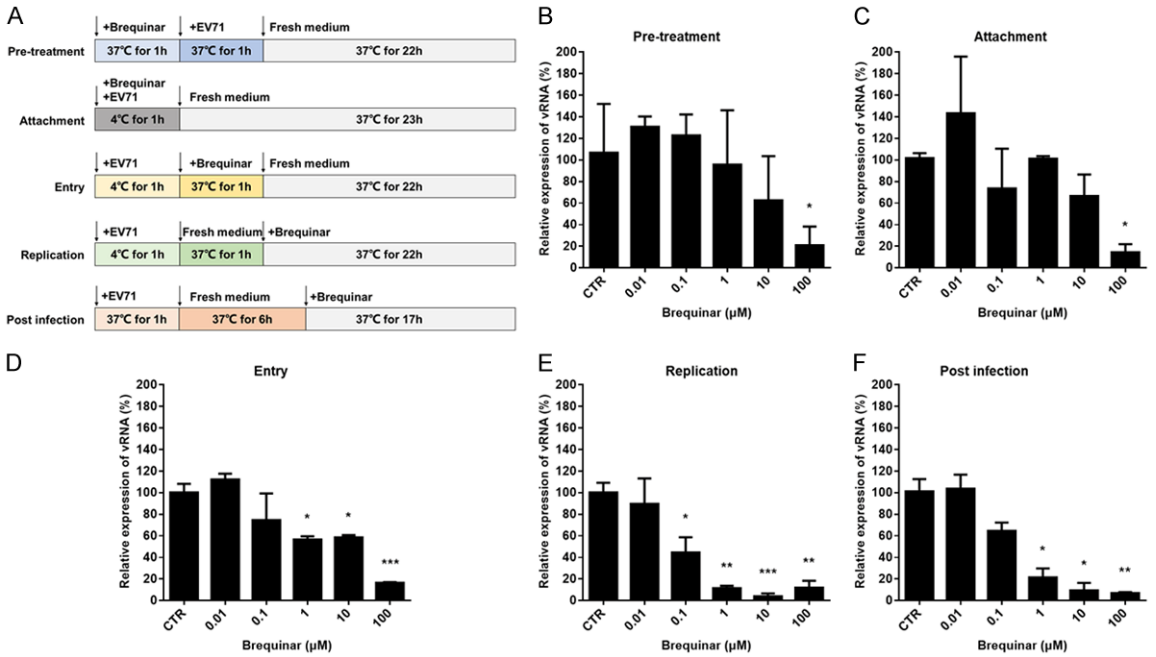

Figure 2. Brequinar inhibits EV71 replication post virus entry. (A) Schematic representation of the different treatment strategy. The RD cells in 12-well plates were incubated with serially diluted Brequinar and EV71 (MOI=0.5) at pre-treatment (B), attachment (C), entry (D), replication (E), and post-infection (F) stages, the EV71 viral RNA copy number was quantified by RT-qPCR (mean ± SD, n=3). *P < 0.05, **P < 0.01, ***P < 0.001.

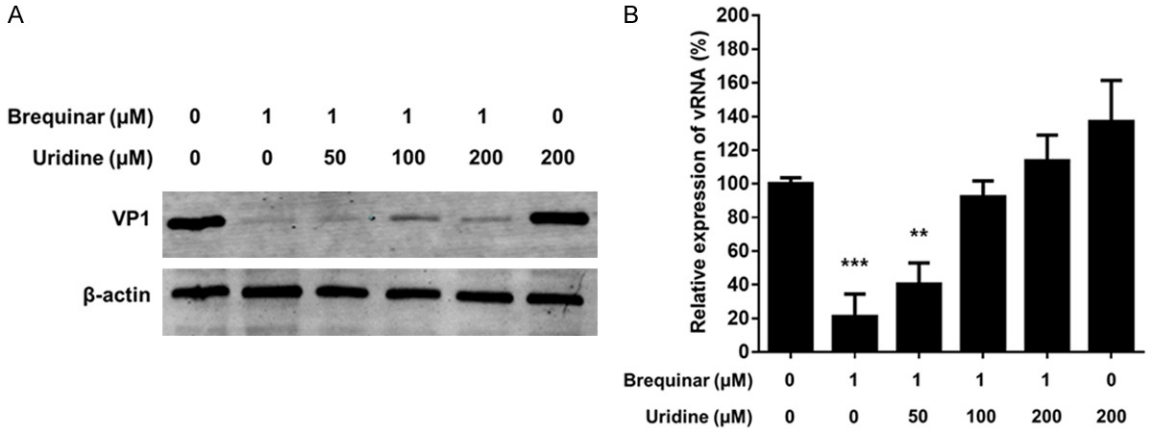

Figure 3. Supplement of exogenous uridine reversed the anti-EV71 effect of Brequinar. A. EV71 VP1 protein level in RD cells was measured by western blot after 24 h of treatment with 1 μM Brequinar by adding serially diluted exogenous uridine, β-actin was set as the loading control. B. EV71 RNA in RD cells was quantified by RT-qPCR after 24 h of treatment with 1 μM Brequinar by adding serially diluted exogenous uridine, data were normalized to the untreated control (set as 100%). Data are presented as means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

ral effect of Brequinar on two enteroviruses (EV70 and CVB3) stocked in our laboratory. The results show that Brequinar potently inhibits viral RNA replication of EV70 (**Figure 4A**) and CVB3 (**Figure 4B**), with an IC50=29.26 nM (**Figure 4C**) and IC50=35.14 nM (**Figure 4D**), respectively. These data demonstrated that Brequinar exhibits potent antiviral efficacy against enteroviruses.

Discussion

EV71 vaccine has been licensed in China for several years and could protect children from HFMD and severe neurological complications [4]. However, due to the low vaccination coverage rate and diverse genogroup of enterovirus, the cases of HFMD caused by EV71 and CAV16 are over 2,000,000 annually in China [3]. It is

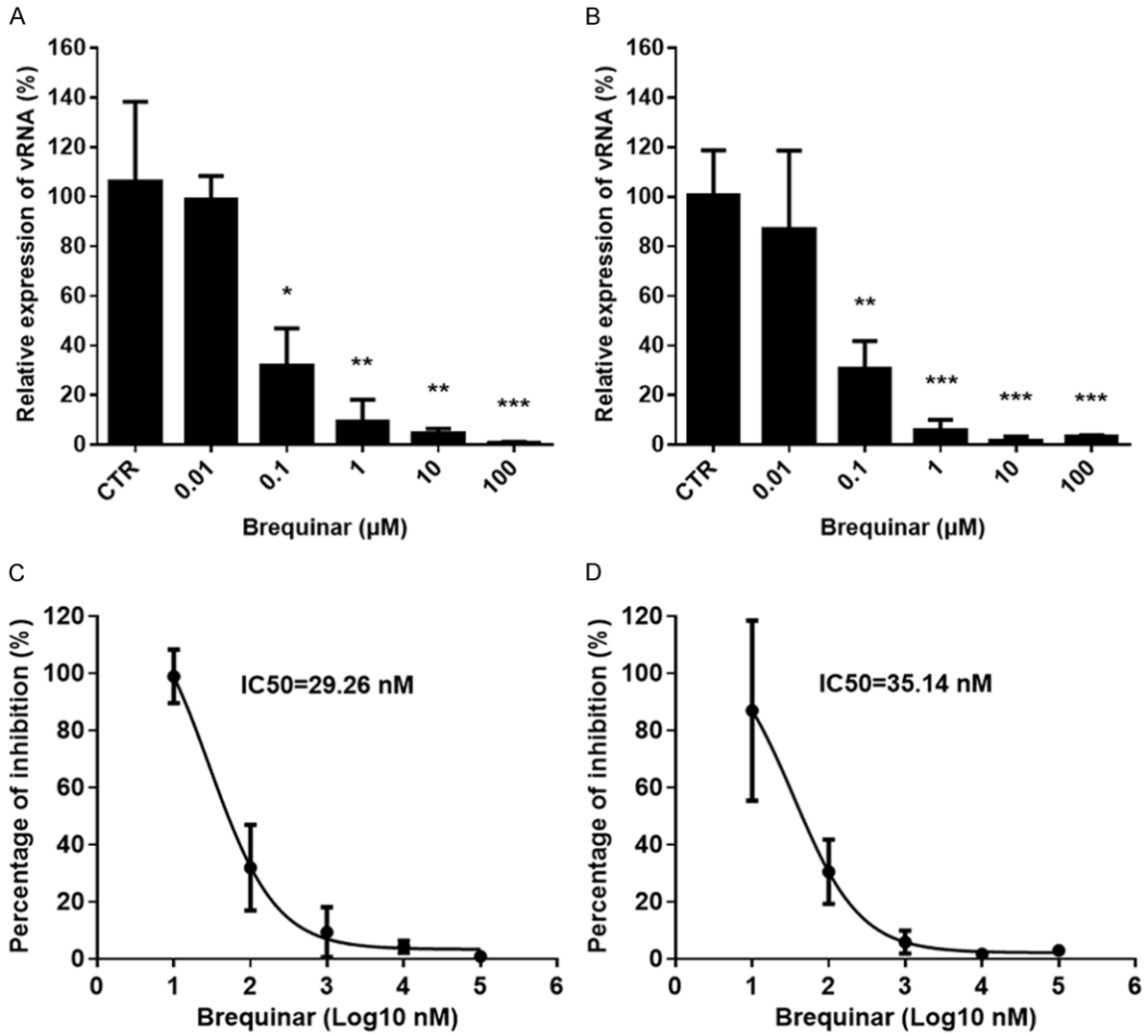

Figure 4. Brequinar exhibits broad-spectrum antiviral activity against enterovirus replication. (A) RD cells were infected with EV70 (MOI=0.5) for 1 h and were incubated with the indicated concentrations of Brequinar for an additional 24 h, the viral RNA copy number was quantified by RT-qPCR. (B) RD cells were infected with CVB3 (MOI=0.5) for 1 h and were incubated with the indicated concentrations of Brequinar for an additional 24 h, the viral RNA copy number was quantified by RT-qPCR. The IC50 of Brequinar against EV70 (C) and CVB3 (D) on RD cells was calculated with GraphPad. Data are presented as means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

urgent to discover safe and effective therapeutics against EV71 and other enteroviruses. In this study, we firstly investigated the potential anti-EV71 activity of Brequinar, a commercial drug used to treat allograft and xenograft rejection following transplantation. As shown in **Figure 1**, Brequinar could potently inhibit EV71 infection by suppressing expression levels of viral RNA and protein in cells and the production of virions in supernatant without cytotoxicity. The antiviral effect may be attribute to the inhibition of DHODH activity by Brequinar targeting pyrimidine biosynthesis [16, 21].

Nucleotides have a very important role in cell metabolism and virus infection. The antiviral effect of many inhibitors targeting de novo pyrimidine biosynthesis has been well studied on Dengue virus, Zika virus, Ebola virus, and other virus infection models [15-23]. In this study, we demonstrated that Brequinar, one of the inhibitors of the DHODH enzyme, has potent antiviral activity against EV71 infection via depleting pyrimidine nucleotide pool (**Figure 3**). The enteroviruses apply the same strategy to replicate their genome. As expected, Brequinar also shows potent antiviral activity ag-

ainst another two enteroviruses, EV70 and CVB3 (**Figure 4**). Further experiments revealed that the antiviral effects of Brequinar begin after virus entry (**Figure 2**), which is affected by the depletion of the pyrimidine pool. Therefore, targeting pyrimidine biosynthesis represents a promising way to develop broad-spectrum antiviral drugs against enteroviruses.

Brequinar is a well-known immunosuppressive agent to treat allograft and xenograft rejection following transplantation [25]. Since the safety and pharmacology of a drug is already known in clinical trials, the drug repurposing approach has many advantages over the development of new drugs to identify new antiviral strategies [8]. Therefore, as the currently used Brequinar in clinic demonstrates considerable antiviral activity against enterovirus in vitro, there should be great potential to expand its application to combating enterovirus infection in clinical use.

Notably, it is reported that Brequinar has some side effects when used in clinic, such as reduced bodyweight gain or bodyweight loss, leukocytopenia, thymic atrophy, thrombocytopenia, cellular depletion of bone marrow and splenic white pulp, and villous atrophy in jejunum [26]. These kinds of clinical observations are comprehensible since all drugs come with side effects. In our study, Brequinar potently inhibits viral RNA replication of EV71, EV70 and CVB3, with an IC50=82.40 nM (**Figure 1C**), IC50=29.26 nM (**Figure 4C**) and IC50=35.14 nM (**Figure 4D**), respectively. The drug dosage used in our study for inhibition of human enteroviruses is much lower than that used in clinical trials [27, 28]. This might make Brequinar a preferable option for treating enterovirus infection, achieving high efficacy with fewer side effects.

In conclusion, this study described the drug repurposing use of Brequinar and revealed that Brequinar inhibited enterovirus infection in vitro at the post-entry stage. Brequinar exerts its antiviral activities by significantly inhibiting DHODH activity, which is sequentially the rate-limiting enzyme in the de novo biosynthesis pathway of pyrimidines. This property of the antiviral mechanism makes Brequinar a promising antiviral drug with broad-spectrum activity against human enteroviruses. Future in vivo studies are needed to demonstrate the antiviral activity of Brequinar for the feasibility of treating enterovirus infections.

Disclosure of conflict of interest

None.

Address correspondence to: Enqing Fu, Department of Pulmonary Disease and Critical Care Medicine, TangDu Hospital, The Air Force Military Medical University, Xi'an, Shaanxi, China. E-mail: fuenqing@sina.com; Shunmin Liu, Department of Respiratory Occupational Diseases, 521 Hospital of Norinco Group, Xi'an, Shaanxi, China. E-mail: shunmliu@163.com

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