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Antiviral activity of brequinar against foot-and-mouth disease virus infection *in vitro* and *in vivo*

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ABSTRACT

Foot-and-mouth disease (FMD) is one of the most highly contagious animal disease that affects cloven-hoofed animals. However, the FMD vaccine does not provide effective protection until adaptive immune protection elicited by the vaccination occurs. Therefore, an alternative application of antiviral agents for inhibition of the FMD virus (FMDV) is needed. Here, we demonstrated that brequinar could exhibit antiviral activity in swine kidney cells (IBRS-2 cells) infected with two different FMDV serotypes. Subsequently, *in vivo* activity of brequinar was confirmed in a mouse model of infection. Specifically, brequinar at a concentration of 50 μg, provided 25% protection for 5 days following FMDV challenge. These results suggested that brequinar could be used as effective antiviral agent against FMD.

1. Introduction

Foot-and-mouth disease (FMD) is one of the most economically and socially devastating diseases that affects cloven-hoof animals including cattle, swine, sheep, goats and many other non-domesticated species [1]. Upon infection with the FMD virus (FMDV), subsequent clinical signs include fever, inappetence, lameness, and the appearance of vesicular lesions in the mouth, snout, teats, and feet [2]. The FMDV belongs to the genus *Aphthovirus* within the family *Picornaviridae*, and is a small, non-enveloped virus, which contains a single-stranded positive-sense RNA genome. The virus exists as seven distinct serotypes comprising O, A, C, Asia-1 and Southern African Territories (SAT) 1–3 and as multiple subtypes due to the high mutation rate of the virus [3]. The viral capsid consists 60 copies of four structural proteins (VP1, VP2, VP3, and VP4).

At present, vaccination is the most common method to prevent FMDV infection, including inactivated whole virus vaccines, new inactivated whole virus marker vaccines and subunit vaccine [4]. However, the FMD vaccine has several problems including low immunogenicity, instability of the antigen, insufficient maintenance of the antibody, and little or no cross-protection across serotypes and subtypes [5–7]. Therefore, finding effective antiviral agent against FMDV infection is necessary.

As an immunosuppressive and antiproliferative drug, brequinar has been shown to be effective against allograft and xenograft rejection after transplantation [8,9]. It has been demonstrated to suppresses T cell proliferation, antibody production and tumor growth [10]. The mechanism of action of brequinar likely relies on inhibition of dihydroorotate dehydrogenase (DHODH) activity, the fourth enzyme and a rate-limiting step in the *de novo* biosynthesis of pyrimidines [11]. In this way, brequinar exerts its effect by depleting the cellular pyrimidine pool, which is needed for RNA and DNA synthesis. Recent studies have shown that brequinar exhibits antiviral activity against Cantagalo virus [12], Dengue virus [13], vesicular stomatitis virus, Zika virus, Ebola virus [14] and hepatitis E virus [15] *in vitro*. However, the antiviral activity of brequinar against FMDV has not yet been reported. In this study, we examined the antiviral efficacy of brequinar *in vitro* and *in vivo*. We show that brequinar severely inhibited the production of infectious FMDV and acted its action at the early stages of viral replication. Furthermore, we assessed the action of BQR on FMDV replication in more detail. This is the first time the antiviral effect of BQR against FMDV *in vitro* and *in vivo* has been demonstrated.

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2. Methods

2.1. Cells, viruses and reagents

Swine kidney cells (IBRS-2 cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% FBS. FMDV, including O/MYA98/BY/2010 and A/GDMM/CHA/2013, were provided by OIE/National Foot-and-Mouth Disease Reference Laboratory of China and were propagated in IBRS-2 cell culture with DMEM supplemented with 2% FBS. The viral titers of the lysates from this study were calculated by the 50% tissue culture infected dose ($TCID_{50}$) using the Reed and Muench method. Brequinar (BQR) and Uridine were purchased from MCE (MedChemExpress) with a purity > 97%, dissolved in 100% DMSO to yield 100 mM stock solution for further in vitro studies. For in vivo studies, BQR was dissolved in 10% DMSO and 5% Tween-80 in PBS. Rabbit hyper-immune serum raised against type O FMDV (O/MYA98/BY/2010) were kindly provided by Guang-qing Zhou (OIE/National Foot-and-Mouth Disease Reference Laboratory). Peroxidase-Conjugated Goat anti-rabbit IgG (H + L), Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or anti-rabbit IgG and SPlink detection kit were obtained from (ZSGB, Beijing, China). PrimeScript™ RT reagent kit containing gDNA Eraser and SYBR Premix Ex Taq™II (Tli RNaseH Plus) were purchased from TaKaRa (Dalian,China). Type O FMDV VP1 rabbit polyclonal antibodies were provided by Hai-xue Zheng (OIE/National Foot-and-Mouth Disease Reference Laboratory).

2.2. Determination of Cytotoxic Activity

To investigate the cytotoxicity of BQR on IBRS-2 cells, the MTS assay (Abcam) was performed according to the manufacturer's instructions. Briefly, IBRS-2 cells at a density of 3×10^4 cells /wells were seeded into 96-well culture plates. The next day, the medium was removed, and the 96-well plates was washed three times with DMEM, and then the media was replaced with media containing serially diluted BQR ranging from 3 μM to 100 μM. As a control, DMSO was added to the cells at a concentration of 100 μM. Cytotoxicity was determined after 72 h of treatment.

2.3. Antiviral assays

The antiviral activity was firstly determined by the MTS assay based on the cytopathic effect (CPE) induced by viral infection, as previously reported [16]. One day before infection, 3×10^4 IBRS-2 cells/well were seeded into a 96-well culture plate and incubated at 37 °C in an atmosphere of 5% CO_2. The following day, after removing the medium, the 96-well plates was washed with DMEM three times. Subsequently, the cells was infected with 100$TCID_{50}$ FMDV for 1 h at 37°C to allow virus entry. After removing the uncombined virus with DMEM, cells were treated with a series of concentrations (3, 6, 12, 25, 50, 75, and 100 μM) of BQR for 48 h at 37°C. The viability of IBRS-2 cells was calculated using the MTS assay, and the absorbance was read at 490 nm by using a microplate reader (Thermo Fisher Scientific, USA). The antiviral activity was expressed as the 50% effective concentration (EC_{50}) defined as the concentration required to reduce virus-induced cytopathogenicity by 50% of the control value and was calculated using GraphPad Prism5 (GraphPad Software). The supernatant of each well was collected and analyzed with replication analysis.

2.4. Uridine reversal

In uridine reversal experiments, 25, 50, and 200 μM uridine was added to growth media at the time of compound dosing.

2.5. Addition of BQR at different times points

The time of addition assay was used to investigate the mechanism of action of BQR. BQR, at a final concentration of 50 μM, was added to IBRS-2 cells either the time of (0 h), or after (2, 4, 8 and 16 h) FMDV infection. After 48-h of incubation, Q-PCR and western blotting analyses were performed to determine the FMDV mRNA and VP1 protein levels, respectively.

2.6. Indirect immunofluorescence assay

Approximately, 3×10^5 IBRS-2 cells were seeded in a 12-well plate. The cells were allowed to form a confluent monolayer and were infected with a 100 $TCID_{50}$ of FMDV and incubated for 1 h. After incubation, cells were treated with a series of concentrations of BQR for 12 h. The cells were washed with PBS with 0.1% Tween-20 (PBST) thrice and fixed with 4% paraformaldehyde in PBS for 15 min and were then permeabilized with 0.2% Triton X-100 in PBS for 10 min. After washing, rabbit hyper-immune serum raised against type O FMDV was then added to the each well and incubated for 1 h at room temperature. A secondary antibody (1:1,000) was then added to the wells and incubated for 1 h at room temperature. After incubation, the cells were stained by 4′,6-diamidino-2-phenylindole, dihydrochloride (a nuclear stain; Solarbio, China) according to instructions of the manufacturer. The fluorescence was observed under a fluorescent microscope Nikon ECLIPSE TS100 fluorescence microscope (Nikon, Japan)

2.7. RNA extraction and Q-PCR

Total RNA was isolated from cultured cells using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The concentrations of the extracted RNA were measured using a NanoDrop. Reverse transcriptase reaction was carried out using PrimeScript™ RT reagent kit (Takara) with 1 μg of total RNA, and Q-PCR targeting the 2B gene of the FMDV genome was carried out as previously established with minor modifications [17], using Agilent Technologies Stratagene Mx3005 P instrument (Agilent, USA) and SYBR Premix Ex Taq (Takara, Dalian, China) according to the instructions of the manufacturer. The reaction was performed in triplicate in 96-well plates under the following conditions; 30 s at 95 °C followed by 40 cycles of 5 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C. β-actin was used as the internal control. The relative mRNA expression levels of the genes were compared with those of β-actin by the $2^{-\Delta\Delta Ct}$ method.

2.8. Western blot assay

Western blots were conducted following the procedure used by Feng et al [18]. Cells were lysed in a Pierce RIPA buffer, and the protein concentration in the lysates was determined using the BCA assay. Proteins were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked for 4 h with 5% nonfat dry milk solution in Tris-buffered saline, blotted with specific primary antibodies, and were incubated with secondary antibodies conjugated with horseradish peroxidase. Proteins were visualized by chemiluminescence and clarity Pierce™ ECL Western Blotting Substrate.

2.9. Antiviral reagent injection, viral challenge, and monitoring in suckling mice

Specific-pathogen-free suckling mice, 3 to 4 days old, weighing 3 to 4 g, which were purchased from the Lanzhou Veterinary research institute, were used to further investigate the antiviral activity of BQR *in vivo*. The FMDV dose was determined through 10-fold serial dilutions of the virus. The 50% lethal dose (LD_{50}) of FMDV (O/MYA98/BY/2010) was estimated by the Reed-Muench method. Suckling mice were

inoculated by intraperitoneal injection with 50 μg of BQR. Mice were treated with PBS containing 10% DMSO and 5% Tween-80 in the same volume as controls. Two hours following BQR injection, the suckling mice were challenged with 100 LD_{50} FMDV in a volume of 100 μL by intraperitoneal injection. The animals were monitored for 5 days. The survival curve was performed using GraphPad Prism5.

2.10. Hematoxylin and eosin (H&E) Staining

The heart tissues of mice were fixed in 10% neutral buffered formalin for 48 h, processed, embedded in paraffin, and prepared as 5 μm sections. Subsequently, the fixed tissues were stained with H&E. Histopathological changes were observed under a Digital microscope (BA400Digital, Motic, China).

2.11. Statistical analyses

All the data are presented as the mean ± standard. One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was used to compare multiple groups computed by GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA). All the experiments were performed in triplicate. Values of $p < 0.05$ were considered to be statistically significant at a 95% confidence interval.

3. Results

3.1. Anti-FMDV effects of BQR in vitro

We first investigated if BQR treatment has cytotoxic effects on IBRS-2 cells as was assessed by viability of treated IBRS-2 cells. IBRS-2 cells treated with BQR (3, 6, 12, 25, 50, 75, and 100 μM), had cell viabilities of 91.41%, 95.92%, 88.38%,89.38%,86.26%,89.21%,and 78.88%, respectively (Fig. 1A). Therefore, the 50% cytotoxicity concentration (CC_{50}) of BQR was estimated over 100 μM. Subsequently, the treatment of FMDV-infected cells with serially diluted BQR for 48 h, resulted in inhibitory rates of 3.50%, 14.95%, 34.40%, 50.99%, 63.08%, 73.73%, and 66.96%, respectively (Fig. 2A). q-PCR analyses showed that the viral mRNA levels declined in a dose-dependent manner (Fig. 2B and 2D). Anti-FMDV potency was then verified using an immunofluorescence assay on the infected cells treated with BQR at serial dilutions (3, 6, 12, 25, 50, 75, and 100 μM) 12 h after infection (Fig. 3). A dose-dependent reduction was detected in the fluorescence signal representing FMDV antigen-expressing cells. BQR thus effectively inhibited FMDV replication, with EC_{50} and SI values of 29.25 μM and 3.41, respectively. The antiviral effects of BQR were further investigated by another representative member FMDV species, A/GDMM/CHA/2013. It was noted that a 92.86% protection rate from CPE was observed in the cells treated with BQR at 3 μM after infection with A/GDMM/CHA/2013 (Fig. 2C). Based on these data, a selectivity index ($SI = CC_{50}/EC_{50}$) of approximately 33.33, was obtained.

3.2. Mechanism of action of BQR

To identify which stage of virus replication BQR inhibited, a time-of-addition experiment was performed. IBRS-2 cells were incubated with BQR at 0, 2, 4, 8, and 16 h post-infection. Infected cells without BQR treatment were used as a control (Fig. 4). Viral mRNA was noticeably inhibited by BQR at 8 h post-infection (p.i.), which was equivalent to BQR treatment at 0 h p.i., indicating that BQR blocked FMDV replication at the early stages of infection. Additionally, no virus protein expression was detected when BQR was added at 8 h p.i., whereas viral antigen expression rose drastically to a high level after BQR addition at 16 h p.i. These data suggested that BQR exerted a strong anti-FMDV effects prior to 8 h p.i., and that the inhibitory effect declined drastically after 16 h p.i.

A previous study has shown that one of the potential antiviral mechanisms of BQR is through the inhibition of DHODH activity. To determine whether this was involved in inhibition of FMDV, uridine was added to infected cells, when treated with BQR. Uridine alone had no effect to the cells and addition of uridine (25, 50, and 200 μM) together with BQR for 48 h completely restored the protection levels of infected cells (Fig. 5). This was accompanied by the recovery of virus protein expression measured by Western blot.

3.3. Antiviral activity of BQR in vivo

To test the anti-FMDV effect of BQR *in vivo*, we injected antiviral agents into suckling mice and then challenged them with a 100 LD_{50} of FMDV O/MYA98/BY/2010. The suckling mice treated with BQR had ruddy skin; responded to touch; and had increased mental awareness compared with the infected groups (Fig. 6B). *In vivo* results showed that BQR significantly prolonged the survival time of infected suckling mice ($P < 0.0001$) (Fig. 6A). On day 5 p.i., there was still a 25% survival rate in the treated group. In contrast, severe pathological changes were observed in FMDV-infected mice, which were characterized by vacuolar degeneration in myocardial mesangial cells and in vacuoles in myocardial fibers. Inflammatory cell infiltration was also observed around the necrotic area and interstitium, as was a decrease and dissolution of the myocardial space. As shown in Fig. 7, BQR treatment significantly alleviated lesions in the heart tissue of FMDV-infected mice ($P < 0.05$). Less damage was observed in BQR-treated mice, although a small amount of inflammatory cell infiltration was found around the interstitial space.

4. Discussion

Foot and mouth disease has been considered a serious disease for centuries and has caused significant losses to the livestock industry since its first appearance. In addition, it is still a constant threat due to its rapid infection rate and high transmission rate [19]. BQR was originally identified and developed as an antimetabolite in cancer and was subsequently demonstrated to exhibit immunosuppressive activity, on the basis that tumor cells rely heavily on *de novo* nucleotide synthesis, lowering pyrimidine synthesis (by use of BQR) and possibly interfering

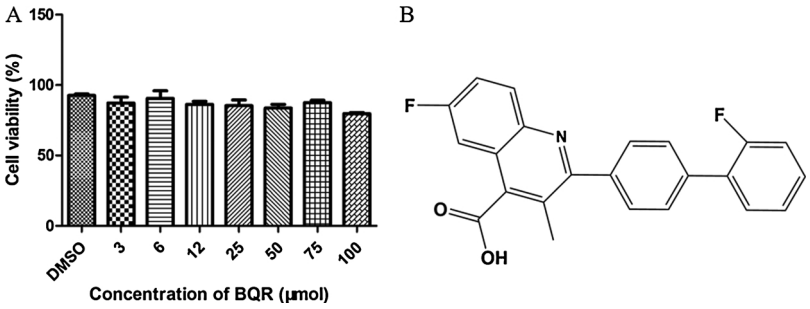

Fig. 1. Cytotoxicity of brequinar in IBRS-2 cells measured using an MTS assay. Cells were incubated with different concentrations of brequinar for 72 h, and a cell viability assay was performed via an MTS assay. Data are expressed as the mean ± S.D. of three independent experiments. (A) The chemical structure of brequinar. (B) The cytotoxic effect of brequinar treatment on IBRS-2 cells.

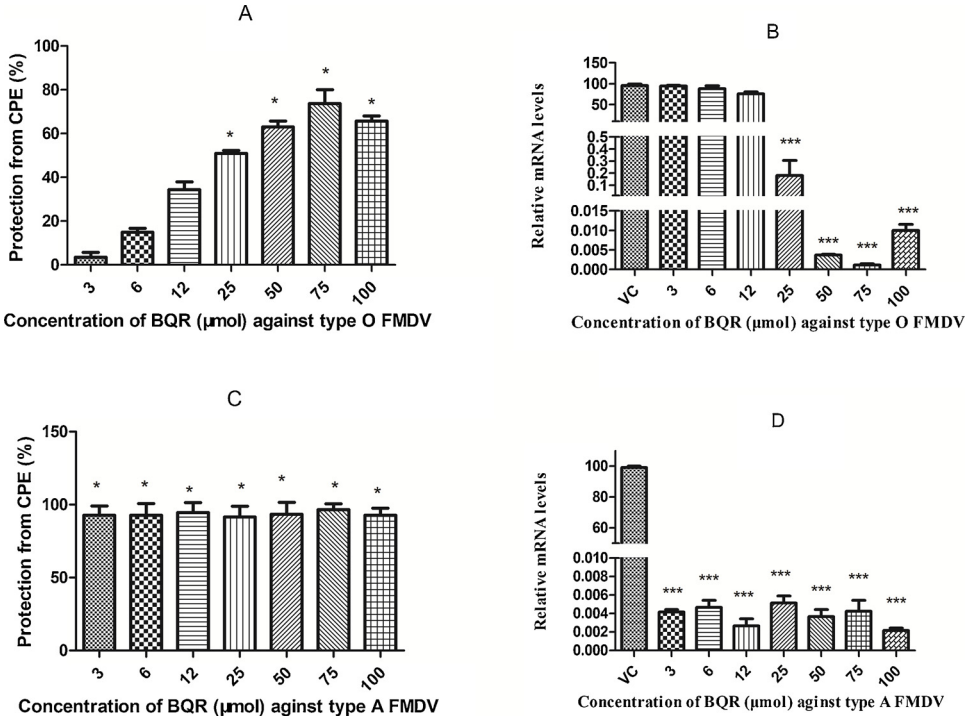

Fig. 2. Antiviral effect of brequinar in IBRS-2 cells. IBRS-2 cells were infected with O/MY98/BY/2010 (A) and A/GD/MM/2013 (C) and incubated with various concentrations of brequinar and cell viability was determined by MTS assay. (B, D) The relative mRNA level was determined by amplifying a portion of FMDV 2B gene using Q-PCR. Relative expression (fold change) in comparison to the control group in the absence of brequinar (set as 100) is illustrated. Data are presented as mean ± S.D. of three independent experiments. *P<0.05, *** P< 0.001 compared to the control.

with the rapid proliferation of lymphocytes [20]. Furthermore, the antiviral effect of BQR has been demonstrated against several DNA and RNA viruses. However, to the best of our knowledge, there have been no previous definitive studies on the antiviral activity of BQR against FMDV.

In the current study, we clarified the antiviral activity of BQR against FMDV. No cytotoxicity was observed at the tested concentrations and treatment with 50 μM BQR provided over 50% protection to IBRS-2 cells from CPE induced by O/MYA98/BY/2010 with an EC_{50} and SI value of 29.25 ± 8.17 μM and 3.41, respectively. Interestingly, 3 μM BQR could provide over 90% protection to IBRS-2 cells infected with A/GD/MM/2013 with an SI value of 33.33. Therefore, A/GD/MM/2013 appeared to be nearly 10 times more sensitive to the effect of BQR than O/MYA98/BY/2010. To investigate the action of BQR on FMDV infection in more detail, we investigated the effect of the drug using a time-of-addition assay. BQR effectively inhibited the expression of FMDV mRNA and protein expression between 0 and 8 h p.i.; however, at 16 h p.i., this inhibition was lost. Collectively, BQR was able to inhibit the early stages of the viral cycle. Previous studies have shown that BQR inhibits the activity of cellular DHODH, a rate-limiting enzyme in the *de novo* synthesis of pyrimidines, leading to depletion of the intracellular pyrimidine pool [15,21]. In agreement, we also found that uridine supplementation attenuated the anti-FMDV effects of BQR.

Interferon-stimulated genes (ISGs) are known as a type of antiviral effector, which are induced by interferons. However, in recent years, researchers have revealed that some nucleotide synthesis inhibitors also trigger expression of ISGs, such as IRF1, DDX58, and IRF7 [22–24]. Yeo et al. found that treatment of cells with BQR alone could activate interferon-stimulated response elements [25]. In the following study, it is needed to investigate that whether antiviral ISGs can be induced upon

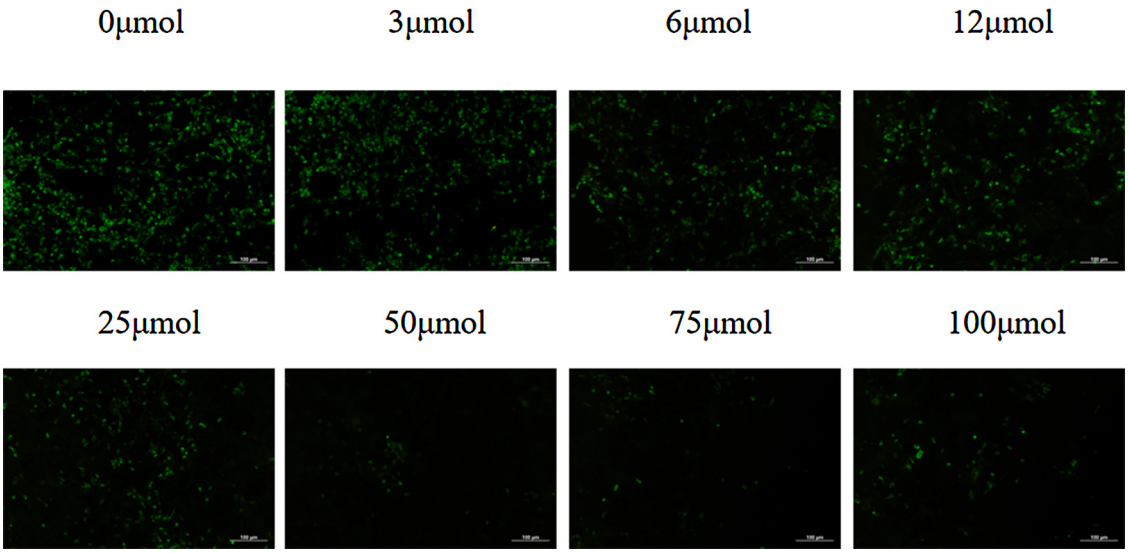

Fig. 3. Anti-FMDV activity of brequinar detected by IFA. A indirect immunofluorescence assay of FMDV-infected IBRS-2 cells was performed to detect the antiviral activity of increasing concentrations of brequinar.

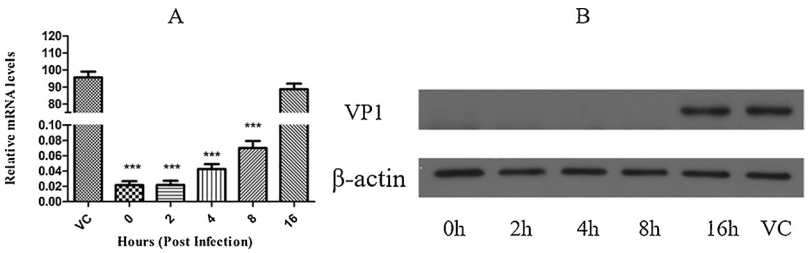

Fig. 4. Inhibitory effects of brequinar at different times post infection. Brequinar was added to the infected IBRS-2 cells at different time post infection. The virus mRNA and VP1 protein levels was examined at 48 h post infection using Q-PCR (A) and Western Blot (B), respectively. Experiments were done in triplicate, and all differences are statistically significant in relation to control (***$P < 0.001$). The data were analyzed by one-way ANOVA.

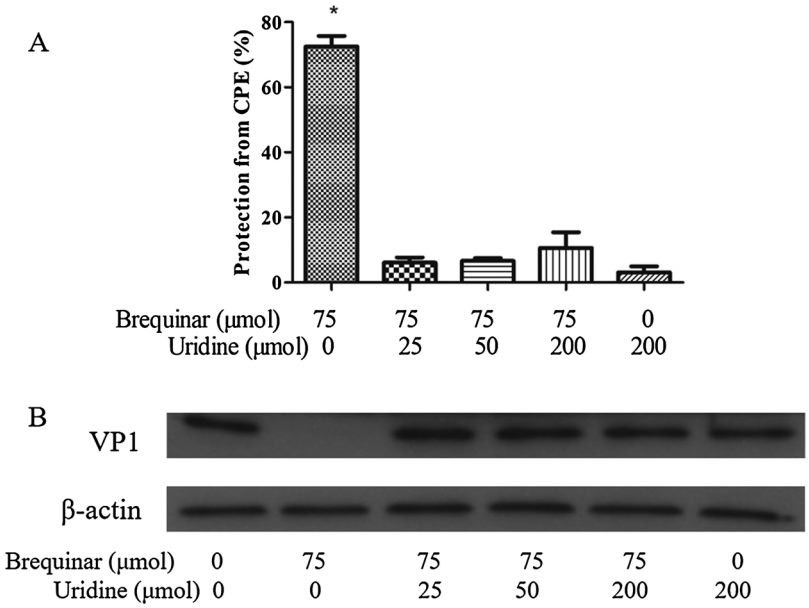

Fig. 5. The attenuated effect by uridine supplementation. A. Effect of uridine supplementation on viral cytopathic effect of FMDV. Data are presented as mean ± S.D. of three independent experiments. B. The viral VP1 protein and β-actin were detected by western blot. β-actin was used as an internal control. 25, 50, 200, 200 μM uridine were added to FMDV infected IBRS-2 cells, when treated with 75 μmol/L brequinar for 48 h. Cell viability and VP1 protein was analysed as described above. Data are the Mean ± SD of three independent experiments.

treatment with BQR.

Currently, combination therapy is commonly practiced as an antiviral treatment to minimize drug resistance. Recently, researcher reported that the combination of nucleoside analogs and nucleoside synthesis inhibitors exhibited strong antiviral synergy. This result suggested that synergy can be achieved with compound pairs in which one compound suppresses the synthesis of the nucleoside for which the other compound is a corresponding nucleoside analog [25]. Therefore, whether BQR and nucleoside analogs have synergistic effects against FMDV needs further investigation.

Previous studies have demonstrated that BQR is effective against several DNA and RNA viruses; however, nearly all of these studies tested the efficacy of BQR *in vitro*. In our study, we demonstrated the antiviral activity of BQR *in vivo*, where suckling mice were challenged with FMDV, with results indicating that 25% of the mice challenged with a 100 LD_{50} of FMDV survived in the BQR-treated group following a period of 5 days; whereas mice in the non-treated groups were died after 60 h p.i.. Further study is necessary to test the antiviral effect of BQR in natural hosts such as swine, cattle, and goats, and against more FMDV strains.

In conclusion, we have demonstrated that BQR has substantial antiviral activities against FMDV *in vitro* and *in vivo*. Specifically, BQR exhibited antiviral effects on FMDV replication at early stages of infection, which were attenuated by uridine supplementation. The results presented here broaden the possibility for future *in vivo* testing of BQR in a natural host of FMDV infection or in other viral diseases.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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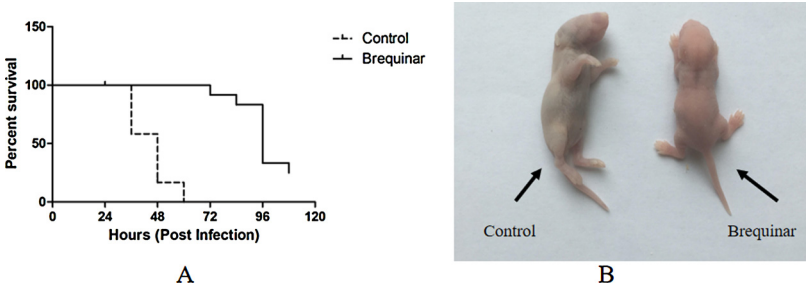

Fig. 6. In vivo activity of brequinar. Suckling mice were inoculated intranasally with 50 μg brequinar or PBS containing 10% DMSO and 5% Tween-80 (Control). 2 h later, all the animals was infected with 100LD_{50} O/MY98/BY/2010. Animal survival was monitored for 5 days. (A) The survival curve was performed using GraphPad Prism5. (B) The morphological observation of treated and non-treated infected mice at 60 hpi.

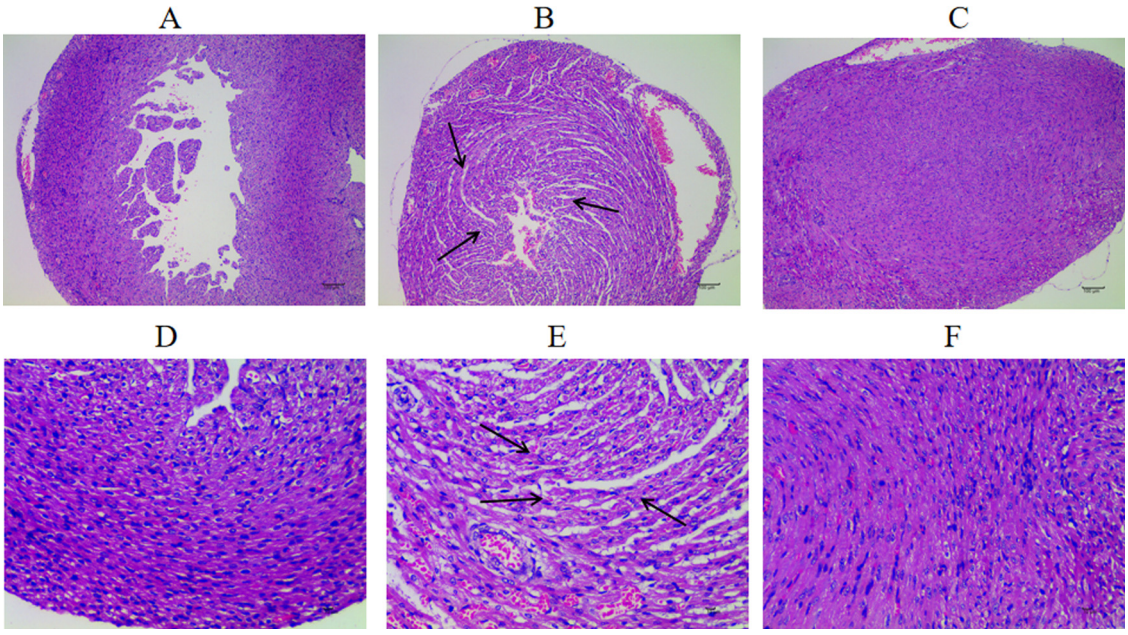

Fig. 7. The pathological changes of heart tissues at 34 hpi were observed after H & E staining. Five mice of each group were sacrificed on 34 h after viral exposures, and tissue samples were obtained for pathological examination. (A, D) Heart tissue from the non-infected mice. (B, E) Heart tissue from non-treated infected mice. (C, F) Heart tissue from treated and infected mice. Tissue damage was identified and is indicated by black arrows. A, B, C (magnification, 100×); D, E, F (magnification, 400×).

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