Contents lists available at ScienceDirect

Antiviral Research

Suppression of pyrimidine biosynthesis by targeting DHODH enzyme robustly inhibits rotavirus replication

Sunrui Chen[a], Shihao Ding[a], Yuebang Yin[a], Lei Xu[b], Pengfei Li[a], Maikel P. Peppelenbosch[a], Qiuwei Pan[a], Wenshi Wang[a,*]

[a] Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, the Netherlands
[b] College of Life Sciences, Northwest A&F University, Yangling, Shaanxi, 712100, China

ABSTRACT

Rotavirus infection remains a great health burden worldwide especially in some developing countries. It causes severe dehydrating diarrhea in infants, young children, as well as immunocompromised and organ transplanted patients. Viral replication heavily relies on the host to supply nucleosides. Thus, host enzymes involved in nucleotide biosynthesis represent potential targets for antiviral development. Dihydroorotate dehydrogenase (DHODH) is the rate-limiting enzyme in the *de novo* biosynthesis pathway of pyrimidines. In this study, we demonstrated that two specific DHODH enzyme inhibitors, brequinar (BQR) and leflunomide (LFM) robustly inhibited rotavirus replication in conventional human intestinal Caco2 cell line as well as in human primary intestinal organoids. The antiviral effect is conserved in both laboratory strain SA11 and rotavirus strain 2011K isolated from clinical sample. Mechanistic study indicated that BQR and LFM exerted their anti-rotavirus effect through targeting DHODH to deplete pyrimidine nucleotide pool. Therefore, targeting pyrimidine biosynthesis represents a potential approach for developing antiviral strategies against rotavirus.

1. Introduction

Rotavirus (RV), a member of *Reoviridae* family, is a non-enveloped virus which has a double-stranded RNA genome of 11 segments surrounded by three concentric protein layers. It has been reported to cause estimated 25% of moderate-to-severe illnesses (Kotloff et al., 2013) and 30% of total diarrheal deaths during the first 2 years from birth (Wang et al., 2016a). The development and deployment of rotavirus vaccines was a breakthrough in the fight against diarrheal diseases. Two rotavirus vaccines, Rotarix™ and Rotateq, are licensed and widely available in several countries (Yin et al., 2018). Nevertheless, due to limited resources, developing countries including India, Nigeria, Pakistan, Ethiopia and the Democratic Republic of the Congo bear the major burden of mortality (Groome et al., 2014). In addition, immunocompromised patients are also under the risk of RV infection which would cause remarkable morbidity and mortality (Lee and Ison, 2014; Sugata et al., 2012; Yin et al., 2015c). For the treatment of rotavirus gastroenteritis, intravenous fluid supply has been used for treatment of dehydration from diarrhea. However, in the severe case of inpatients and immunocompromised patients who are suffering from prolonged diarrhea and fever, virus-specific treatment will be expected, if possible. In fact, there is still no FDA-approved medication available against rotavirus disease. Therefore, to ensure that the remaining burden of mortality and morbidity can be fully addressed in the future, research on the development of novel antiviral strategies is highly needed.

Cellular nucleotides, composed of purines and pyrimidines, play a vital role in constituting nucleic acids RNA and DNA. *De novo* synthesis and salvage pathway are the two pathways for nucleic acid synthesis *in vivo* (Evans and Guy, 2004). Viral replication heavily relies on host supply of nucleoside biosynthesis. Therefore, host enzymes involved in nucleoside biosynthesis represent potential targets for antiviral development. Ribavirin, the most well-known antiviral drug, is such an inhibitor that suppresses guanine biosynthesis via inhibition of cellular IMP dehydrogenase (IMPDH). Several studies have indicated that depletion of cellular GTP pool is the primary mechanism by which ribavirin inhibits virus replication (e.g. flaviviruses and hepatitis E virus) (García et al., 2018; Nicolini et al., 2018). Along this line, we aim to investigate whether inhibitors of the pyrimidine biosynthesis pathway could be targeted for potential antiviral development against rotavirus.

Dihydroorotate dehydrogenase (DHODH) is sequentially the fourth and the rate-limiting enzyme in the *de novo* biosynthesis pathway of pyrimidines. It is located in the inner membrane of mitochondria, where it plays a role of converting dihydroorotate to orotate (Munier-Lehmann et al., 2013). Then, the multifunctional UMP synthase uses orotate to produce UMP, one of the essential precursors for synthesis of all other pyrimidine nucleotides. Several studies have reported that inhibition of DHODH enzyme suppresses a range of different viruses
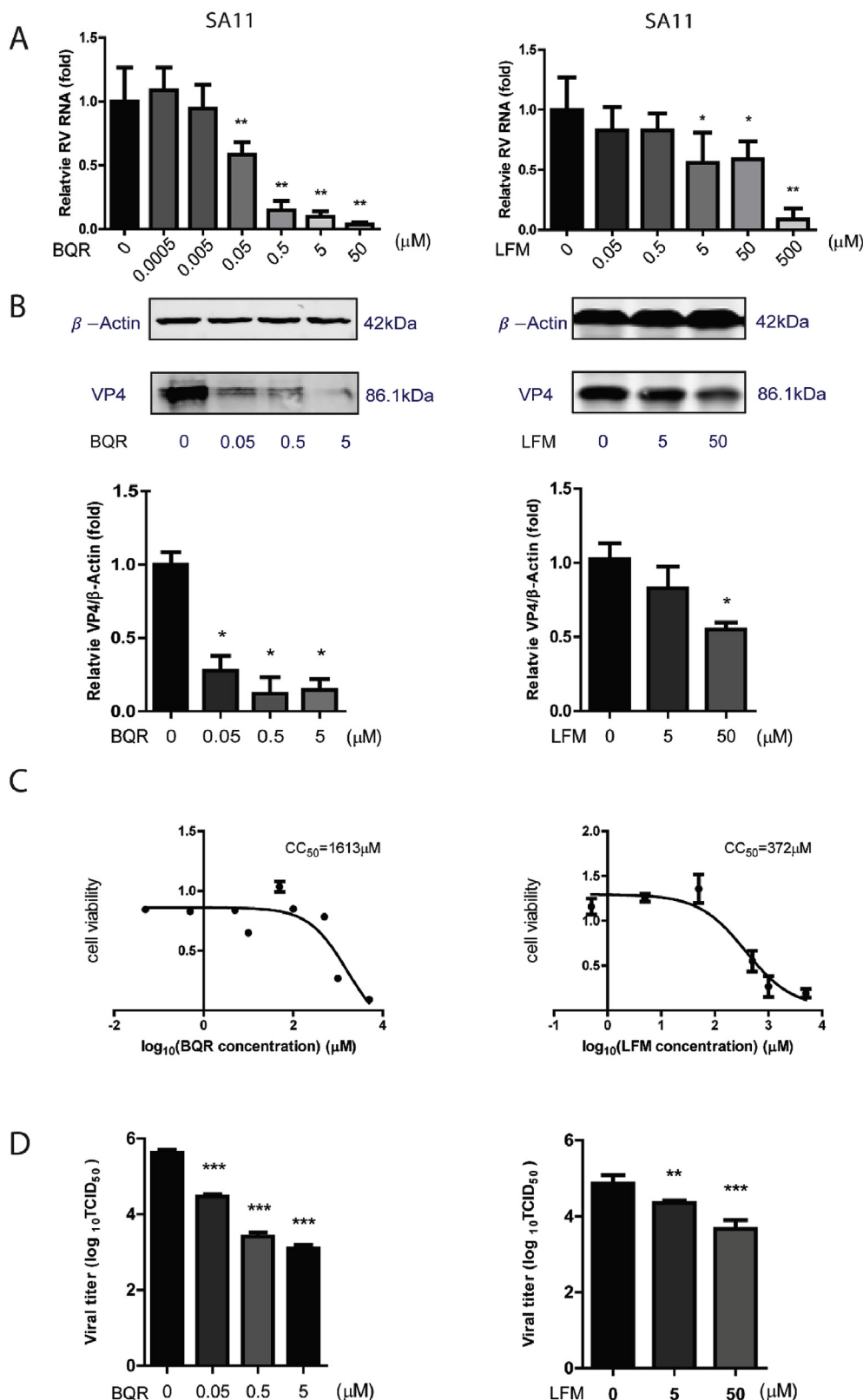
Fig. 1. BQR and LFM exert potent antiviral activity against rotavirus SA11 on Caco2 cells. Caco2 cells were infected with SA11 rotavirus. After infection, cells were treated with various concentrations of BQR or LFM. (A) qRT-PCR analysis indicated that BQR (n = 6) and LFM (n = 6) exerted potent antiviral effect against rotavirus replication. Data were normalized to housekeeping genes *GAPDH* and presented relative to the control (CTR) (set as 1). Data represent means ± SEM. *, $P < 0.05$; **, $P < 0.01$. (B) Western blot analysis showed that BQR (n = 4, 0.05–5 μM) and LFM (n = 4, 50 μM) inhibited rotavirus replication. Data represent means ± SEM. *, $P < 0.05$. (C) 50% cytotoxic concentration (CC_{50}) curves of BQR (left panel) and LFM (right panel) were determined by MTT assays. (D) The supernatant of each well was harvested after freeze and thaw for three times, virus titer from different groups was measured (n = 4. Data represent means ± SEM. ***, $P < 0.001$).

replication (Hoffmann et al., 2011; Luthra et al., 2018; Tan et al., 2005; Wang et al., 2011, 2016b).

In this study, we report that brequinar (BQR) and leflunomide (LFM), two DHODH inhibitors, potently inhibit rotavirus replication in human intestinal cell line as well as three dimensional (3D) cultured primary intestinal organoids. Mode-of-action studies demonstrate that their antiviral activity is mainly achieved via the inhibition of DHODH, resulting in the depletion of intracellular pyrimidine pools. Collectively, our study has stressed the concept that targeting pyrimidine biosynthesis represents a potential approach for antiviral development against rotavirus.
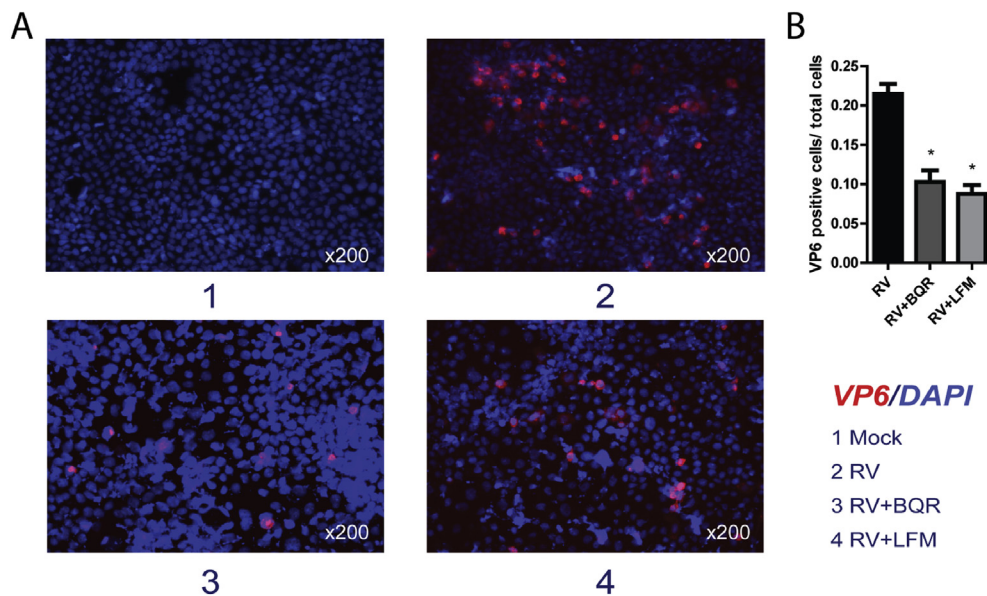
Fig. 2. BQR and LFM inhibit the expression of rotavirus VP6 protein. (A)Indirect fluorescence microscope analysis of viral structural protein VP6 (red) upon treatment with BQR (0.05 μM) and LFM (5 μM). Nuclei were visualized by DAPI (blue). (B) The ratio of VP6 positive cells/total cell number was quantified (n = 4). Data represent means ± SEM. *, $P < 0.05$.

VP6/**DAPI**

1 Mock
2 RV
3 RV+BQR
4 RV+LFM

2. Materials and methods

2.1. Reagents

Brequinar (BQR) sodium salt hydrate (Method Detection Limit [MDL] no. MFCD21363375), leflunomide (LFM) (Chemical Abstracts Service [CAS] no. 75706-12-6), 6-azauracil (6-AU) (MDL no. MFCD00006456) and uridine (CAS no. 58-96-8), were purchased from Sigma. All the reagents were dissolved in dimethyl sulfoxide (DMSO).

2.2. Viruses

Simian rotavirus SA11, a broadly used and well-studied laboratory strain (Cecílio et al., 2012), was gifted by Karen Knipping from Nutricia Research Utrecht, The Netherland. In the study, rotavirus SA11 was prepared as described (Knipping et al., 2012). Stool sample collected from rotavirus infected patient was obtained from the Erasmus MC biobank, Department of Virosience, Erasmus Medical Center, Rotterdam. This stool sample 2011K was taken during patient diarrhea period and tested for enterovirus, parechovirus, norovirus genogroups I and II, rotavirus, adenovirus, astrovirus and sapovirus by PCR. Sample 2011K is only positive to rotavirus and negative to other virus mentioned above. Sample information was also described in previous studies (Corless et al., 2002; Hoek et al., 2013; van Maarseveen et al., 2010).

2.3. Cell lines and human primary intestinal organoids

Human colon cancer cell line Caco2 and human embryonic kidney cell line 293T (HEK 293T) were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium) containing 20% (vol/vol) heat-inactivated fetal calf serum (FCS, Sigma–Aldrich, St. Louis USA) and 100 U/ml Penicillin/Streptomycin (P/S, Gibco, Grand Island, USA) solution. A humidified incubator was used for cells culturing at 37 °C in 5% CO_2. Cells were analyzed by genotyping and confirmed to be mycoplasma negative.

Human primary small intestinal organoids culture was performed as described previously (Yin et al., 2015a). Fragments of intestinal biopsies or surgically resected intestinal tissues were treated with complete chelating solution (CCS, MilliQ H_2O was supplemented with 1.0 g/L of $Na_2HPO_4-2H_2O$, 1.08 g/L of KH_2PO_4, 5.6 g/L of NaCl, 0.12 g/L of KCl, 15 g/L of Sucrose, 10 g/L of D-Sorbitol and 80 μg/L of DL-dithiothreitol) followed by 8 mM EDTA. Crypts were finally collected and suspended in

40 μL growth factor reduced phenol-red free Matrigel (Corning, Bedford, USA). Then, they were incubated at 37 °C with 5% CO_2 with culture medium containing CMGF, 2% (vol/vol) of B-27® Supplements (Gibco, Grand Island, USA), 1% (vol/vol) of N2® Supplements (Gibco, Grand Island, USA), 500 pg/mL of EGF, 1 mM n-Acetyl Cysteine, 10 mM Nicotinamide, 0.5 μM A83-01 (TGF-b inhibitor), 3 μM SB202190 (p38 inhibitor), 20% (vol/vol) of R-Spondin 1 (conditioned medium), 10% (vol/vol) of Noggin (conditioned medium) and 50% (vol/vol) of Wnt3a (conditioned medium). Culture medium was refreshed every 2–3 days, and HIOs were passaged every 6–7 days.

2.4. Inoculation of SA11 rotavirus and clinical derived strain 2011K

Caco2 cells were washed, suspended in T75 flask and subsequently seeded into a 48-well plate (5×10^4 cells/well). Culture medium was discarded when cell confluence was approximately 80%, and cell monolayers were washed twice with PBS. 100 μL of serum-free DMEM medium, then rotavirus (MOI = 0.7) with 5 μg/mL of trypsin (Gibco, Paisley, UK) were added and incubated at 37° C with 5% CO_2 for 60 min for infection, followed by 4 times washing with PBS to remove un-attached viruses Then, cells were incubated with culture medium containing 5 μg/ml of trypsin at 37 °C with 5% CO_2.

2.5. Virus production assay

Virus, cell line and HIOs were treated as previous work (Yin et al., 2015b). In short, caco2 cells were inoculated with SA11 rotavirus (MOI = 0.7) then harvested after 48 h incubation with serum-free medium to perform assays as followed. In parallel, human primary intestinal organoids were infected with 10 times higher concentration of SA11 rotavirus than cell infection for 1.5 h followed by 4 times washing with PBS. Afterwards, HIOs with no Matrigel remain were spun down at 500 g for 10 min to adhered to the bottom of 24-well plate coated with Collagen R solution (SERVA, Heidelberg, Germany). Organoids culture medium was added gently and HIOs were incubated at 37 °C with 5% CO_2. Culture medium and HIOs were harvested respectively after 48 h to detect and enumerate rotavirus. Virus titers from supernatants were determined by calculating the $\log_{10}TCID_{50}/mL$ in Ma104 cells using the method developed by Reed and Muench (Reed and Muench, 1938).

2.6. RNA isolation, cDNA synthesis and qRT-PCR

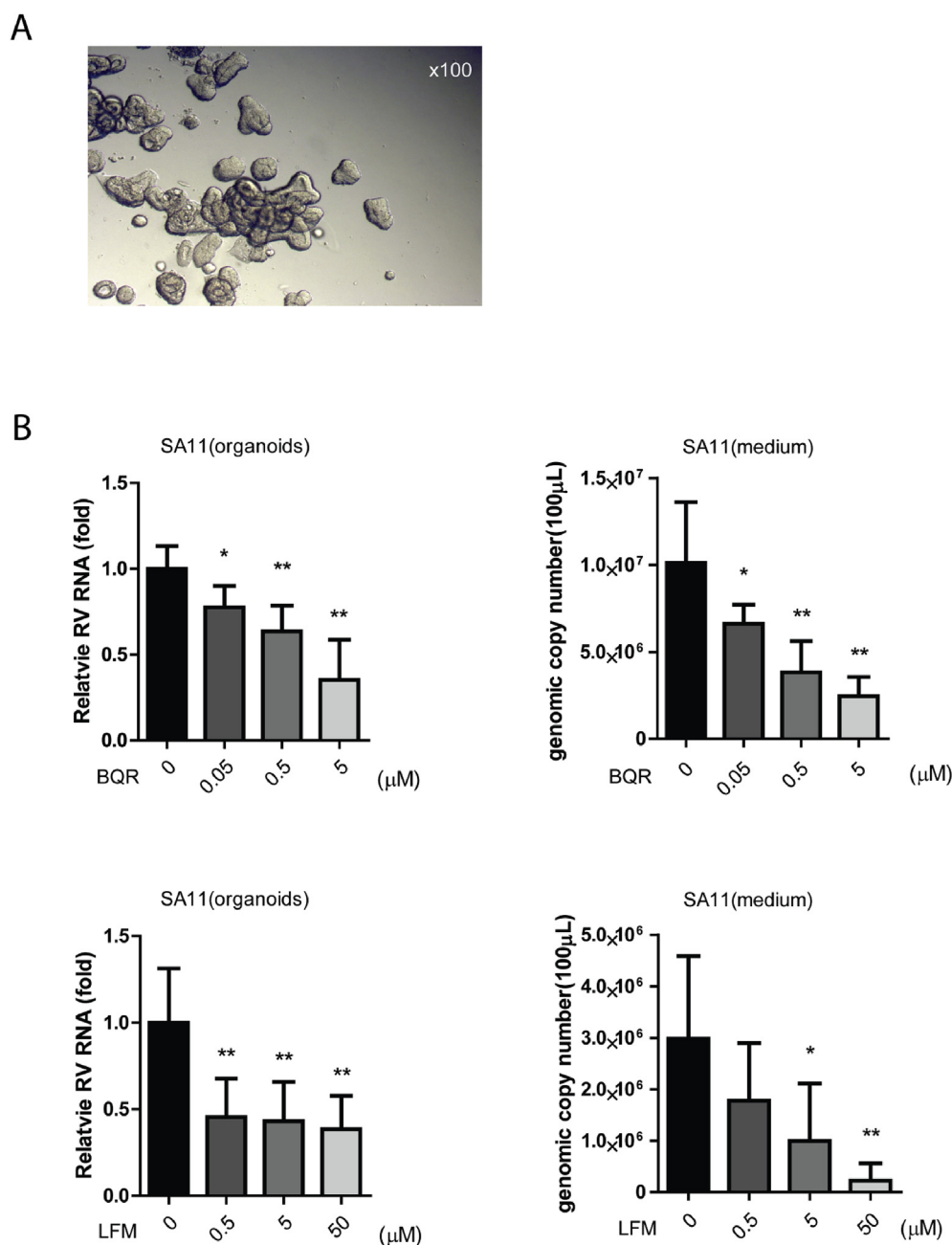Total RNA was isolated using Macherey-Nagel NucleoSpin® RNA II

Fig. 3. BQR and LFM exert potent antiviral activity against rotavirus SA11 on human intestinal organoids. (A) Representative microscopy image of cultured human small intestinal organoids. (B) BQR (n = 6) as well as LFM (n = 6) inhibit SA11 replication and virus production after 48 h incubation. The relative rotavirus RNA and genomic copy number were analyzed by qRT-PCR. Data represent means ± SEM. *, $P < 0.05$; **, $P < 0.01$ (Data of standard curve is in Supplementary Fig. S1).

kit (Bioke, Leiden, Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). Total RNA reverse transcription was performed by using a cDNA Synthesis kit (TAKARA BIO INC.) with random hexamer primers. Real-time PCR reactions (50 °C for 2 min, 95 °C for 10 min, followed by 50 or 60 cycles of 95 °C for 15 s and 58 °C for 30 s and 72 °C for 10 min) were performed with SYBRGreen-based real-time PCR (Applied Biosystems®, Austin, USA) according to the manufacturer's instruction. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as housekeeping gene. Relative gene expressions were normalized to GAPDH using formula $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = \Delta CTsample - \Delta CTcontrol$). Template control and reverse transcriptase control were included in all RT-qPCR experiments. All qRT-PCR primers are listed in Supplementary Table 1.

2.7. Quantification of rotavirus genome copy numbers

An amplicon of the SA11 (a fragment of VP6 gene from 564 to 718) was cloned into the pCR2.1-TOPO vector (Invitrogen, San Diego, CA) to generate a template for quantifying rotavirus genome copy number. The plasmid was extracted by Quick Plasmid Miniprep Kit (Invitrogen, Lohne, Germany) following manufacturer's instructions. A series of dilutions (from 10^{-2} to 10^{-10}) were prepared and then were amplified and quantified by qRT-PCR to generate a standard curve. This standard curve was generated by plotting the log copy number versus the cycle threshold (CT) value (Fig. S1). Copy numbers were calculated by using the following equation: Copy number (molecules/μl) = [concentration (ng/μl) × $6.022 × 10^{23}$ (molecules/mol)]/[length of amplicon × 640 (g = /mol) × 10^9 (ng/g).
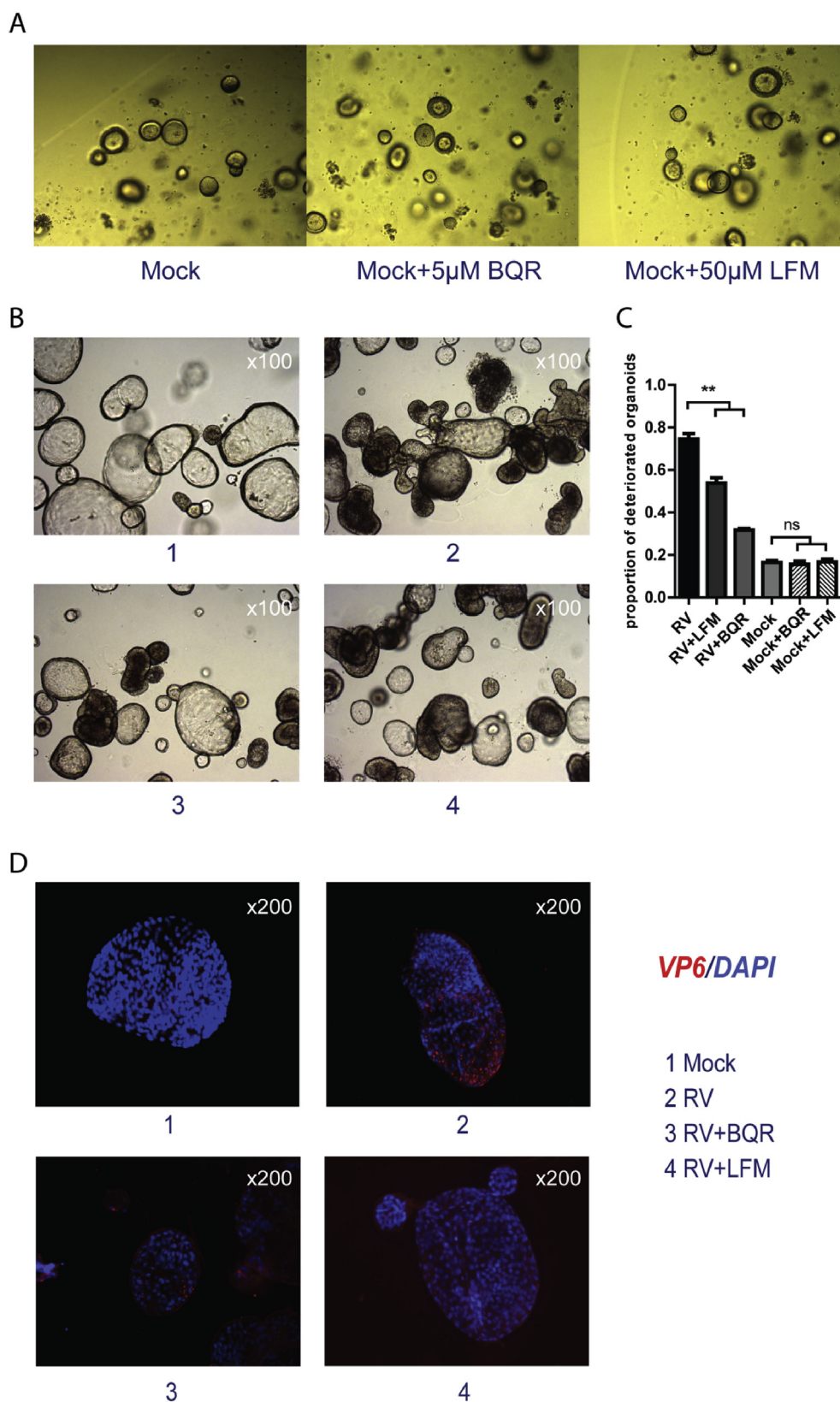
Fig. 4. Microscopy image analysis indicated that BQR and LFM exert potent antiviral activity against rotavirus SA11 on human intestinal organoids (HIOs). (A) Microscopy image analysis of HIOs treated with 5 μM BQR and 50 μM LFM. (B) Microscopy image analysis of HIOs infected by rotavirus SA11 and treated with CTR, 5 μM BQR or 50 μM LFM. (C) Scoring of proportion of deteriorated HIOs in different random visions, n = 6. Data represent means ± SEM. **, *P* < 0.01. (D) 5 μM BQR and 50 μM LFM inhibited rotavirus SA11 replication on HIOs. Rotavirus structural protein VP6 (red). Nuclei were visualized by DAPI (blue).
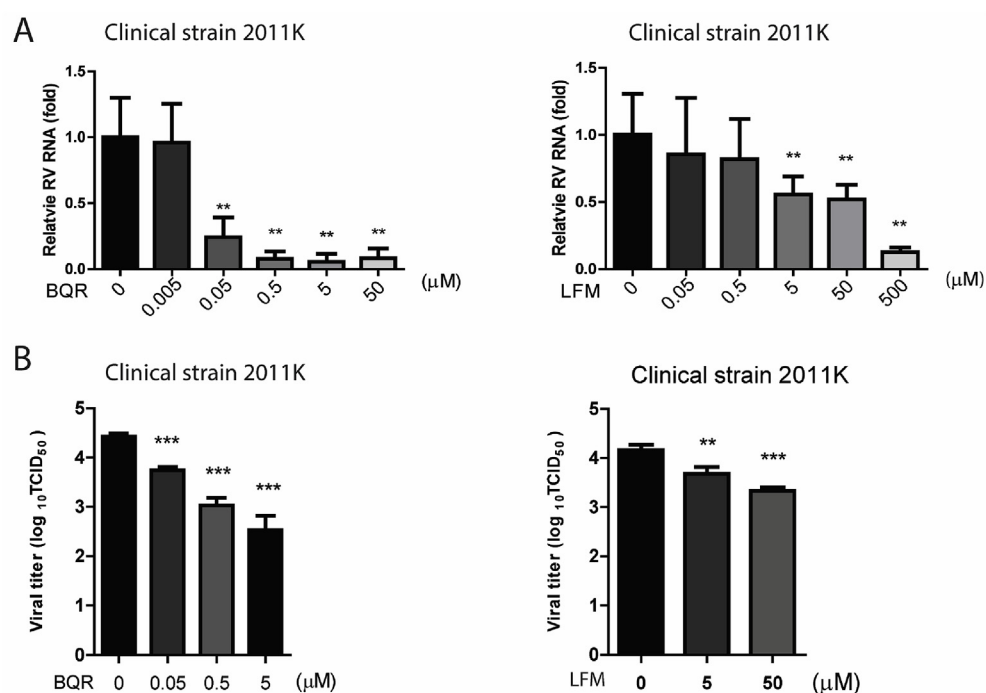
A

Clinical strain 2011K

Clinical strain 2011K

B

Clinical strain 2011K

Clinical strain 2011K

Fig. 5. BQR and LFM showed antiviral effect against clinical-derived rotavirus strains. (A) qRT-PCR analysis indicated that BQR (n = 6) and LFM (n = 6) exerted strong antiviral effect against rotavirus 2011K. Data were normalized to house-keeping genes *GAPDH* and are presented relative to the control (CTR) (set as 1). Data represent means ± SEM. *, $P < 0.05$; **, $P < 0.01$. (B) Virus titer of rotavirus 2011K were measured upon BQR and LFM treatment respectively (n = 4). Data represent means ± SEM. ***, $P < 0.001$.

2.8. Determination of compromised organoids scoring

The scoring process was performed as previously (Grabinger et al., 2014). In short, minimum of 100 organoids were counted after 3 days of passaging. After 1.5 h incubation with SA11 rotavirus, HIOs were cultured in matrigel and treated with different agents for 48 h, and HIOs were counted and defined as viable or dead via their morphological appearance in the optimal microscope (Axiovert 40 CFL, Zeiss, Oberkochen, Germany) in at least three random visions in different parallel wells, the description of this scoring system is showed in Fig. S3. The proportion of deteriorated organoids was calculated as (viable/total) %.

2.9. Gene knockdown assays

Lentiviral shRNA vectors, targeting dihydroorotate dehydrogenase (DHODH) gene or non-targeted control lentivirus were produced in 293T cells as previous work (Qu et al., 2018). Caco2 cells were inoculated with lentivirus to generate stable gene knockdown cells. Cells were subsequently selected with 5 μg/mL puromycin (Sigma). After pilot study, two shRNA vectors exerting optimal gene knockdown were selected. Knockdown and control Caco2 cells were incubated with rotavirus as described above. All shRNA primers were listed in Supplementary Table 2.

2.10. Western blot assay

Lysed cells were subjected to SDS-PAGE, and proteins were transferred to PVDF membrane (Immobilon-FL). DHODH (ab 54621, 1:1000, mouse monoclonal, Abcam), SA11 rotavirus VP4 (1:1000, HS-2, mouse monoclonal; provided by professor Harry Greenberg, Stanford University School of Medicine, USA) was detected by western blot analysis and β-actin protein was detected as loading control (sc-47778, 1:1000, mouse monoclonal; Santa Cruz). The intensity of the immunoreactive bands of blotted protein was quantified by the Odyssey V3.0 software.

2.11. Immunofluorescence analysis

After rotavirus infection and BQR treatment, HIOs were harvested and fixed in 4% paraformaldehyde in PBS at 4 °C for 10 min. Fixed HIOs were added into the CytoSpin II Cytocentrifuge (Shandon Scientific Ltd, Runcorn, England), then spined down at 1000 rpm for 2 min. The slides containing HIOs were rinsed 3 times with PBS for 5 min each, followed by treatment with 0.1% (vol/vol) Tritonx100 for 4 min. Subsequently, the slides were twice rinsed with PBS for 5 min, followed by incubation with milk-tween-glycine medium (0.05% tween, 0.5% skim milk and 0.15% glycine) to block background staining for 30 min. Slides were incubated in a humidity chamber with anti-rotavirus antibodies (1:250, mouse monoclonal; Abcam) diluted in milk-tween-glycine medium at 4 °C overnight. Slides were washed 3 times for 5 min each in PBS prior to 1 h incubation with 1:1000 dilutions of the anti-mouse IgG (H + L, Alexa Fluor® 594) secondary antibody. Nuclei were stained with DAPI (4, 6-diamidino-2-phenylindole; Invitrogen). Images were detected using EVOS FL cell imaging system (Thermo Fisher).

2.12. MTT assay

The CC_{50} values of BQR and LFM (1613 μM for BQR and 372 μM for LFM) were determined by MTT assay (Fig. 1C) as previous study (Qu et al., 2018). Approximately 1×10^4 Caco2 cells were seeded per well in 96-well plate. After 48 h, cells were incubated with 10 μL 5 mg/ml MTT for 3 h, then replaced with 100 ml dimethyl sulfoxide (DMSO) medium (Sigma). Absorbance (490 nm) was analyzed accordingly.

2.13. Statistics

All numerical results were performed at least two technical replicates in twice biological replicates and are reported as Mean ± SEM. The statistical significance of differences between means was assessed with the Mann-Whitney test (GraphPad Prism 5; GraphPad Software Inc., La Jolla, CA). The threshold for statistical significance was defined as P ≤ 0.05.
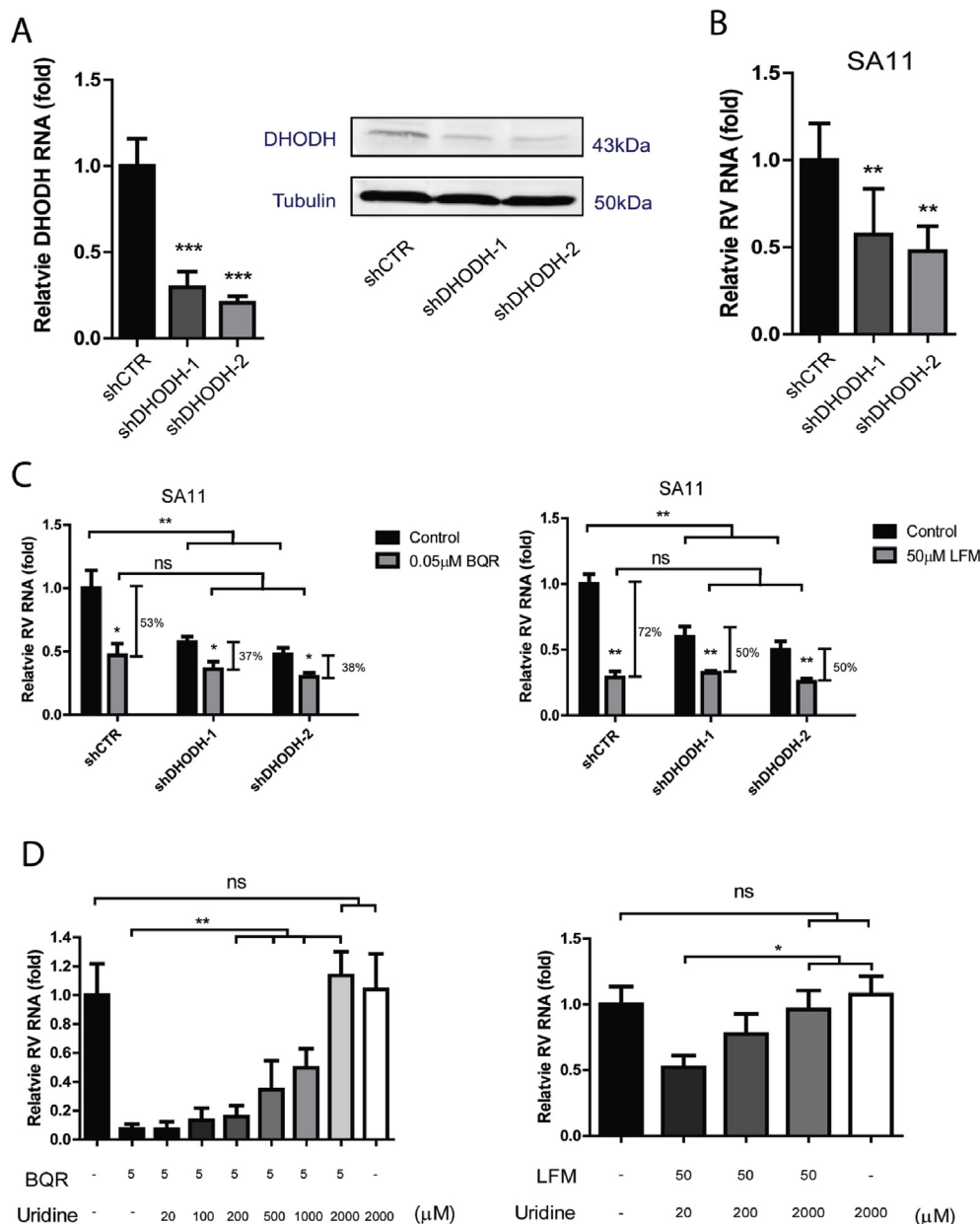
Fig. 6. BQR and LFM exert its anti-rotavirus effect through targeting DHODH to deplete pyrimidine nucleotide pool. (A) qRT-PCR and western blot analysis confirmed the knockdown of DHODH in Caco2 cells (n = 9). Data represent means ± SEM. ***, $P < 0.001$. (B) Knockdown of DHODH significantly inhibits SA11 rotavirus replication in Caco2 cells (n = 6). Data represent means ± SEM. **, $P < 0.01$. (C) Anti-rotavirus effect of BQR and LFM was partially blocked in two clones of DHODH knockdown cells (n = 6). Data represent means ± SEM. *, $P < 0.05$. (D) Supplementation of exogenous uridine attenuates the anti-rotavirus effect of BQR and LFM in Caco2 cells (n = 6). Data represent means ± SEM. **, $P < 0.01$.

3. Result

3.1. BQR and LFM potently inhibit the replication of SA11 and patient-derived rotavirus strain 2011K

BQR is an immunosuppressive and antiproliferative compound which has been evaluated in multiple clinical trials as a potential treatment for cancer (Madak et al., 2017; Makowka et al., 1993). Similarly, LFM is also an immunosuppressive drug used in active moderate-to-severe rheumatoid arthritis and psoriatic arthritis (Davis et al., 1996). So we tried to investigate whether these two inhibitors of the pyrimidine biosynthesis pathway have potential anti-rotavirus effect. After infection by simian rotavirus SA11, Caco2 cells were treated with various concentrations of BQR and LFM for 48 h. BQR and LFM potently inhibited SA11 rotavirus replication in a dose-dependent manner as measured in both RNA and protein levels (Fig. 1A and B), while cell viability was not affected (Fig. 1C). The IC$_{50}$ values of BQR and LFM are 0.04917 μM and 48.98 μM respectively, while the CC$_{50}$ values are 1613 μM and 372 μM, indicating a high specificity of these two

compounds on rotavirus (Fig. 1C and Fig. S2). Consistently, SA11 rotavirus titer was also significantly decreased by BQR and LFM treatment (Fig. 1D). Indirect fluorescence microscopy analysis further confirmed that BQR and LFM could inhibit rotavirus replication (Fig. 2A and B). 3D cultured primary human intestinal organoids (HIO) represent state-of-art model for investigating intestinal physiology and pathology. Therefore, we further tested the antiviral activity of BQR and LFM based on the HIO model. Importantly, BQR and LFM reduced the levels of cellular viral RNA and inhibited virus production significantly, while no cytotoxicity effects were observed (Fig. 3A and B, Fig. S3A). For instance, with 5 μM of BQR, cellular viral RNA was decreased by 65% (n = 6, p < 0.01), and virus production was inhibited by 76% (n = 6, p < 0.01). Since rotavirus infection causes pathological and morphological changes on organoids, we randomly selected three scopes and quantified the proportion of compromised organoids upon different treatments. Both BQR and LFM could strongly decrease the proportion of compromised organoids (Fig. 4A–C). This notion was further supported by the evidence that BQR and LFM led to the decreased levels of rotavirus VP6 protein (Fig. 4D). Next, we expand our study to patient-
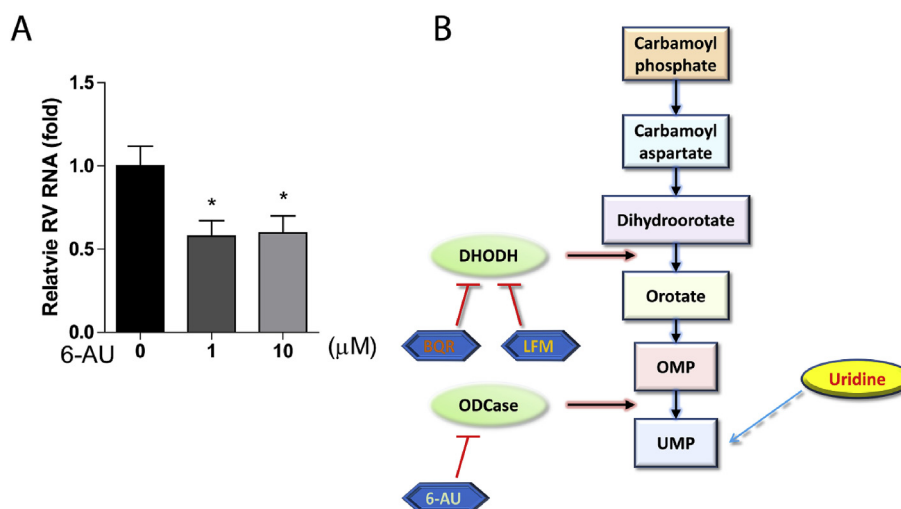
Fig. 7. Depleting pyrimidine nucleotide pool inhibits rotavirus replication. (A) qRT-PCR analysis showed that 6-AU, a specific inhibitor of ODCase, inhibited rotavirus replication (n = 6). Data represent means ± SEM. *, $P < 0.05$. (B) Schematic overview of *de novo* pyrimidine biosynthesis pathway.

derived rotavirus strain 2011K. Consistently, BQR and LFM profoundly inhibited virus replication of this rotavirus strain (Fig. 5). Collectively, these results demonstrated that BQR and LFM could potently inhibit rotavirus replication.

3.2. BQR and LFM, two DHODH inhibitors, inhibit rotavirus replication through depletion of the cellular pyrimidine nucleotide pool

Classically, BQR and LFM are two well-established DHODH inhibitors. Upon inhibition of DHODH, cellular pyrimidine biosynthesis pathway will be blocked, resulting in the depletion of pyrimidine nucleotide pool. To investigate whether these two compounds exert a similar mode-of-action against rotavirus, we employed both BQR and LFM for further mechanistic study. We first used an RNAi-based loss-of-function assay to study the role of DHODH. Two successful knock-down clones were confirmed by both qRT-PCR and western blot (Fig. 6A). And the silencing of DHODH resulted in compromised rotavirus replication (Fig. 6B). The specific effect was further confirmed when we added BQR and LFM to DHODH knockdown or control cell lines infected with SA11. Knockdown of DHODH abrogated the anti-rotavirus effect rate of BQR from 53% to 37% and 38% respectively. As for LFM, the effect rate was decreased from 72% to 50% on both knockdown cell lines (Fig. 6C). To further validate whether the antiviral effect of BQR and LFM was achieved via the depletion of pyrimidine pool, exogenous uridine was supplemented to cell culture medium. Of note, supplementation of pyrimidine dose-dependently abolished the anti-rotavirus effect of BQR and LFM. At the concentration of 2000 μM, BQR and LFM-related anti-rotavirus effect was totally abolished (Fig. 6D). Taken together, these results indicated that BQR and LFM exerts their anti-rotavirus effect through targeting DHODH to deplete pyrimidine nucleotide pool. Orotidine 5′-monophosphate decarboxylase (ODCase) serves as the downstream enzyme of DHODH, which catalyzes OMP to UMP. 6-azauracil (6-AU), a potent inhibitor of ODCase, was evaluated for its potential effect on rotavirus replication. Consistently, 6-AU also inhibits rotavirus replication (Fig. 7A). Conceptually, targeting pyrimidine biosynthesis represents a potential approach for antiviral development against rotavirus.

4. Discussion

Though rotavirus vaccine has been introduced, and the vaccine remarkably reduced the burden of rotavirus gastroenteritis in many developed countries, however, due to prevalence and diversity of the virus, one third to half of the children are still suffering from

vaccination failure in some developing countries (Macartney et al., 2000). Antiviral treatment shall serve as complement to vaccine. A drug repurposing approach offers many advantages for the identification of new antiviral strategies over the development of new drugs, since safety and pharmacology in humans are already well known. Therefore, if a drug, currently in clinical use, demonstrates considerable antiviral activity against rotavirus, there should be great potential to expand its application to combating rotavirus infection.

Of note, BRQ and LFM are two well-known immunosuppressive agents (Batt, 1999). As for LFM, it was reported as immunosuppressive therapy for bone marrow (Avery et al., 2004) and renal (Chon and Josephson, 2011) transplantation. Accumulating evidence indicated that organ transplant patients irrelevant to their ages are more vulnerable to rotavirus infection. Thus, the potential clinical prospects of BQR and LFM or their analogues may be of achieving "one stone two birds" effects: exerting immunosuppressive function and anti-rotavirus effect at the same time. Of note, BQR was reported to have some side effects, such as leukocytopenia, thrombocytopenia, reduced body weight gain or body weight loss, thymic atrophy, cellular depletion of bone marrow and splenic white pulp, and villous atrophy in jejunum (Pally et al., 1998). Similarly, LFM also has side effects, including gastrointestinal symptoms, rash or allergic reactions and alopecia (Emery et al., 2000; Mladenovic et al., 1995; Smolen et al., 1999; Strand et al., 1999). These kinds of clinical observation are comprehensible based on the fact that all drugs come with side effects. Importantly, the drug dosages of both drugs used in our study for inhibition of rotavirus are much lower than their previous clinical trial dosages (Maroun et al., 1993; Natale et al., 1992; Smolen et al., 1999). This might make BQR or LFM a preferable option for treating rotavirus infection, achieving high efficacy with less side effects. In addition, pyrimidine biosynthesis (Fig. 7B) represents a potential host pathway for novel antiviral drug development. These specific inhibitors of this pathway may represent as a starting point for the development of efficient anti-rotavirus drugs with more broad applications.

Intestinal carcinoma-derived cell lines such as Caco2 cell line are widely used as *in vitro* model to investigate rotavirus infection (Cuadras et al., 2002; Frias et al., 2012). Compared to such two-dimensional (2D) single cell type culture systems, the three-dimensional (3D) model of human primary intestinal organoids surpass 2D models by cell diversity and spatial structure on rotavirus infection (Yin et al., 2015a). Specifically, these primary intestinal organoids consist of many undifferentiated and heterogeneous cell types with similar functions as in the organ/tissue of origin (Sato and Clevers, 2012, 2013; Sato et al., 2009, 2011), including enterocytes, enteroendocrine cells, goblet cells,

Paneth cells and stem cells (Saxena et al., 2015). We have validated our results observed in conventional cell culture model also in the primary human organoids. Moreover, the use of rotavirus strains isolated from clinical samples leads our study one step closer to clinical therapy against rotavirus infection.

Nucleotides play an important role in host cell metabolism and are essential for virus infection. Many inhibitors targeting *de novo* pyrimidine biosynthesis (Fig. 7B) have been well studied on dengue virus, HBV, HEV and other virus infection models (Beardsley et al., 1989; Greene et al., 1995; Hoppe-Seyler et al., 2012; Nelson et al., 1975; Qing et al., 2010; Silva et al., 1997; Wang et al., 2011, 2016b). In this study, we demonstrated that both BQR and LFM, inhibitors of DHODH enzyme, have potent antiviral activity against rotavirus infection. Mechanistic study demonstrated that BQR and LFM exert their anti-rotavirus effect through targeting DHODH to deplete pyrimidine nucleotide pool. Therefore, targeting pyrimidine biosynthesis represents a potential approach for developing antivirals against rotavirus.

Conflicts of interest

The authors declare that they have no competing interests.

Grant support

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.04.005.

References

Avery, R.K., Bolwell, B.J., Yen-Lieberman, B., Lurain, N., Waldman, W.J., Longworth, D.L., Taege, A.J., Mossad, S.B., Kohn, D., Long, J.R., 2004. Use of leflunomide in an allogeneic bone marrow transplant recipient with refractory cytomegalovirus infection. Bone Marrow Transplant. 34, 1071.

Batt, D.G., 1999. Inhibitors of dihydroorotate dehydrogenase. Expert Opin. Ther. Pat. 9, 41–54.

Beardsley, G.P., Moroson, B.A., Taylor, E.C., Moran, R.G., 1989. A new folate antimetabolite, 5, 10-dideaza-5, 6, 7, 8-tetrahydrofolate is a potent inhibitor of de novo purine synthesis. J. Biol. Chem. 264, 328–333.

Cecílio, A.B., de Faria, D.B., de Carvalho Oliveira, P., Caldas, S., de Oliveira, D.A., Sobral, M.E.G., Duarte, M.G.R., de Souza Moreira, C.P., Silva, C.G., de Almeida, V.L., 2012. Screening of Brazilian medicinal plants for antiviral activity against rotavirus. J. Ethnopharmacol. 141, 975–981.

Chon, W.J., Josephson, M.A., 2011. Leflunomide in renal transplantation. Expert Rev. Clin. Immunol. 7, 273–281.

Corless, C.E., Guiver, M., Borrow, R., Edwards-Jones, V., Fox, A.J., Kaczmarski, E.B., Mutton, K.J., 2002. Development and evaluation of a 'real-time'RT-PCR for the detection of enterovirus and parechovirus RNA in CSF and throat swab samples. J. Med. Virol. 67, 555–562.

Cuadras, M.A., Feigelstock, D.A., An, S., Greenberg, H.B., 2002. Gene expression pattern in Caco-2 cells following rotavirus infection. J. Virol. 76, 4467–4482.

Davis, J.P., Cain, G.A., Pitts, W.J., Magolda, R.L., Copeland, R.A., 1996. The immunosuppressive metabolite of leflunomide is a potent inhibitor of human dihydroorotate dehydrogenase. Biochemistry 35, 1270–1273.

Emery, P., Breedveld, F., Lemmel, E., Kaltwasser, J., Dawes, P., Gömör, B., Van den Bosch, F., Nordström, D., Bjørneboe, O., Dahl, R.J.R., 2000. A comparison of the efficacy and safety of leflunomide and methotrexate for the treatment of rheumatoid arthritis. 39, 655–665.

Evans, D.R., Guy, H.I., 2004. Mammalian pyrimidine biosynthesis: fresh insights into an ancient pathway. J. Biol. Chem. 279, 33035–33038.

Frias, A.H., Jones, R.M., Fifadara, N.H., Vijay-Kumar, M., Gewirtz, A.T., 2012. Rotavirus-induced IFN-β promotes anti-viral signaling and apoptosis that modulate viral replication in intestinal epithelial cells. Innate Immun. 18, 294–306.

García, C.C., Quintana, V.M., Castilla, V., Damonte, E.B., 2018. Towards host cell-targeting therapies to treat dengue virus infections. Front. Anti-Infect. Drug Discov. 7 7, 45.

Grabinger, T., Luks, L., Kostadinova, F., Zimberlin, C., Medema, J.P., Leist, M., Brunner,

T., 2014. Ex vivo culture of intestinal crypt organoids as a model system for assessing cell death induction in intestinal epithelial cells and enteropathy. Cell Death Dis. 5, e1228.

Greene, S., Watanabe, K., Braatz-Trulson, J., Lou, L., 1995. Inhibition of dihydroorotate dehydrogenase by the immunosuppressive agent leflunomide. Biochem. Pharmacol. 50, 861–867.

Groome, M.J., Moon, S.-S., Velasquez, D., Jones, S., Koen, A., Niekerk, N.v., Jiang, B., Parashar, U.D., Madhi, S.A., 2014. Effect of breastfeeding on immunogenicity of oral live-attenuated human rotavirus vaccine: a randomized trial in HIV-uninfected infants in Soweto, South Africa. Bull. World Health Organ. 92, 238–245.

Hoek, R.A.S., Paats, M.S., Pas, S.D., Bakker, M., Hoogsteden, H.C., Boucher, C.A.B., van der Eerden, M.M., 2013. Incidence of viral respiratory pathogens causing exacerbations in adult cystic fibrosis patients. Scand. J. Infect. Dis. 45, 65–69.

Hoffmann, H.-H., Kunz, A., Simon, V.A., Palese, P., Shaw, M.L., 2011. Broad-spectrum antiviral that interferes with de novo pyrimidine biosynthesis. Proc. Natl. Acad. Sci. Unit. States Am. 108, 5777–5782.

Hoppe-Seyler, K., Sauer, P., Lohrey, C., Hoppe-Seyler, F., 2012. The inhibitors of nucleotide biosynthesis leflunomide, FK778, and mycophenolic acid activate hepatitis B virus replication in vitro. Hepatology 56, 9–16.

Knipping, K., Garssen, J., van't Land, B., 2012. An evaluation of the inhibitory effects against rotavirus infection of edible plant extracts. Virol. J. 9, 1.

Kotloff, K.L., Nataro, J.P., Blackwelder, W.C., Nasrin, D., Farag, T.H., Panchalingam, S., Wu, Y., Sow, S.O., Sur, D., Breiman, R.F., 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382, 209–222.

Lee, L.Y., Ison, M.G., 2014. Diarrhea caused by viruses in transplant recipients. Transpl. Infect. Dis. 16, 347–358.

Luthra, P., Naidoo, J., Pietzsch, C.A., De, S., Khadka, S., Anantpadma, M., Williams, C.G., Edwards, M.R., Davey, R.A., Bukreyev, A., 2018. Inhibiting pyrimidine biosynthesis impairs Ebola virus replication through depletion of nucleoside pools and activation of innate immune responses. Antivir. Res. 158, 288–302.

Macartney, K.K., Baumgart, D.C., Carding, S.R., Brubaker, J.O., Offit, P.A., 2000. Primary murine small intestinal epithelial cells, maintained in long-term culture, are susceptible to rotavirus infection. J. Virol. 74, 5597–5603.

Madak, J.T., Cuthbertson, C.R., Chen, W., Showalter, H.D., Neamati, N., 2017. Design, synthesis, and characterization of brequinar conjugates as probes to study DHODH inhibition. Chem.–A Eur. J. 23, 13875–13878.

Makowka, L., Sher, L.S., Cramer, D.V., 1993. The development of brequinar as an immunosuppressive drug for transplantation. Immunol. Rev. 136, 51–70.

Maroun, J., Ruckdeschel, J., Natale, R., Morgan, R., Dallaire, B., Sisk, R., Gyves, J.J.C.c., pharmacology, 1993. Multicenter phase II study of brequinar sodium in patients with advanced lung cancer. 32, 64–66.

Mladenovic, V., Domljan, Z., Rozman, B., Jajic, I., Mihajlovic, D., Dordevic, J., Popovic, M., Dimitrijevic, M., Zivkovic, M., Campion, G.J.A., Rheumatology, R.O.J.o.t.A.C.o., 1995. Safety and effectiveness of leflunomide in the treatment of patients with active rheumatoid arthritis. 38, 1595–1603.

Munier-Lehmann, H.l.n., Vidalain, P.-O., Tangy, F.d.r., Janin, Y.L., 2013. On dihydroorotate dehydrogenases and their inhibitors and uses. J. Med. Chem. 56, 3148–3167.

Natale, R., Wheeler, R., Moore, M., Dallaire, B., Lynch, W., Carlson, R., Grillo-Lopez, A., Gyves, L.J.A.o.o., 1992. Multicenter phase II trial of brequinar sodium in patients with advanced melanoma. 3, 659–660.

Nelson, J.A., Carpenter, J.W., Rose, L.M., Adamson, D.J., 1975. Mechanisms of action of 6-thioguanine, 6-mercaptopurine, and 8-azaguanine. Cancer Res. 35, 2872–2878.

Nicolini, L.A., Zappulo, E., Viscoli, C., Mikulska, M., 2018. Management of chronic viral hepatitis in the hematological patient. Expert Rev. Anti-infect. Ther. 16, 227–241.

Pally, C., Smith, D., Jaffee, B., Magolda, R., Zehender, H., Dorobek, B., Donatsch, P., Papageorgiou, C., Schuurman, H.-J.J.T., 1998. Side effects of brequinar and brequinar analogues, in combination with cyclosporine. In: The Rat, vol. 127. pp. 207–222.

Qing, M., Zou, G., Wang, Q.-Y., Xu, H.Y., Dong, H., Yuan, Z., Shi, P.-Y., 2010. Characterization of dengue virus resistance to brequinar in cell culture. Antimicrob. Agents Chemother. 54, 3686–3695.

Qu, C., Zhang, S., Wang, W., Li, M., Wang, Y., van der Heijde-Mulder, M., Shokrollahi, E., Hakim, M.S., Raat, N.J., Peppelenbosch, M.P., 2018. Mitochondrial electron transport chain complex III sustains hepatitis E virus replication and represents an antiviral target. 33, 1008–1019.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. 27, 493–497.

Sato, T., Clevers, H., 2012. Primary Mouse Small Intestinal Epithelial Cell Cultures, Epithelial Cell Culture Protocols. Springer, pp. 319–328.

Sato, T., Clevers, H., 2013. Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. Science 340, 1190–1194.

Sato, T., Vries, R.G., Snippert, H.J., Van De Wetering, M., Barker, N., Stange, D.E., Van Es, J.H., Abo, A., Kujala, P., Peters, P.J., 2009. Single Lgr5 stem cells build crypt–villus structures in vitro without a mesenchymal niche. Nature 459, 262.

Sato, T., Stange, D.E., Ferrante, M., Vries, R.G.J., Van Es, J.H., Van Den Brink, S., Van Houdt, W.J., Pronk, A., Van Gorp, J., Siersema, P.D., 2011. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology 141, 1762–1772.

Saxena, K., Blutt, S.E., Ettayebi, K., Zeng, X.-L., Broughman, J.R., Crawford, S.E., Karandikar, U., Sastri, N.P., Conner, M.E., Opekun, A., 2015. Human intestinal enteroids: a new model to study human rotavirus infection, host restriction and pathophysiology. J. Virol. JVI. 01930-01915.

Silva, H.T., Cao, W., Shorthouse, R.A., Loffler, M., Morris, R.E., 1997. In Vitro and in Vivo Effects of Leflunomide, Brequinar, and Cyclosporine on Pyrimidine Biosynthesis, Transplantation Proceedings. Elsevier Science Publishing Company, Inc., pp.

1292–1293.

Smolen, J.S., Kalden, J.R., Scott, D.L., Rozman, B., Kvien, T.K., Larsen, A., Loew-Friedrich, I., Oed, C., Rosenburg, R., 1999. Efficacy and safety of leflunomide compared with placebo and sulphasalazine in active rheumatoid arthritis: a double-blind, randomised, multicentre trial. Lancet, E.L.S.G.J.T. 353, 259–266.

Strand, V., Cohen, S., Schiff, M., Weaver, A., Fleischmann, R., Cannon, G., Fox, R., Moreland, L., Olsen, N., Furst, D., Caldwell, J., 1999. Treatment of active rheumatoid arthritis with leflunomide compared with placebo and methotrexate. 159, 2542–2550.

Sugata, K., Taniguchi, K., Yui, A., Nakai, H., Asano, Y., Hashimoto, S., Ihira, M., Yagasaki, H., Takahashi, Y., Kojima, S., 2012. Analysis of rotavirus antigenemia in hematopoietic stem cell transplant recipients. Transpl. Infect. Dis. 14, 49–56.

Tan, Y.H., Driscoll, J.S., Mui, S.M., 2005. Dihydroorotate Dehydrogenase Inhibitors for the Treatment of Viral-Mediated Diseases. Patent No: US 6841561 B1.

van Maarseveen, N.M., Wessels, E., de Brouwer, C.S., Vossen, A.C.T.M., Claas, E.C.J., 2010. Diagnosis of viral gastroenteritis by simultaneous detection of Adenovirus group F, Astrovirus, Rotavirus group A, Norovirus genogroups I and II, and Sapovirus in two internally controlled multiplex real-time PCR assays. J. Clin. Virol. 49, 205–210.

Wang, Q.-Y., Bushell, S., Qing, M., Xu, H.Y., Bonavia, A., Nunes, S., Zhou, J., Poh, M.K., de Sessions, P.F., Niyomrattanakit, P., 2011. Inhibition of dengue virus through suppression of host pyrimidine biosynthesis. J. Virol JVI. 02510-02510.

Wang, H., Naghavi, M., Allen, C., Barber, R.M., Bhutta, Z.A., Carter, A., Casey, D.C., Charlson, F.J., Chen, A.Z., Coates, M.M., 2016a. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015. The Lancet 388, 1459–1544.

Wang, Y., Wang, W., Xu, L., Zhou, X., Shokrollahi, E., Felczak, K., Van Der Laan, L.J.W., Pankiewicz, K.W., Sprengers, D., Raat, N.J.H., 2016b. Crosstalk between Nucleotide Synthesis Pathways with Cellular Immunity in Constraining Hepatitis E Virus Replication. Antimicrobial Agents and Chemotherapy. AAC. 02700-02715.

Yin, Y., Bijvelds, M., Dang, W., Xu, L., van der Eijk, A.A., Knipping, K., Tuysuz, N., Dekkers, J.F., Wang, Y., de Jonge, J., 2015a. Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. Antivir. Res. 123, 120–131.

Yin, Y., Bijvelds, M., Dang, W., Xu, L., van der Eijk, A.A., Knipping, K., Tuysuz, N., Dekkers, J.F., Wang, Y., de Jonge, J.J.A.r., 2015b. Modeling rotavirus infection and antiviral therapy using primary intestinal organoids. 123, 120–131.

Yin, Y., Metselaar, H.J., Sprengers, D., Peppelenbosch, M.P., Pan, Q., 2015c. Rotavirus in organ transplantation: drug-virus-host interactions. Am. J. Transplant. 15, 585–593.

Yin, Y., Dang, W., Zhou, X., Xu, L., Wang, W., Cao, W., Chen, S., Su, J., Cai, X., Xiao, S., 2018. PI3K-Akt-mTOR axis sustains rotavirus infection via the 4E-BP1 mediated autophagy pathway and represents an antiviral target. Virulence 9, 83–98.