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LETTER TO THE EDITOR

The IMPDH inhibitors, ribavirin and mycophenolic acid, inhibit peste des petits ruminants virus infection

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Introduction

Peste des petits ruminants virus (PPRV), as a causative agent of peste des petits ruminants (PPR), belongs to the genus Morbillivirus in the family Paramyxoviridae. PPR is a highly contagious viral disease affecting domestic as well as some wild small ruminants, in particular goats and sheep (Baron et al. 2017). This disease can lead to a high mortality up to 100% in immunologically susceptible populations which show the symptoms of pneumonia, erosive and necrotizing stomatitis, oculonasal discharges, and enteritis. This contributes to huge economic loss (Baron et al. 2017).

The first outbreak of PPR in sheep and goats was happened in Tibet, China in 2007 (Wang et al. 2009), followed in free-living bharals in 2008 in the same region (Bao et al. 2011). Despite no further spread of PPR in China due to multiple measures of control, a severe epidemic transmitted from neighbor countries has occurred in 2013, spreading to many provinces of China (Wang et al. 2009; Wu et al. 2016; Bao et al. 2017). Although the government has urged the development of vaccines to prevent PPR epidemic (Diallo 2004; Sen et al. 2010; Li et al. 2014), these programs are currently facing challenges to move forward.

We hypothesize that discovery and repurposing existing drugs for treating PPRV represents a cost-effective solution at least to partially circumvent this emerging burden. Ribavirin is a generic antiviral medication for treating various types of viral infections, in particular for patients with chronic hepatitis C virus infection (McCormick et al. 1986; Livak and Schmittgen 2001; Dixit and Perelson 2006; Graci and Cameron 2006; Borden and Culjkovic-Kraljacic 2010; Paeshuyse et al. 2011; Cholongitas and Papatheodoridis 2014). It has also been investigated to treat the infection of animal viruses, such as Canine distemper virus (Lanave et al. 2017). It is a competitive inhibitor of inosine-5′-monophosphate dehydrogenase (IMPDH), a key enzyme in purine nucleotide synthesis, In this study, we have evaluated the potential anti-PPRV effects and the mechanism-of-action of both ribavirin andmycophenolic acid (MPA), an uncompetitive IMPDH inhibitor.

Materials and methods

Cells and viruses

African green monkey (Vero) cells were cultured in Dulbecco's Modified Eagles's Medium (DMEM, Min Hai, Lan Zhou, China) supplemented with 10 heat-inactivated New born calf serum (NBS, Min Hai, Lan Zhou, China) at 37 °C in 5% CO2.Live attenuated PPRV vaccine strain Nig 75/1 was obtained from the China Institute of Veterinary Drug Control.

For experiments, Vero cells were seeded into 6-well cell culture plates (Costar, Coring, New York, USA) and grew for 24 h until cell layers were 70% confluent before infected with virus. Then, the monolayers were infected with PPRV, absorption for 2 h, the virus was removed. Finally, cells were continually cultured in DMEM, supplemented with 2 heat-inactivated New born calf serum at 37 °C in 5% CO2.

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Compounds

Powdered ribavirin, and Guanosine were purchased from Sigma-Aldrich(St.Louis,Mo,USA). Stock solutions of the compounds were made in dimethyl sulfoxide (DMSO) and stored at −20 °C. The 1.5 M Mycophenolic acid (MPA) stock was a gift from Kan Chen (Zhejiang Sci-Tech University, Hangzhou, China).

Cytotoxicity assay

Potential cytotoxic effect of ribavirin on Vero cells was tested using the cell counting Kit-8assay (Biodragon, BeiJing, China). Vero cells were seeded in 96-well microplates (Costar, Coring, New York, USA) for 24 h. Then, the medium was replaced with 100 μl of 10% DMEM supplemented with various concentrations of ribavirin (0.1 μmol/L, 1 μmol/L, 10 μmol/L or 100 μmol/L). The 10% DMEM without ribavirin was represented as control. The cells were incubated for another 72 h or120 hours respectively. After that, cck8 assay was performed according to the manufacturer's recommendation (http://www.bofonesci.com). In brief, 10 μl of cck8 stock solution was added to each well and incubated at 37 °C for 4 h. Optical density at 450 nm was measured for each well. The percentage of cell viability was calculated as follow:

$$\% \text{ viability} = \times 100\%.$$

Quantification of PPRV replication

For quantification of PPRV replication, intracellular viral RNA was extracted using TRIzol reagent (Invitrogen) and quantified using Biospectrometer (Eppendorf Germany) according to the manufacturer's protocol. cDNA was prepared from total RNA using a cDNA Synthesis Kit (Promega). The PPRV RNA level was quantified using a SYBR Green-based real-time PCR assay (Promega) according to the manufacturer's instructions. The PCR program was performed at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 30s at 58 °C, and 30s at 72.Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene to normalize gene expression. Relative gene expression was normalized to GAPDH by the formula $2^{-\triangle\triangle CT}(\triangle CT_{sample}-\triangle CT_{control})$(Livak and Schmittgen 2001; Schmittgen and Livak 2008). The PPRV gene primer sequences were as follows: PPRV-F, 5'-CTGAATACCAACATTGAG-3′; PRPRV-R, 5'-GAGGAACTTAATCTTATCG-3';GAPDH-F,5'-TCTGGGTAAAGTGGATATTGT-3′; GAPDH-R, 5'TTCCAGTATGATTCCACC-3′.

Statistical analysis

Statistical analysis was performed using One Way ANVOA test (GraphPad Prism version 5.01; GraphPad Software). $P \leq 0.05$ was considered statistically significant.

Results

Ribavirin significantly inhibits PPRV infection

To evaluate the potential anti-PPRV effect of ribavirin, cultured Vero cells were used for PPRV infection. Treatment with serial concentrations of ribavirin (0.1, 1, 10 or 100 μmol/L) for 120 h dose-dependently inhibit viral replication as quantified by the relative levels of the PPRV H gene expression (Fig. 1a). Consistently, the number PPRV positive cell foci were decreased in a dose-dependent manner accordingly, as observed by cellular morphologies (Fig. 1b).

To profile the dynamics in response to ribavirin, PPRV infected Vero cells were treated with 100 μmol/L ribavirin for 24, 48, 72, 96 or 120 h, and infected Vero cells without treatment were used as control. Clear antiviral effects were observed at different time points (Fig. 1c). Apparently, treatment for 72 h appears already to exert optimal antiivral effect (Fig. 1c). We thus further validated the anti-PPRV effects of 1, 10 or 100 μmol/L ribavirin for treating 72 h (Fig. 1d). Importantly, the concentrations of ribavirin tested in our study do not have clear cytotoxic effect up to 100 μmol/L (Fig. 1e).

Guanosine supplementation attenuates the anti-PPRV effects of ribavirin

One of the well-recognized antiviral mechanisms of ribavirin is through the inhibition of IMPDH to deplete the intracellular GTP pools. To determine whether this is involved in inhibition of PPRV, 1 μg/ml or10μg/ml guanosine was added to infected cells, when treated with ribavirin. Guanosine alone has no effect but significantly attenuated the anti-PPRV effect of ribavirin (Fig. 2).

MPA exerts potent anti-PPRV effect, but attenuated by guanosine supplementation

We next examined the effect of MPA, an uncompetitive IMPDH inhibitor, on PPRV infection. MPA as an immunosuppressive agent is widely used for treating rejection after organ transplantation. It has been shown to inhibit a variety of viral infections (Pan et al. 2012a; Wang et al. 2014b; Yin et al. 2016; Dang et al. 2017). We found that treatment with 1 or 10 μg/ml MPA for 72 h profoundly inhibited PPRV infection (Fig. 3a). Similarly, the antiviral activity of MPA was

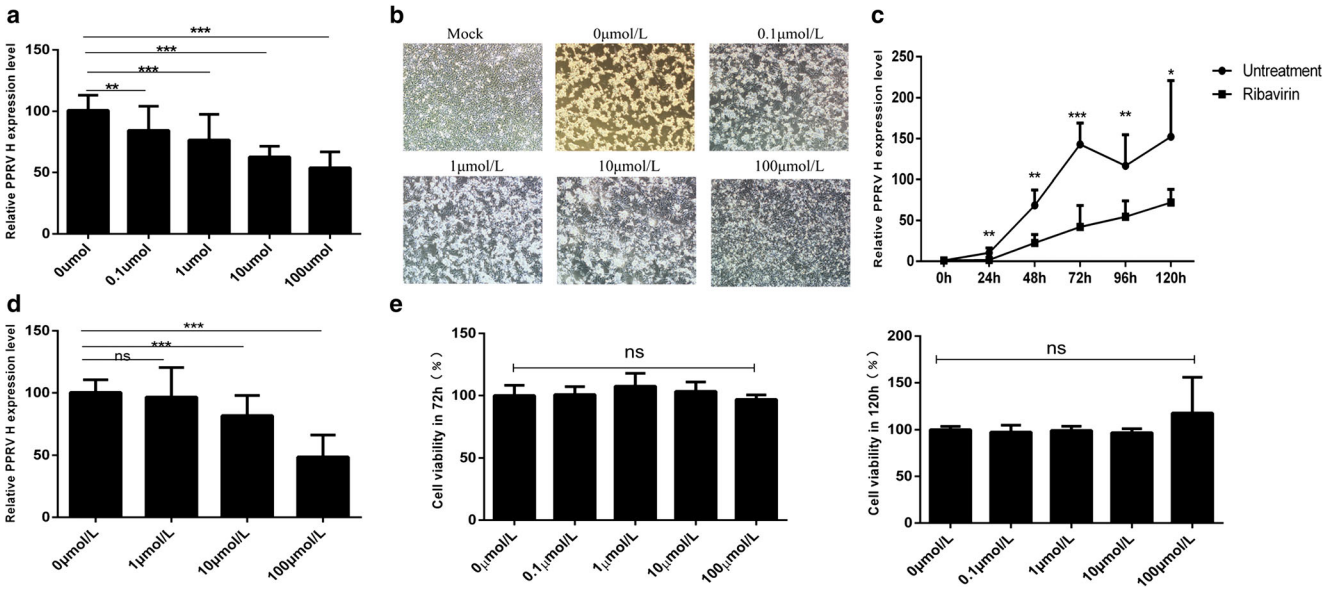

Fig. 1 Antiviral effects of ribavirin against PPRV in cell culture. a PPRV infected Vero cells were treated with different concentrations of ribavirin for 120 h, total RNA was extracted and reserve transcripted, H gene and GAPDH were quantified by Real-time PCR. **b** Vero cells were mock treated or infected with 1 MOI PPRV. Ribavirin were applied at different concentration at 24 h. Cellular morphology was observed under light microscope at 120 h after treatment. **c** Vero cells infected with 1 MOI PPRV treated with 100 μmol/L ribavirin for 24, 48, 72, 92 or 120 h. H gene was analyzed as described above. **d** PPRV infected Vero cells were treated with different concentrations of ribavirin for 72 h. H gene was analysed as described above. **e** Vero cells were incubated for 72 h and 120 h and the viability of Vero cells were tested by cck8 kit. Data are the Mean ± SD of three independent experiments.*$p < 0.05$;**, $p < 0.01$ ***, $p < 0.001$; ns, not significant

attenuated by guanosine supplementation in a dose-dependent manner (Fig. 3b).

Discussion

A variety of nucleoside analogues have been extensively utilized in curing viral infections due to their potent antiviral functions. Ribavirin asa type of guanosine analogue, is widely used to treat acute and chronicviral infection, including hepatitis E virus (Debing et al. 2013, 2014; Dao Thi et al. 2016; Qu et al. 2017), hepatitis C virus (McCormick et al. 1986; Dixit and Perelson 2006; Borden and Culjkovic-Kraljacic 2010), and parainfluenza virus (Casey et al. 2013; Kihira et al. 2014). In this study we have demonstrated that ribavirin dose-dependently inhibits PPRV infection at concentrations that relevant to the blood levels in treated patients (Jain et al. 2005). This is reflected by both inhibition of the viral H gene expression and formation of PPRV positive cell foci. Importantly, no cytotoxicity was observed on the host cells across the serial concentrations of ribavirin used in this study. Several antiviral mechanisms have been proposed for ribavirin. These include: (i) depletion of intracellular GTP pools; (ii) immunomodulatory effects; (iii) lethal mutagenesis through anerror catastrophe; (iv) direct inhibition of the viral polymerase, and (V) inhibit eukaryotic initiation factor 4E to interfere with cap-dependent translation (Paeshuyse et al. 2011; Dong et al. 2012; Thomas et al. 2012). Our study has revealed that purine nucleotide depletion by ribavirin is an important mechanism in inhibiting PPRV infection, although we do not exclude the potential involvement of other mechanisms. This was further supported by the potent anti-PPRV effect of MPA, an uncompetitive IMPDH inhibitor. Consistently, this effect was also alleviated by guanosine supplementation.

In fact, the anti-PPRV effects of MPA with clinically achievable doses are more potent that ribavirin treatment as we have demonstrated in this study. It has previously been demonstrated a broad antiviral spectrum of MPA in vitro (Pan et al. 2012b). However, its antiviral efficacy in patients

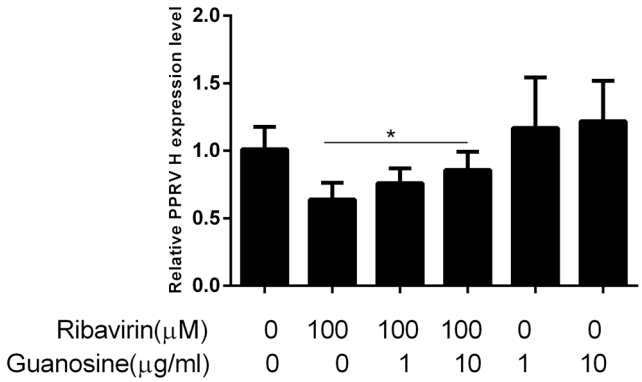

Fig. 2 Guanosine supplementation attenuated the anti-PPRV effect of ribavirin 1 μg/ml and 10 μg/ml guanosine were added to PPRV infected Vero cells, when treated with 100 μmol/L ribavirin for 72 h. H gene was analysed as described above. Data are the Mean ± SD of three independent experiments.*$p < 0.05$;**, $p < 0.01$ ***, $p < 0.001$; ns, not significant

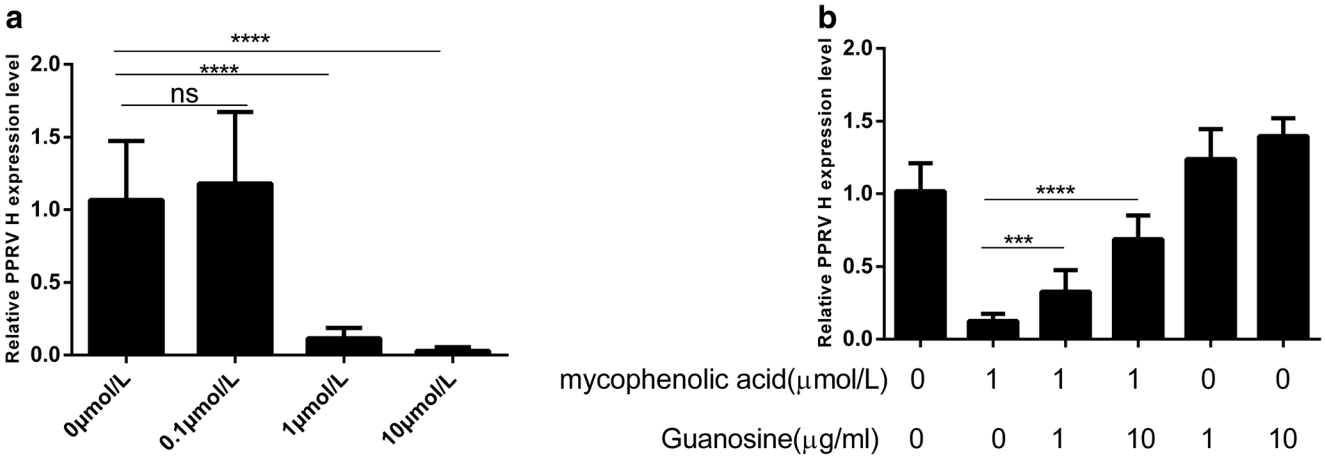

Fig. 3 Potent anti-PPRV effect of MPA through depletion of cellular GTP pools. a 0.1,1 or 10 μmol/L MPA were added to PPRV infected Vero cells for 72 h. H gene was analyzed as described above. **b** 1or 10 μg/ml guanosine were added to PPRV infected Vero cells, when treated with 1 μmol/LMPA for 72 h. H gene was analyzed as described above. Data are the Mean ± SD of three independent experiments.*$p < 0.05$;**, $p < 0.01$ ***, $p < 0.001$; ns, not significant

remains under debating. This is likely because of its immunosuppressive effect, which will profoundly affect the host antiviral immunity (Wang et al. 2014a). Thus, future research is required to further clarify the in vivo antiviral efficacy of MPA.

In conclusion, we have demonstrated that the two IMPDH inhibitors, ribavirin and MPA, effectively inhibit PPRV infection in cell culture. Mechanistically, depletion of cellular purine nucleotide is essential for the anti-PPRV effects. Both regimens, as generic medications, are safe and cheap, and have been used in the clinic to treat patients for decades. We believe that repurposing these antiviral drugs represents a cost-effective scenario to combat PPRV infection in domestic and wild animals. Nevertheless, it is essentially required to validate the efficacy in animal models, before wide application for the treatment of infected animal patients.

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Compliance with ethical standards

In this study, all experiments have carried out without any humans or animals.

Conflict of interest All authors have no conflict of interest in this study.

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