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Ribavirin efficiently suppresses porcine nidovirus replication

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine epidemic diarrhea virus (PEDV) are porcine nidoviruses that represent emerging viral pathogens causing heavy economic impacts on the swine industry. Although ribavirin is a well-known antiviral drug against a broad range of both DNA and RNA viruses in vitro, its inhibitory effect and mechanism of action on porcine nidovirus replication remains to be elucidated. Therefore, the present study was conducted to determine whether ribavirin suppresses porcine nidovirus infection. Our results demonstrated that ribavirin treatment dose-dependently inhibited the replication of both nidoviruses. The antiviral activity of ribavirin on porcine nidovirus replication was found to be primarily exerted at early times post-infection. Treatment with ribavirin resulted in marked reduction of viral genomic and subgenomic RNA synthesis, viral protein expression, and progeny virus production in a dose-dependent manner. Investigations into the mechanism of action of ribavirin against PRRSV and PEDV revealed that the addition of guanosine to the ribavirin treatment significantly reversed the antiviral effects, suggesting that depletion of the intracellular GTP pool by inhibiting IMP dehydrogenase may be essential for ribavirin activity. Further sequencing analysis showed that the mutation frequency in ribavirin-treated cells was similar to that in untreated cells, indicating that ribavirin did not induce error-prone replication. Taken together, our data indicate that ribavirin might not only be a good therapeutic agent against porcine nidovirus, but also a potential candidate to be evaluated against other human and animal coronaviruses.

1. Introduction

The *Nidovirales* are an order of enveloped single-stranded positive-sense RNA viruses with animal hosts that include the families *Arteriviridae*, *Coronaviridae*, and *Roniviridae* (Cavanagh, 1997; Mayo, 2002; Spaan et al., 2005). Despite striking differences in genome size and virion morphology, the genome organization and expression strategy of the two groups belonging to the *Nidovirales* order were found to be comparable. The nidovirus genome contains two large ORFs, 1a and 1b, comprising the 5′ two-thirds of the viral genome encoding non-structural proteins (NSPs) and the remaining ORFs located in the 3′ terminal region coding for structural proteins (Lai et al., 2007; Snijder and Spaan, 2007). The initial translation from ORF1a and ORF1b yields the 1a and lab replicase polyproteins, respectively, which are then proteolytically processed into functional NSPs including the viral RNA-dependent RNA polymerase (RdRp) (Bautista et al., 2002; van Aken et al., 2006; Ziebuhr et al., 2000). The RdRp-containing replication complex mediates genomic RNA replication and subgenomic (sg) mRNA transcription, eventually generating a nested set of 3′-coterminal sg mRNAs that are individually translated to structural proteins (Lai et al., 2007; Snijder and Spaan, 2007).

Porcine reproductive and respiratory syndrome virus (PRRSV), a pathogenic macrophage-tropic arterivirus of pigs, is the etiological agent of acute respiratory illness in young piglets and reproductive failure in pregnant sows (Albina, 1997). PRRSV primarily replicates in porcine alveolar macrophages (PAMs) and can establish persistent infection in lymphoid tissues of infected pigs that lasts for several months (Albina et al., 1994; Christopher-Hennings et al., 1995; Duan et al., 1997; Wills et al., 2003). As a result, PRRSV infection results in suppression of normal macrophage functions and immune responses, which may render pigs susceptible to secondary bacterial or viral infections, leading to more severe disease than either agent alone (Allan et al., 2000; Feng et al., 2001; Harms et al., 2001; Wills et al., 2000). Porcine epidemic diarrhea virus (PEDV), a pathogenic enterocyte-tropic coronavirus of swine, is the etiological agent of acute enteritis, which is characterized by lethal watery diarrhea followed by dehydration leading to death with a high mortality rate in suckling piglets (Debouck and Pensaert, 1980). These two viruses, PRRSV and PEDV, are devastating porcine nidoviral pathogens that have still continued to plague swine-producing nations, causing tremendous economic losses to the global and Asian pork industries (Neumann et al., 2005; Pensaert and Yeo, 2006).

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Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, also known as Virazole) is a synthetic guanosine analog that exhibits broad-spectrum antiviral activity in vitro (Sidwell et al., 1972). It has been used experimentally against a wide range of both DNA and RNA viruses, including GB virus B, Hantaan virus, Hendra virus, respiratory syncytial virus, Lassa fever virus, Norwalk virus, and West Nile virus (Chang and George, 2007; Cooper et al., 2003; Day et al., 2005; McCormick et al., 1986; Lanford et al., 2001; Rockx et al., 2010; Severson et al., 2003). Most notably, ribavirin is used in combination with interferon-α for treatment of chronic hepatitis C virus (HCV) infections (Cummings et al., 2001; Davis et al., 1998). However, there is still no report regarding an antiviral effect of ribavirin during the replication cycle of porcine nidoviruses. In the present study, therefore, we tried to investigate the antiviral activity of ribavirin and its mechanism of action in target cells upon porcine nidovirus infection. Independent treatment of target cells with ribavirin significantly impaired PRRSV and PEDV infection. Further experiments revealed that suppression of ribavirin affects post-entry steps of the replication cycle of PRRSV and PEDV, including viral genomic and sg RNA synthesis, viral protein expression, and virus production. The addition of guanosine to the ribavirin treatment resulted in moderate reversal of the antiviral effects, suggesting that ribavirin activity is involved in the depression of cellular guanosine triphosphate (GTP) levels. Sequencing analysis of the PRRSV and PEDV genomes in the ribavirin-treated and non-treated groups revealed that the mutation rates were similar and indicated that ribavirin did not induce catastrophic mutations during the replication of porcine nidoviruses. Altogether, our results suggest that ribavirin may be an excellent therapeutic option for nidovirus infection in a human or veterinary subject.

2. Materials and methods

2.1. Cells, viruses, reagents, and antibodies

PAM-pCD163 cells (Lee et al., 2010) were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), antibiotic–antimycotic solutions (100×; Invitrogen), 10 mM HEPES (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and nonessential amino acids (100×; Invitrogen) in the presence of 50 μg/ml Zeocin (Invitrogen). Vero cells were cultured in alpha minimum essential medium (α-MEM, Invitrogen) with 10% FBS and antibiotic–antimycotic solutions. The cells were maintained at 37 °C in a humidified 5% CO_2 incubator. PRRSV strain VR-2332 was propagated in PAM-pCD163 cells as described previously (Lee and Lee, 2010). PEDV strain SM98-1 was kindly provided by the Korean Animal, Plant and Fisheries Quarantine and Inspection Agency and propagated in Vero cells as described previously (Hofmann and Wyler, 1988). Ribavirin and mycophenolic acid (MPA) were purchased from Sigma (St. Louis, MO) and dissolved in distilled water (DW) or dimethyl sulfoxide (DMSO), respectively. A monoclonal antibody (MAb; SDOW17) against the PRRSV N protein was purchased from Rural Technologies (Brookings, SD). The PEDV spike (S) glycoprotein-specific and N protein-specific monoclonal antibodies (MAbs) were kind gifts from Sang-Geon Yeo (Kyungpook National University, Daegu, South Korea). The anti-β-actin antibody and horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell viability assay

The cytotoxic effects of reagents on PAM-pCD163 and Vero cells were analyzed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) detecting cell viability. Briefly, PAM-pCD163 and Vero cells were grown at 1×10^4 cells/well in a 96-well tissue culture plate with ribavirin or MPA treatment for 24 h. After one day of incubation, 50 μl of MTT solution (1.1 mg/ml) was added to each well and the samples were incubated for an additional 4 h. The supernatant was then removed from each well, after which 150 μl of DMSO was added to dissolve the color formazan crystal produced from the MTT. The absorbance of the solution was measured at 540 nm using an enzyme-linked immunosorbent assay plate reader. All MTT assays were performed in triplicate.

2.3. Immunofluorescence assay (IFA)

PAM-pCD163 and Vero cells grown on microscope coverslips placed in 6-well tissue culture plates were pretreated with ribavirin or MPA for 1 h and mock infected or infected with PRRSV and PEDV at a multiplicity of infection (MOI) of 01, respectively. The virus-infected cells were further grown in the presence of ribavirin until 48 hpi, fixed with 4% paraformaldehyde for 10 min at room temperature (RT) and permeabilized with 0.2% Triton X-100 in PBS at RT for 10 min. The cells were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at RT and then incubated with N-specific MAb 7 for 2 h. After being washed five times in PBS, the cells were incubated for 1 h at RT with a goat anti-mouse secondary antibody conjugated with Alexa Fluor 488 (Molecular Probes, Carlsbad, CA), followed by counterstaining with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). The coverslips were mounted on microscope glass slides in mounting buffer (60% glycerol and 0.1% sodium azide in PBS) and cell staining was visualized using a fluorescent Leica DM IL LED microscope (Leica, Wetzlar, Germany).

2.4. Western blot analysis

PAM-pCD163 and Vero cells were grown in 6-well tissue culture plates for 1 day and were mock infected or infected with PRRSV or PEDV at an MOI of 0.1. At the indicated times, cells were harvested in 50 μl of lysis buffer (0.5% TritonX-100, 60 mM β-glycerophosphate, 15 mM ρ-nitro phenyl phosphate, 25 mM MOPS, 15 mM, $MgCl_2$, 80 mM NaCl, 15 mM EGTA [pH 7.4], 1 mM sodium orthovanadate, 1 μg/ml E64, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF) and sonicated on ice five times for 1 s each. Homogenates were lysed for 30 min on ice, and clarified by centrifugation at 15,800 × *g* (Eppendorf centrifuge 5415R, Hamburg, Germany) for 30 min at 4 °C. The protein concentrations of the cell lysates were determined by a BCA protein assay (Pierce, Rockford, IL). The cell lysates were mixed with 4× NuPAGE sample buffer (Invitrogen) and boiled at 70 °C for 10 min. The proteins were separated on NuPAGE 4–12% gradient Bis–Tris gel (Invitrogen) under reducing conditions, and electrotransferred onto Immunobilon-P (Millipore, Billerica, MA). The membranes were subsequently blocked with 3% powdered skim milk (BD Biosciences, Belford, MA) in TBS (10 mM Tris–HCl [pH 8.0], 150 mM NaCl) with 0.05% Tween-20 (TBST) at 4 °C for 2 h, and reacted at 4 °C overnight with the primary antibody against PRRSV N, PEDV S or β-actin. The blots were then incubated with the secondary horseradish peroxidase (HRP)-labeled antibody (Santa Cruz Biotechnology) at a dilution of 1:5000 for 2 h at 4 °C. Proteins were visualized by enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. To quantify viral protein production, band densities of PRRSV N and PEDV S proteins were quantitatively analyzed using a computer densitometer with the Wright Cell Imaging Facility (WCIF) version of the ImageJ software package (http://www.uhnresearch.ca/facilities/wcif/imagej/) based on the density value relative to β-actin gene.

Table 1
List of primers used in this study.

Virus	Primer name	Nucleotide sequences (5′–3′)	Purpose	Location (nt)
PRRSV	Genomic RNA-Fwd	TGTCCGTTCGTGAAACCC	Real-time RT-PCR	1400–1417
	Genomic RNA-Rev	CGCAGGGAGTCTGAGGATTTGG	Real-time RT-PCR	1623–1644
	sg mRNA-Fwd	GGAGCTGTGACCATTGGCA	Real-time RT-PCR	46–64
	sg mRNA-Rev	TCATGCTGAGGGTGATGC	Real-time RT-PCR & PCR	15,073–15,090
	ORF5-Fwd	GTGGGCAACTGTTTTAGCCT	RT-PCR	13,559–14,736
	Porcine β-actin-Fwd	GACCACCTTCAACTCGATCA	Real-time RT-PCR	920–939
	Porcine β-actin-Rev	GTGTTGGCGTAGAGGTCCTT	Real-time RT-PCR	963–982
PEDV	Genomic RNA-Fwd	AGTACGGGGCTCTAGTGCAG	Real-time RT-PCR	12,611–12,630
	Genomic RNA-Rev	GCTTATCCAAATTCTTCAGGCG	Real-time RT-PCR	12,741–12,762
	sg mRNA-Fwd	AGACCTTGTCTACTCAATTCAACT	Real-time RT-PCR	44–67
	sg mRNA-Rev	TGCTGGTCCTTATTCCCCTT	Real-time RT-PCR	26,461–26,480
	N gene-Fwd	ATGGCTTCTGTCAGC	RT-PCR	26,335–26,349
	N gene-Rev	TTAATTTCCTGTGTC	RT-PCR	27,646–27,660
	Monkey GAPDH-Fwd	TCAACAGCGACACCCACTC	Real-time RT-PCR	959–967
	Monkey GAPDH-Rev	CTTCCTCTTGTGCTCTTGCTG	Real-time RT-PCR	1131–1151

2.5. Time course of ribavirin treatment

PAM-pCD163 and Vero cells were infected with PRRSV or PEDV, respectively, at an MOI of 0.1 as described above. At −1, 0, 1, 2, 4, 6, 8, 10, 12, or 24 hpi, ribavirin was added to give the indicated final concentration for the remainder of the time course experiment. The virus-infected and ribavirin-treated cells were further maintained and the infection was terminated at 48 hpi by fixing the monolayers with 4% paraformaldehyde for 10 min at RT. The fixed cells were subjected to IFA to assess the presence of PRRSV or PEDV infection as described above.

2.6. Virus internalization assay

An internalization assay was performed as described previously (Cai et al., 2007). Briefly, PAM-pCD163 and Vero cells grown in 6-well culture plates were infected with PRRSV and PEDV, respectively, at an MOI of 0.1 at 4 °C for 1 h, respectively. Unbound viruses were then washed with PBS and the cells were either incubated at 4 °C or 37 °C in the presence ribavirin for 1 h, followed by treatment with protease K (0.5 mg/ml) at 4 °C for 45 min to remove the bound but uninternalized virus particles. The PRRSV-infected cells were then serially diluted in RPMI and added onto fresh PAM-pCD163 cell monolayers in 96-well tissue culture plates. At 48 h post-incubation, internalized viruses were titrated through IFA as described above and the 50% tissue culture infectious dose ($TCID_{50}$) was calculated. For PEDV, the serially diluted infected cells were added onto uninfected Vero cells and after 48 h, internalized viruses were titrated using a plaque assay as described previously (Nam and Lee, 2010), and the plaques were counted after 1% crystal violet staining.

2.7. Quantitative real-time RT-PCR

PAM-pCD163 and Vero cells were incubated with ribavirin for 1 h prior to infection and then inoculated with PRRSV or PEDV at an MOI of 1 for 1 h at 37 °C. The virus inoculum was subsequently removed and the infected cells were maintained in fresh medium containing ribavirin for 48 h. Total RNA was extracted from lysates of the infected cells at 48 hpi using TRIzol reagent (Invitrogen) and treated with DNase I (TaKaRa) according to the manufacturer's protocols. The concentrations of the extracted RNA were measured using a NanoVue spectrophotometer (GE Healthcare, Piscataway, NJ). Quantitative real-time RT-PCR was conducted using a Thermal Cycler Dice Real Time System (TaKaRa) with gene-specific primer sets (Table 1) as described previously (Sagong and Lee, 2011). The RNA levels of viral genes were normalized to that of mRNA for the β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and relative quantities (RQ) of mRNA accumulation were evaluated using the $2^{-\Delta\Delta Ct}$ method. To detect alteration of genomic RNA and sg mRNA levels in the presence of ribavirin during porcine nidovirus infection, the results obtained using ribavirin-treated samples were compared with vehicle-treated results.

2.8. Virus titration

PAM-pCD163 and Vero cells were PRRSV or PEDV infected with treatment of ribavirin as described above. The culture supernatant was collected at different time points (6, 12, 24, 36, and 48 hpi) and stored at −80 °C. The titer of PRRSV was measured by limiting dilution on PAM-pCD163 cells through IFA as described above and then quantified as the $TCID_{50}$ per ml. The PEDV titer was determined by a plaque assay using Vero cells and quantified as plaque-forming units (PFU) per ml.

2.9. Supplementation of guanosine in the presence of ribavirin

PAM-pCD163 and Vero cells were preincubated with 100 μM guanosine for 6 h before ribavirin was added to the medium at various final concentrations and then inoculated with PRRSV or PEDV as described above. The virus inoculum was removed and the infected cells were maintained in fresh medium containing ribavirin and guanosine. At 48 h dpi, the virus-infected cells were fixed and subjected to IFA to evaluate the presence of PRRSV or PEDV infection as described above.

2.10. Sequencing analysis of the nidoviral genome in the presence or absence of ribavirin

The N gene coding regions for PRRSV and PEDV were sequenced to monitor mutation frequency. Cells were PRRSV or PEDV infected and treated with ribavirin as described above. At 48 hpi, total RNA was extracted by TRIzol, and RT-PCR was performed to amplify the region encoding the ORF5 to ORF7 genes of PRRSV or the N gene of PEDV using gene-specific primer sets (Table 1). The corresponding PCR product was then cloned into the pGEM-T vector (Promega, Madison, WI). For sequencing of the gene in the recombinant vector, we selected 20 independent bacterial colonies per group and analyzed mutations in the region.

2.11. Statistical analysis

A Student's *t* test was used for all statistical analyses and *P*-values of less than 0.05 were considered statistically significant.

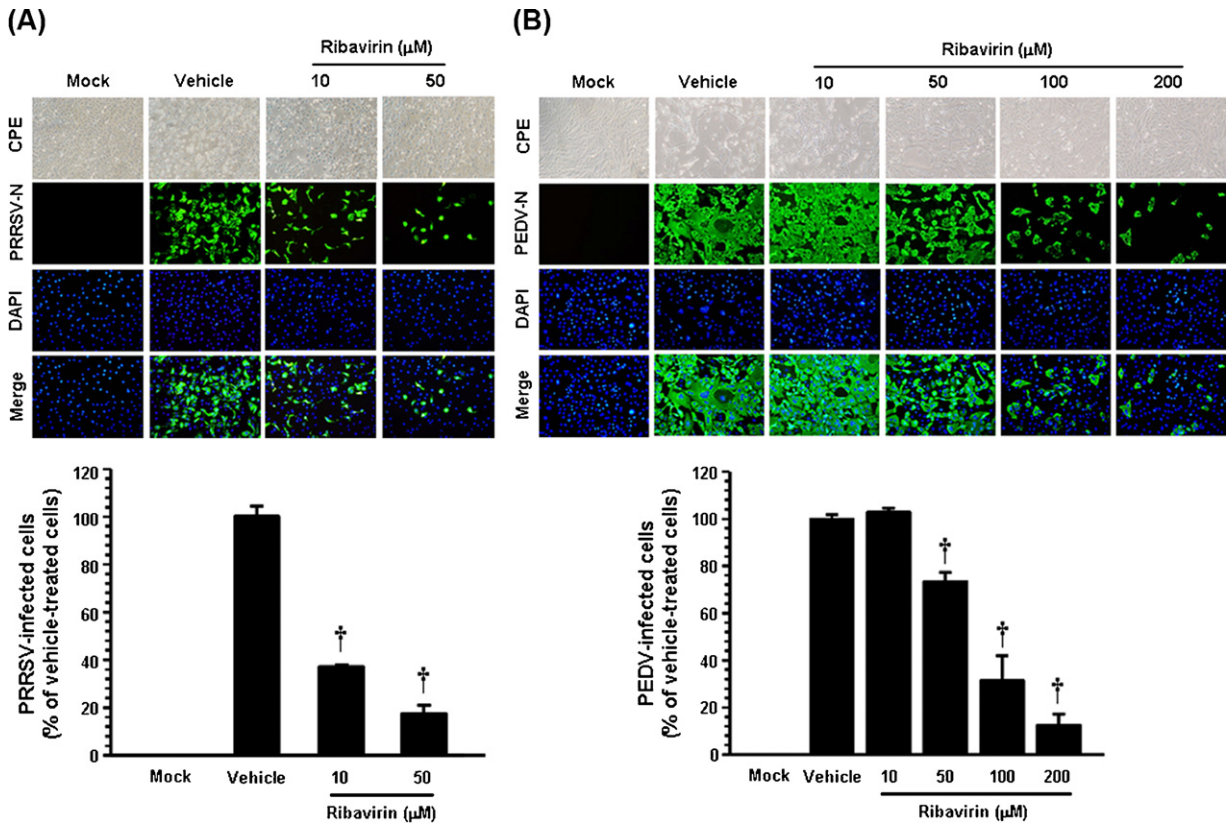

Fig. 1. Effect of ribavirin on the replication of porcine nidoviruses. PAM-pCD163 and Vero cells were preincubated with ribavirin at indicated concentrations for 1 h prior to infection and were mock-infected or infected with PRRSV (A) or PEDV (B) at an MOI of 0.1. Virus-infected cells were further maintained for 48 h in the presence of vehicle or ribavirin. Virus-specific CPEs were observed daily and were photographed at 48 hpi using an inverted microscope at a magnification of 100× (first panels). For immunostaining, infected cells were fixed at 48 hpi and incubated with MAb against the N protein of PRRSV or PEDV followed by Alexa green-conjugated goat anti-mouse secondary antibody (second panels). The cells were then counterstained with DAPI (third panels) and examined using a fluorescent microscope at 200× magnification. Viral productions in the presence of ribavirin were measured by quantifying the number of cells expressing N proteins through IFA. Five fields at 200× magnification were counted per each condition and the total number of cells per field as determined by DAPI staining was similar in all fields. Values are representative of the mean of three independent experiments and error bars represent standard deviations. $^{*}P = 0.001–0.05$; $^{\dagger}P < 0.001$.

3. Results

3.1. Suppression of porcine nidovirus replication by ribavirin

To examine the effect of ribavirin on porcine nidovirus replication, PRRSV and PEDV were selected because they are important viral pathogens that economically affect the swine industry. Based on MTT assay, none of the doses of ribavirin tested in the present study caused detectable cell death of PAM-pCD163 and Vero cells (data not shown). PAM-pCD163 and Vero cells were pretreated with ribavirin at concentrations of 10–50 μM or 10–200 μM, respectively, or with DW as a vehicle control for 1 h prior to infection. Ribavirin was present during the entire period of infection. Viral production was initially measured by monitoring the cytopathic effect (CPE) after infection and confirmed by immunofluorescence using N protein-specific MAb at 48 hpi (Fig. 1). In vehicle-treated control cells, visible CPE appeared at 24 hpi and became predominant by 48 hpi, and N-specific staining was clearly evident in many cell clusters, indicating infection and the spread of the virus to neighboring cells. In contrast, ribavirin had an obvious inhibitory effect on virus propagation. As shown in Fig. 1A and B, ribavirin significantly decreased virus-induced CPE and viral gene expression of PRRSV and PEDV at concentrations of ~10 μM and ~50 μM, respectively. N protein staining revealed that the number of cells expressing viral antigen, as quantified by N protein staining results, was also reduced during ribavirin treatment, resulting in a maximum of ~80% inhibition in response to 50 μM and 200 μM for PRRSV and PEDV, respectively (Fig. 1A and B). Moreover, the effective doses for inhibiting 50% (ED_{50}) of the replication of PRRSV and PEDV were determined to be about 21 μM and 73 μM, respectively. Taken together, these data demonstrate that ribavirin efficiently suppresses the replication of porcine nidoviruses.

To further determine at which point ribavirin acted during the porcine nidovirus infection period, PAM-pCD163 and Vero cells were treated independently with ribavirin at various time points post-infection. At 48 hpi, the levels of PRRSV or PEDV replication were measured indirectly as viral antigen production by quantifying cells expressing the N protein through IFA (Fig. 2). Treatment of cells with 50 μM ribavirin for up to 4 hpi resulted in more than 80–90% decrease in PRRSV production when compared to the untreated control. Addition of ribavirin between 6 and 12 hpi led to 75–38% inhibition of PRRSV infectivity. Similarly, treatment with 200 μM ribavirin at −1, 0, and 1 hpi was found to lead to 83–61% suppression of PEDV production, while its treatment at 2–12 hpi resulted in 49–28% reduction in PEDV infectivity. In contrast, no significant impairment of porcine nidovirus propagation was observed when ribavirin was added at 24 hpi. These results demonstrate that the inhibitory effect of ribavirin against the replication of PRRSV and PEDV was exerted primarily during the initial period of infection, suggesting that its action occurs mainly at early time points after porcine nidovirus infection.

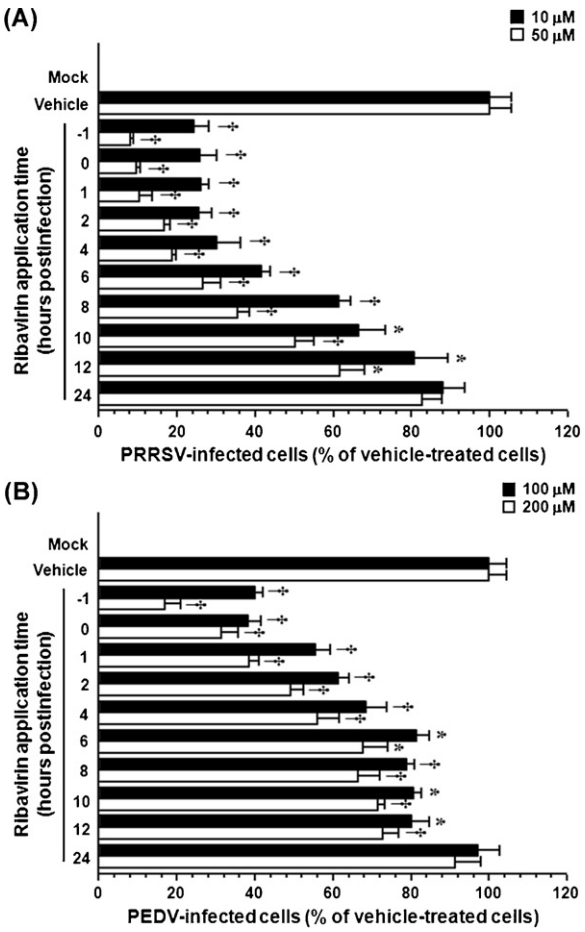

Fig. 2. Suppression of nidovirus propagation by ribavirin at early times post-infection. PAM-pCD163 and Vero cells were pretreated with ribavirin and were infected with PRRSV (A) or PEDV (B), respectively. At the indicated times at post-infection, ribavirin was added to achieve a final concentration. At 48 hpi, virus-infected cells were fixed and virus infectivity was determined by quantifying the number of cells expressing N proteins through IFA. Data are representative of the mean of three independent experiments and error bars represent standard deviations. $^{*}P = 0.001–0.05$; $^{\dagger}P < 0.001$.

3.2. Inhibition of infectious viral progeny production by ribavirin

To further assess the antiviral activity of ribavirin against porcine nidovirus replication, virus yield was determined during treatment of ribavirin. Upon infection, viral supernatants were collected at 48 hpi and viral titers were measured. As Fig. 3A shows, the presence of ribavirin reduced the release of viral progeny in a dose-dependent manner. The peak viral titer was determined to be 10^{6} $TCID^{50}$/ml and $10^{6.34}$ PFU/ml in the vehicle-treated control for PRRSV and PEDV, respectively. However, the addition of 50 μM or 200 μM ribavirin decreased titers of PRRSV and PEDV to $10^{3.53}$ $TCID^{50}$/ml and $10^{3.52}$ PFU/ml, respectively (almost 3 log reduction compared with the control). The growth kinetics study further demonstrated that the overall process of porcine nidovirus replication was significantly delayed when cells were treated with ribavirin (Fig. 3B). Consequently, these findings revealed that ribavirin inhibits optimal progeny virus release within the natural host cells.

3.3. Effect of ribavirin on virus internalization process

To evaluate which steps of the replication cycle of porcine nidoviruses were targeted by ribavirin, we started focusing on the earliest step, virus entry upon the ribavirin treatment. To address this issue, an internalization assay was performed as described previously (Cai et al., 2007). PAM-pCD163 and Vero cells were inoculated with PRRSV and PEDV, respectively, at 4 °C for 1 h to allow virus attachment and further maintained either at 4 °C or 37 °C to permit virus internalization in the presence of ribavirin. The samples were then treated with protease K to remove the remaining viruses from the cell surface. The serially diluted infected cells were subsequently subjected to an infectious center assay on uninfected PAM-pCD163 and Vero cell monolayers and virus titers were measured 2 days later through IFA or by plaque assay. As shown in Fig. 3C, the titers of PRRSV and PEDV were virtually the same in cells treated with ribavirin or without ribavirin at 37 °C and determined to be $10^{1.7}$–$10^{2.2}$ $TCID_{50}$/ml and 2.2×10^{2}–3.2×10^{2} PFU/ml, respectively, indicating no difference between those cells. In contrast, only minimal viral productions of about $10^{1.3}$ $TCID_{50}$/ml (PRRSV) and 4.5×10^{1} PFU/ml (PEDV) were observed in cells maintained at 4 °C, which was likely due to inefficient removal of the bound virus. These results indicated that ribavirin has no inhibitory effect on the virus entry process.

3.4. Inhibition of nidoviral protein translation by ribavirin

Like all positive-sense RNA viruses, following the uncoating process, the nidovirus genome is released into the cytoplasm and immediately serves as a template for viral translation by the same cellular cap-dependent mechanism. Early nidovirus translation produces replicase polyproteins that are posttranslationally cleaved into NSPs by viral proteases. Subsequently, the nonstructural replicase proteins mediate de novo synthesis of viral RNA, including genomic RNA replication and sg mRNA transcription. The nidovirus structural proteins are translated lately from respective sg mRNA transcripts. Thus, to determine the inhibitory mechanism of ribavirin on the postentry steps of the nidovirus life cycle, we first investigated whether viral protein translation was affected by ribavirin. To accomplish this, PAM-pCD163 and Vero cells were treated with ribavirin for 1 h prior to infection, and the drug was allowed to remain during infection and subsequent incubation. The expression levels of the PRRSV N and PEDV S proteins in the presence or absence of ribavirin were evaluated at 48 hpi by western blot analysis. The production of both proteins productions was drastically diminished during ribavirin treatment (Fig. 4). Densitometric analysis of the western blots revealed that the intracellular expression of both N and S proteins was reduced by ribavirin, with a maximum of about 90% inhibition at the concentration of 50 μM and 200 μM, respectively (Fig. 4). This marked reduction was probably not the result of a nonspecific decrease in the translation mechanism, since Ponceau S staining results indirectly indicated that the expression levels of the overall cellular proteins remained similar following treatment (data not shown). Taken together, these results demonstrated the inhibitory effects of ribavirin specifically against viral translation during porcine nidovirus replication.

3.5. Suppression of porcine nidovirus transcription by ribavirin

For positive-strand RNA viruses, synthesis of the viral nonstructural proteins is required for viral RNA replication and transcription. Thus, it is conceivable that impaired viral protein expression in would be caused by inhibition of viral RNA synthesis. Since nidovirus infection produces two RNA entities including genomic versus subgenomic, we therefore determined if ribavirin specifically affected genome replication and sg mRNA transcription. To answer this question, the relative levels of both genomic RNA and sg mRNA were assessed by quantitative real-time strand-specific RT-PCR in the presence or absence of ribavirin upon porcine nidovirus infection. As shown in Fig. 5A, ribavirin exhibited a maximal 70% and 80% reduction in the synthesis of PRRSV genomic RNA and sg

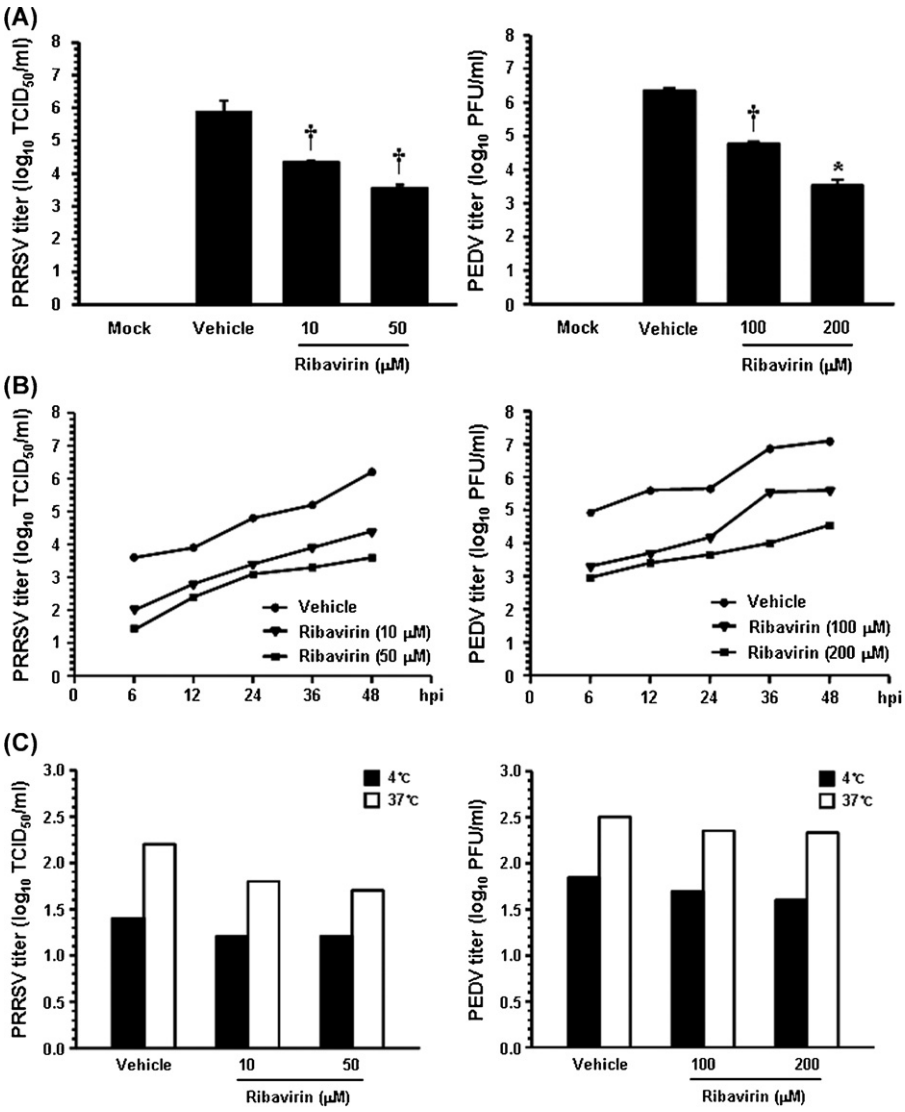

Fig. 3. Reduction of viral progeny production by ribavirin. (A) PAM-pCD163 and Vero cells were pretreated with ribavirin for 1 h and were mock or virus infected (MOI of 0.1). Ribavirin was present in the medium throughout the infection. At 48 hpi, the virus supernatants were collected and the titers of PRRSV (left) and PEDV (right) were determined. (B) Growth kinetics of PRRSV (left) and PEDV (right) upon treatment with ribavirin was assessed exactly as for panel A. At the indicated time points post-infection, culture supernatants were harvested and virus titers were measured. (C) Virus internalization assay. PAM-pCD163 and Vero cells were infected at an MOI of 0.1 at 4 °C for 1 h. After washing with cold PBS, infected cells were maintained in the presence or absence of ribavirin either at 4 °C or 37 °C for an additional hour. Bound but uninternalized virus particles were removed by treatment with protease K. The infected cells were then serially diluted and plated onto fresh target cells. At 2 days post-incubation, internalized viruses were titrated by IFA and plaque assay for PRRSV (left) and PEDV (right), respectively. Results are expressed as the mean values from triplicate wells and error bars represent standard deviations. $^{*}P = 0.001–0.05$; $^{\dagger}P < 0.001$.

mRNA at a concentration of 50 μM, respectively, when compared with untreated infected cells. Furthermore, a similar inhibitory effect of ribavirin on genome replication and sg mRNA transcription of PEDV was observed. The amounts of PEDV genomic RNA and sg mRNA detected in cells treated with 200 μM ribavirin were only about 13% and 11% of the untreated level (Fig. 5B). The decreases in viral RNA levels after the addition of ribavirin were not due to nonspecific inhibition of transcription, as mRNA levels of the internal control (β-actin or GAPDH) remained unchanged in all samples (data not shown). Altogether, these results indicated that ribavirin suppresses the synthesis of the nidoviral genomic RNA and sg mRNA.

3.6. Mechanisms of action of ribavirin against porcine nidoviruses

Several mechanisms of action for the antiviral activity of ribavirin have been suggested, including a reduction in cellular GTP pools via inosine monophosphate dehydrogenase (IMPDH) inhibition and increased mutation frequency on the virus genome leading to error catastrophe (Graci and Cameron, 2006). To assess these known mechanisms, we first examined whether replenishment of guanosine affected the antiviral effect of ribavirin against porcine nidovirus infection. The addition of 100 μM guanosine to the ribavirin-treated and virus-infected cells was found to moderately reverse the antiviral activity of ribavirin. The incubation of ribavirin alone reduced PRRSV production to 29% and 17% at 10 μM and 50 μM, respectively, whereas supplementation of guanosine to ribavirin inhibited virus production to 40% and 26% at the same concentrations (Fig. 6A, left panel). Likewise, while PEDV production was declined to 71%, 37%, and 12% in the presence of ribavirin alone at 50 μM, 100 μM, and 200 μM, respectively, it decreased to 100%, 48%, and 31% at the same concentrations when guanosine was added to the ribavirin treatment (Fig. 6A, right panel). To verify these results, we also tested the effects of MPA, a potent inhibitor of cellular IMPDH, on the replication of PRRSV and PEDV. As shown in Fig. 6B, MPA efficiently reduced porcine nidovirus propagation

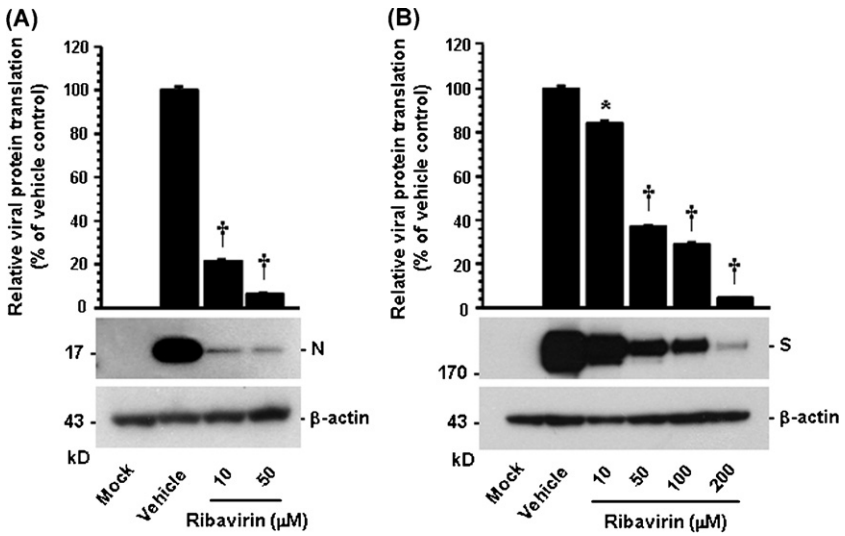

Fig. 4. Inhibition of viral protein translation by ribavirin. Ribavirin-treated PAM-pCD163 and Vero cells were mock-infected or infected with PRRSV (A) or PEDV (B) for 1 h and were further cultivated in the presence or absence of ribavirin. At 48 hpi, cellular lysates were collected, resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted by using the antibody that recognizes the PRRSV N protein or the PEDV S protein. The blot was also reacted with mouse MAb against β-actin to verify equal protein loading. Each viral protein expression was quantitatively analyzed by densitometry in terms of the relative density value to the β-actin gene and ribavirin-treated sample results were compared to vehicle-control results. Values are representative of the mean from three independent experiments and error bars denote standard deviations. $^{*}P = 0.001–0.05$; $^{\dagger}P < 0.001$.

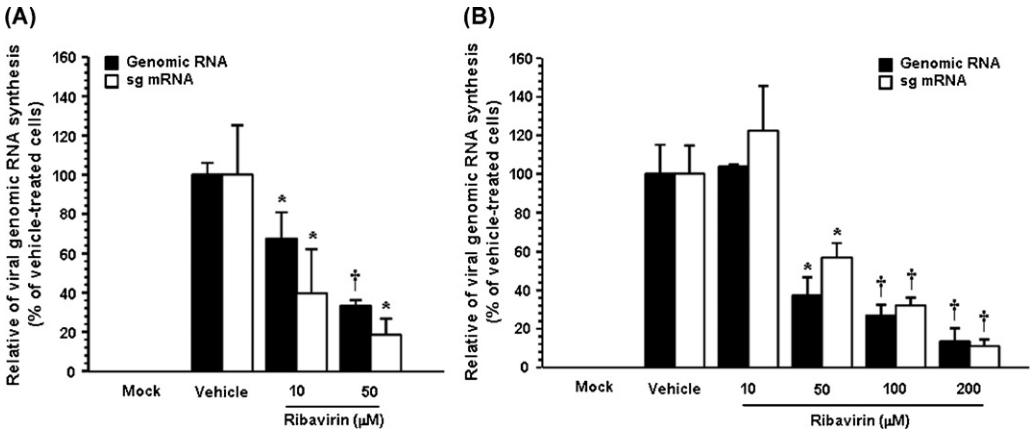

Fig. 5. Inhibition of viral RNA transcription by ribavirin. PAM-pCD163 and Vero cells pretreated with ribavirin were mock-infected or infected with PRRSV (A) or PEDV (B) for 1 h and were incubated in the presence of ribavirin. Total cellular RNA was extracted at 48 hpi, and strand-specific viral genomic RNAs (black bars) and sg mRNAs (white bars) of PRRSV and PEDV were amplified by quantitative real-time RT-PCR. Viral positive-sense genomic RNA and sg mRNA were normalized to mRNA for porcine β-actin or monkey GAPDH and relative quantities (RQ) of mRNA accumulation were evaluated. Ribavirin-treated sample results were compared with untreated results. Values are representative of the mean from three independent experiments and error bars denote standard deviations. $^{*}P = 0.001–0.05$; $^{\dagger}P < 0.001$.

at concentrations greater than 0.5 μM and 0.1 μM in a dose-dependent manner for PRRSV and PEDV, respectively. Sequence analysis of the porcine nidovirus genome in the presence or absence of ribavirin was conducted to investigate whether it triggers catastrophic mutations. We analyzed 20 recombinant sequences of the ORF5 to ORF7-coding region corresponding to nucleotide numbers 13,559–15,090 (PRRSV) and the N gene region corresponding to nucleotide numbers 26,335–27,660 (PEDV) from each virus grown in the presence of ribavirin. As controls, 20 colonies were also obtained individually from untreated PRRSV- and PEDV-infected

Table 2
Summary of mutations noted in sequence analysis of wild-type and ribavirin-treated PRRSV and PEDV N genes.

Virus	Treatment	Groups (%)[a]	Mutation category		
			Transitions	Transversions	Total[b]
PRRSV	No ribavirin	17/20 (85)	1.18	0.51	1.69
	Ribavirin	17/20 (85)	1.08	0.41	1.49
PEDV	No ribavirin	17/20 (85)	1.09	0.15	1.24
	Ribavirin	17/20 (85)	1.51	0.15	1.66

[a] The PRRSV ORF5-ORF7 coding region (1532 bp) and the PEDV N (1326 bp) gene were RT-PCR amplified and the amplicon product was then cloned into the pGEM-T vector. For sequencing of the gene in the recombinant vector, 20 clones per group were selected and analyzed the number (rate) of mutations.
[b] A total of 30,640 nt and 26,520 nt for PRRSV and PEDV were sequenced and a mutation frequency was calculated per 1000 nt, respectively.

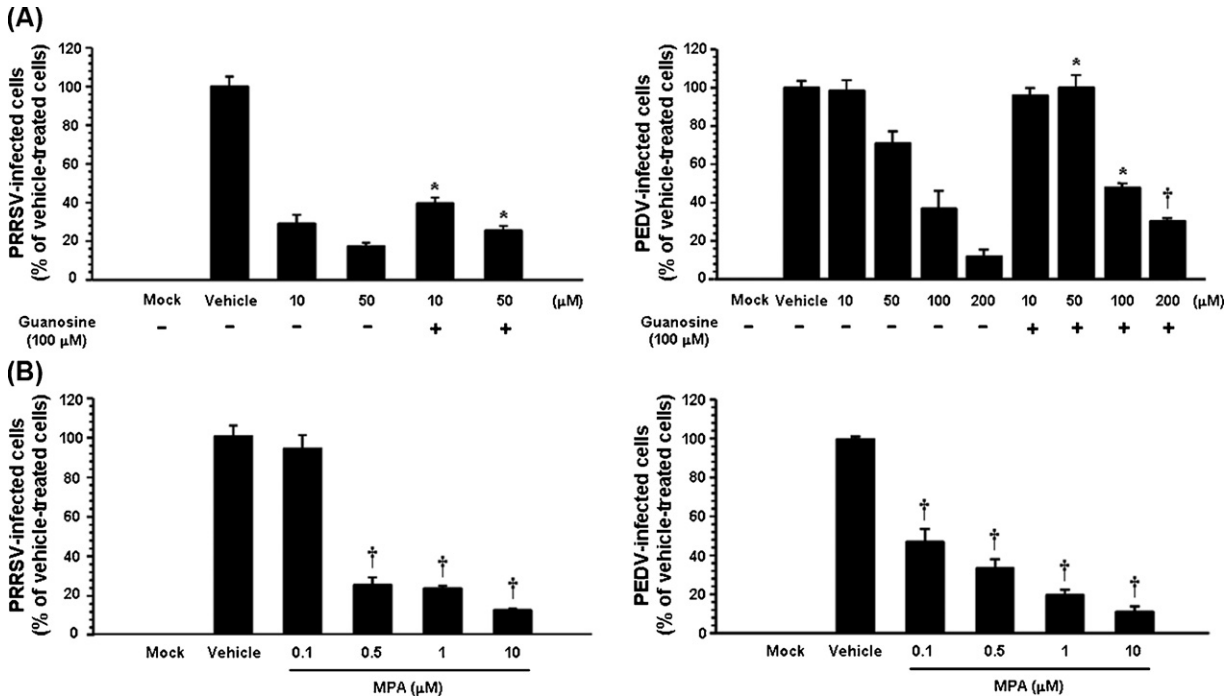

Fig. 6. Effects of supplementation of guanosine in ribavirin treatment and MPA on porcine nidovirus infection. (A) Effect of ribavirin alone or with guanosine (100 μM) on the replication of PRRSV (left) and PEDV (right). PAM-pCD163 and Vero cells were preincubated with various concentrations of ribavirin with (+) or without 100 (−) μM guanosine. Virus-infected cells were fixed at 48 hpi and subjected to immunofluorescence assay. Viral productions were measured by quantifying the number of cells expressing N proteins through IFA as described above. Viral productions in cells treated by guanosine and ribavirin that were significantly different from those in cells treated with ribavirin alone are indicated. (B) Effect of MPA on the replication of PRRSV (left) and PEDV (right). PAM-pCD163 and Vero cells were preincubated with various concentrations of MPA ranging from 0 (mock) to 10 μM and infected with PRRSV or PEDV. At 48 hpi, virus-infected cells were subjected to immunofluorescence assay, and viral productions were measured by quantifying the number of cells expressing N proteins through IFA as described above. Data are representative of the mean values from three independent experiments and error bars represent standard deviations. $^{*}P = 0.001–0.05$; $^{\dagger}P < 0.001$.

cells. In the ribavirin-treated groups, point mutations occurred in 17 (85%) plasmids for both PRRSV and PEDV, which corresponded to a mutation frequency of 1.49 and 1.66 per 1000 nt, respectively. Interestingly, mutations were also found in 17 (85%) plasmids in the untreated groups for PRRSV and PEDV, which were calculated as a mutation frequency of 1.69 and 1.24 per 1000 nt, respectively (Table 2). These sequencing results indicated that there were no significant differences in the mutation rates of ribavirin-treated and non-treated groups upon porcine nidovirus infection.

4. Discussion

The *Nidovirales* are an order of enveloped, positive-strand RNA viruses with animal hosts, which synthesize a nested set of multiple sg mRNAs. This order includes three families *Arteriviridae*, *Coronaviridae*, and *Roniviridae*, which have similar genome organization and replication strategy, but different virion morphology and genome length (Mayo, 2002; Lai et al., 2007; Snijder and Spaan, 2007). Porcine nidoviruses are not only devastating viral pathogens to the pig population, but can also be applied as an animal virus model to study human or veterinary-important nidoviruses. Among these, despite tremendous efforts to control the diseases, PRRSV and PEDV have continuously plagued pig-producing countries, leading to significant economic impacts on the global or Asian swine industry, respectively. This is partially due to a lack of efficient vaccines capable of conferring full protection against viral infections and antiviral agents for treating porcine nidoviruses. Although ribavirin has an antiviral effect on a variety of DNA and RNA viruses (Sidwell et al., 1972), its efficacy and mode of action on porcine nidovirus replication are currently unknown. The present study demonstrated that ribavirin also exerts very effective antiviral activity against PRRSV and PEDV in vitro, as indicated by ED_{50} values of approximately 21 μM (5.1 μg/ml) and 73 μM (17.8 μg/ml), respectively. Ribavirin can potentially act via inhibition of various steps of the virus life cycle, including viral translation, genome or transcript capping, RNA synthesis, and progeny virus spread (Graci and Cameron, 2006). Treatment of cells with ribavirin resulted in significant attenuation of postentry steps during the replication of porcine nidovirus, as determined by lower progeny production, diminished viral protein expression, and decreased synthesis of genomic RNA and sg mRNA. It was previously reported that the tier of murine norovirus 1 (MNV-1) in the presence of ribavirin dropped about 10-fold after virus infection, but remained similar after 48 h of virus infection (Chang and George, 2007). In this study, growth kinetics experiments also indicated that the reduced rate of nidoviral titers in the presence of ribavirin was found to be comparable between 24 and 48 hpi. Since ribavirin should be converted to its monophosphate active form (RMP) to exert antiviral activity, it appears that virus infection may disrupt the normal metabolic processes, leading to interference with the conversion of ribavirin (Parker, 2005). Taken together, our data indicate that ribavirin potently elicits antiviral activity against PRRSV and PEDV in target cells.

An important aspect of the antiviral activity of ribavirin may be attributed to the ability to act via multiple mechanisms simultaneously. Although the mechanisms of action of ribavirin still remain controversial, five primary mechanisms have been proposed depending on the particular virus. These include reduction in cellular GTP pools by inhibiting IMPDH, enhanced mutation frequency via its incorporation into the viral genome leading to catastrophic error, modulation of host immune responses, inhibition of 5′ mRNA capping, and interference with viral RNA polymerase. Therefore, the mechanisms of action of ribavirin may differ among viruses, and its antiviral activity may be operated

predominantly via one or two mechanisms (Graci and Cameron, 2006; Parker, 2005). To elucidate potential mechanisms responsible for the antiviral effect of ribavirin on porcine nidoviruses, we first tested whether the antinidoviral activity of ribavirin is involved in inhibition of IMPDH and subsequent depression of cellular GTP levels. In previous studies, the addition of guanosine to the culture medium efficiently reversed the antiviral effects of ribavirin against norovirus, yellow fever virus, and human parainfluenza virus 3 (Chang and George, 2007; Leyssen et al., 2005). The present study so investigated the effects replenishing GTP by adding guanosine to ribavirin-treated cells upon virus infection. Consistent with previous reports, the addition of guanosine to the ribavirin treatment significantly reversed the antiviral activity of ribavirin in porcine nidoviruses. Furthermore, a noncompetitive IMPDH inhibitor, MPA, was found to strongly inhibit the replication of PRRSV and PEDV at concentrations above 0.5 μM and 0.1 μM, respectively. In contrast to ribavirin, which has to be converted to the active RMP form to elicit the antiviral activity, MPA does not require metabolic activation to exert its function. Thus, the efficient antinidoviral activity of MPA suggests that ribavirin may directly lead to the inhibition of intracellular IMPDH levels. Since RNA viruses replicate with high genetic variation, they exist as viral quasispecies that are complex and dynamic mutant distributions that share a dominant nucleotide sequence and a mutant spectrum (Ruiz-Jarabo et al., 2000). Therefore, RNA viruses reside on the edge of mutation crisis, and the increased average error frequency can cause defective genetic information and reduced viability termed error catastrophe (Crotty et al., 2000; Day et al., 2005). Ribavirin has been shown to trigger catastrophic mutations including lethal mutations in a variety of viruses and these may accumulate as a virus goes through multiple rounds of replication, resulting in extinction of the viral population (Crotty et al., 2001; Contreras et al., 2002; Day et al., 2005; Lanford et al., 2001; Severson et al., 2003). However, sequence analysis of the conserved N gene regions of PRRSV and PEDV in the ribavirin-treated or untreated groups revealed that the two groups produced similar mutation frequencies (1.89/1.66 versus 2.42/1.24). These results were not surprising since RNA genomes mutate at average rates of 10^{-3}–10^{-5} per nucleotide copied during replication of RNA viruses by the virus-encoded RNA-dependent RNA polymerase (RdRp) lacking proofreading functions (Drake and Holland, 1999). Our data indicated that the antiviral activity of ribavirin against porcine nidoviruses may not be associated with error-prone replication by increasing the probability of ribavirin incorporation into the viral genome.

In conclusion, our findings described here demonstrated that ribavirin effectively prevents the replication of porcine nidoviruses in target cells at subcytotoxic doses. Additionally, the highest doses of ribavirin used in this study did not increase the frequency of mutations in the nidoviral genome, and instead affected intracellular GTP levels via IMPDH inhibition. Thus, we propose that one of the modes of action of ribavirin to elicit the antiviral effects against porcine nidoviruses occurs via GTP depletion, which may not work in concert with catastrophic mutations. Although further studies based on in vivo assessment are needed to evaluate the efficacy and safety of ribavirin, the results presented here indicate that it is a good candidate of choice for antiviral approach against porcine nidoviral diseases.

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References

Albina, E., Madec, F., Cariolet, R., Torrison, J., 1994. Immune response and persistence of the porcine reproductive and respiratory syndrome virus in infected pigs and farm units. Veterinary Record 134, 567–573.
Albina, E., 1997. Epidemiology of porcine reproductive and respiratory syndrome (PRRS): an overview. Veterinary Microbiology 55, 309–316.
Allan, G.M., McNeilly, F., Ellis, J., Krakowka, S., Meehan, B., McNair, I., Walker, I., Kennedy, S., 2000. Experimental infection of colostrum deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. Archives of Virology 145, 2421–2429.
Bautista, E.M., Faaberg, K.S., Mickelson, D., McGruder, E.D., 2002. Functional properties of the predicted helicase of porcine reproductive and respiratory syndrome virus. Virology 298, 258–270.
Cai, Y., Liu, Y., Zhang, X., 2007. Suppression of coronavirus replication by inhibition of the MEK signaling pathway. Journal of Virology 81, 446–456.
Cavanagh, D., 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Archives of Virology 142, 629–633.
Chang, K.O., George, D.W., 2007. Interferons and ribavirin effectively inhibit Norwalk virus replication in replicon-bearing cells. Journal of Virology 81, 12111–12118.
Christopher-Hennings, J., Nelson, E.A., Hines, R.J., Nelson, J.K., Swenson, S.L., Zimmerman, J.J., Chase, C.L., Yaeger, M.J., Benfield, D.A., 1995. Persistence of porcine reproductive and respiratory syndrome virus in serum and semen of adult boars. Journal of Veterinary Diagnostic Investigation 7, 456–464.
Contreras, A.M., Hiasa, Y., He, W., Terella, A., Schmidt, E.V., Chung, R.T., 2002. Viral RNA mutations are region specific and increased by ribavirin in a full-length hepatitis C virus replication system. Journal of Virology 76, 8505–8517.
Cooper, A.C., Banasiak, N.C., Allen, P.J., 2003. Management and prevention strategies for respiratory syncytial virus (RSV) bronchiolitis in infants and young children: a review of evidence-based practice interventions. Pediatric Nursing 29, 452–456.
Crotty, S., Maag, D., Arnold, J.J., Zhong, W., Lau, J.Y., Hong, Z., Andino, R., Cameron, C.E., 2000. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nature Medicine 6, 1375–1379.
Crotty, S., Cameron, C.E., Andino, R., 2001. RNA virus error catastrophe: direct molecular test by using ribavirin. Proceedings of the National Academy of Sciences of the United States of America 98, 6895–6900.
Cummings, K.J., Lee, S.M., West, E.S., Cid-Ruzafa, J., Fein, S.G., Aoki, Y., Sulkowski, M.S., Goodman, S.N., 2001. Interferon and ribavirin vs interferon alone in the re-treatment of chronic hepatitis C previously nonresponsive to interferon: a meta-analysis of randomized trials. Journal of the American Medical Association 285, 193–199.
Davis, G.L., Esteban-Mur, R., Rustgi, V., Hoefs, J., Gordon, S.C., Trepo, C., Shiffman, M.L., Zeuzem, S., Craxi, A., Ling, M.H., Albrecht, J., 1998. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. New England Journal of Medicine 339, 1493–1499.
Day, C.W., Smee, D.F., Julander, J.G., Yamshchikov, V.F., Sidwell, R.W., Morrey, J.D., 2005. Error-prone replication of West Nile virus caused by ribavirin. Antiviral Research 67, 38–45.
Debouck, P., Pensaert, M., 1980. Experimental infection of pigs with a new porcine enteric coronavirus, CV 777. American Journal of Veterinary Research 41, 219–223.
Drake, J.W., Holland, J.J., 1999. Mutation rates among RNA viruses. Proceedings of the National Academy of Sciences of the United States of America 96, 13910–13913.
Duan, X., Nauwynck, H.J., Pensaert, M.B., 1997. Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). Veterinary Microbiology 56, 9–19.
Feng, W., Laster, S.M., Tompkins, M., Brown, T., Xu, J.S., Altier, C., Gomez, W., Benfield, D., McCaw, M.B., 2001. In utero infection by porcine reproductive and respiratory syndrome virus is sufficient to increase susceptibility of piglets to challenge by *Streptococcus suis* type II. Journal of Virology 75, 4889–4895.
Graci, J.D., Cameron, C.E., 2006. Mechanisms of action of ribavirin against distinct viruses. Reviews in Medical Virology 16, 37–48.
Harms, P.A., Sorden, S.D., Halbur, P.G., Bolin, S.R., Lager, K.M., Morozov, I., Paul, P.S., 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Veterinary Pathology 38, 528–539.
Hofmann, M., Wyler, R., 1988. Propagation of the virus of porcine epidemic diarrhea in cell culture. Journal of Clinical Microbiology 26, 2235–2239.
Lanford, R.E., Chavez, D., Guerra, B., Lau, J.Y., Hong, Z., Brasky, K.M., Beames, B., 2001. Ribavirin induces error-prone replication of GB virus B in primary tamarin hepatocytes. Journal of Virology 75, 8074–8081.
Lai, M.M., Perlman, S., Anderson, L.J., 2007. Coronaviridae. In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia, pp. 1305–1335.
Lee, Y.J., Lee, C., 2010. Porcine reproductive and respiratory syndrome virus replication is suppressed by inhibition of the extracellular signal-regulated kinase (ERK) signaling pathway. Virus Research 152, 50–58.
Lee, Y.J., Park, C.K., Nam, E., Kim, S.H., Lee, O.S., Lee, D.S., Lee, C., 2010. Generation of a porcine alveolar macrophage cell line for the growth of porcine reproductive and respiratory syndrome virus. Journal of Virological Methods 163, 410–415.
Leyssen, P., Balzarini, J., De Clercq, E., Neyts, J., 2005. The predominant mechanism by which ribavirin exerts its antiviral activity in vitro against flaviviruses and

paramyxoviruses is mediated by inhibition of IMP dehydrogenase. Journal of Virology 79, 1943–1947.
Mayo, M.A., 2002. A summary of taxonomic changes recently approved by the ICTV. Archives of Virology 147, 1655–1656.
McCormick, J.B., King, I.J., Webb, P.A., Scribner, C.L., Craven, R.B., Johnson, K.M., Elliott, L.H., Belmont-Williams, R., 1986. Lassa fever. Effective therapy with ribavirin. New England Journal of Medicine 314, 20–26.
Nam, E., Lee, C., 2010. Contribution of the porcine aminopeptidase N (CD13) receptor density to porcine epidemic diarrhea virus infection. Veterinary Microbiology 144, 41–50.
Neumann, E.J., Kliebenstein, J.B., Johnson, C.D., Mabry, J.W., Bush, E.J., Seitzinger, A.H., Green, A.L., Zimmerman, J.J., 2005. Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. Journal of the American Veterinary Medical Association 227, 385–392.
Pensaert, M.B., Yeo, S.G., 2006. Porcine epidemic diarrhea. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.), Diseases of Swine. Wiley-Blackwell, Ames, pp. 367–372.
Parker, W.B., 2005. Metabolism and antiviral activity of ribavirin. Virus Research 107, 165–171.
Rockx, B., Bossart, K.N., Feldmann, F., Geisbert, J.B., Hickey, A.C., Brining, D., Callison, J., Safronetz, D., Marzi, A., Kercher, L., Long, D., Broder, C.C., Feldmann, H., Geisbert, T.W., 2010. A novel model of lethal Hendra virus infection in African green monkeys and the effectiveness of ribavirin treatment. Journal of Virology 84, 9831–9839.
Ruiz-Jarabo, C.M., Arias, A., Baranowski, E., Escarmis, C., Domingo, E., 2000. Memory in viral quasispecies. Journal of Virology 74, 3543–3547.
Sagong, M., Lee, C., 2011. Porcine reproductive and respiratory syndrome virus nucleocapsid protein modulates interferon-β production by inhibiting IRF3 activation in immortalized porcine alveolar macrophages. Archives of Virology 156, 2187–2195.
Severson, W.E., Schmaljohn, C.S., Javadian, A., Jonsson, C.B., 2003. Ribavirin causes error catastrophe during Hantaan virus replication. Journal of Virology 77, 481–488.
Sidwell, R.W., Huffman, J.H., Khare, G.P., Allen, L.B., Witkowski, J.T., Robins, R.K., 1972. Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. Science 25, 705–706.
Snijder, E.J., Spaan, W.J.M., 2007. Arteriviridae. In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia, pp. 1337–1361.
Spaan, W.J.M., Brian, D., Cavanagh, D., de Groot, R.J., Enjuanes, L., Gorbalenya, A.E., Holmes, K.V., Masters, P.S., Rottier, P.J., Taguchi, F., Talbot, P., 2005. Family Coronaviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberg, U., Ball, L.A. (Eds.), Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, London, pp. 947–964.
van Aken, D., Zevenhoven-Dobbe, J., Gorbalenya, A.E., Snijder, E.J., 2006. Proteolytic maturation of replicase polyprotein pp1a by the nsp4 main proteinase is essential for equine arteritis virus replication and includes internal cleavage of nsp7. Journal of General Virology 87, 3473–3482.
Wills, R.W., Gray, J.T., Fedorka-Cray, P.J., Yoon, K.J., Ladely, S., Zimmerman, J.J., 2000. Synergism between porcine reproductive and respiratory syndrome virus (PRRSV) and Salmonella choleraesuis in swine. Veterinary Microbiology 71, 177–192.
Wills, R.W., Doster, A.R., Galeota, J.A., Sur, J.H., Osorio, F.A., 2003. Duration of infection and proportion of pigs persistently infected with porcine reproductive and respiratory syndrome virus. Journal of Clinical Microbiology 41, 58–62.
Ziebuhr, J., Snijder, E.J., Gorbalenya, A.E., 2000. Virus-encoded proteinases and proteolytic processing in the Nidovirales. Journal of General Virology 81, 853–879.