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Ribavirin efficiently suppresses porcine nidovirus replication

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A R T I C L E   I N F O  
Article history:  
Received 31 July 2012  
Received in revised form 19 October 2012  
Accepted 19 October 2012  
Available online 27 October 2012  
Keywords:  
Porcine nidovirus  
PRRSV  
PEDV  
Ribavirin  
Antiviral activity

A B S T R A C T  
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.

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1. Introduction  
The *Nidovirales* are an order of enveloped single-stranded positive-sense RNA viruses with animal hosts that include the families *Arterviridae, 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 *Nidovi- rales* 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 etiologic 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 etiologic 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).
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 (Cummins 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 nivoviruses. In the present study, therefore, we tried to investigate the antiviral activity of ribavirin and its mechanism of action in target cells upon porcine nivovirus 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 nivoviruses. Altogether, our results suggest that ribavirin may be an excellent therapeutic option for nivovirus 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% CO2 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 0.1, 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 Iysis buffer (0.5% Triton X-100, 60 mM β-glycerophosphate, 15 mM p-nitro phenyl phosphate, 25 mM MOPS, 15 mM, MgCl2, 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.uhmcresearch.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       | TGCCTCGTCTGATGACCC          | Real-time RT-PCR          | 1400–1417     |
|           | Genomic RNA-Rev       | CGACCAGATTGATTGGAAGAGG      | Real-time RT-PCR          | 1623–1644     |
|           | sg mRNA-Fwd           | GCAGCAGAGGACGATGGGAGAGG     | Real-time RT-PCR          | 46–64         |
|           | sg mRNA-Rev           | TGATGTCGGAGTGTAG            | Real-time RT-PCR & PCR    | 15,073–15,090 |
|           | ORF5-Fwd              | GTGAGAAGGTTCGTTACCT         | RT-PCR                   | 13,559–14,736 |
|           | Porcine β-actin-Fwd   | GACACCTTTCACTGCACTCA        | Real-time RT-PCR          | 920–939       |
|           | Porcine β-actin-Rev   | GGTGTGCGGATAGCCCTCTT       | Real-time RT-PCR          | 963–982       |
|           | Genomic RNA-Fwd       | AGTTGGGGCTTTAGTGCGG         | Real-time RT-PCR          | 12,611–12,630 |
| PEDV      | Genomic RNA-Rev       | GCTTTACAAATTTCCAGGCCG       | Real-time RT-PCR          | 12,741–12,762 |
|           | sg mRNA-Fwd           | GCCCTCTTGTTCTTCAACTCC       | Real-time RT-PCR          | 44–67         |
|           | sg mRNA-Rev           | TGCCTTAGCTTTATCCCTT         | Real-time RT-PCR          | 26,461–26,480 |
| N gene    | Fwd                   | ATGGCTTCTGTCAGC             | RT-PCR                   | 26,335–26,349 |
| N gene    | Rev                   | TTAATTTCTCGCTCT             | RT-PCR                   | 27,646–27,660 |
| Monkey    | GAPDH-Fwd             | TCAACGGCCAGACCCACTCT         | Real-time RT-PCR          | 959–967       |
| Monkey    | GAPDH-Rev             | CTCTCTCTGCTGTCTGCTG         | 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 unintegrated 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 (TCID50) 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−ΔΔCt 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 TCID50 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 OR5S 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.
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% (ED50) 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.
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 10^5 TCID_{50}/ml and 10^6 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 10^5.53 TCID_{50}/ml and 10^5.32 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 late 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 mRNA.
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.
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 ORFs 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

![Image](https://example.com/image1)

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 cultured 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; †P<0.001.

![Image](https://example.com/image2)

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; †P<0.001.

| Virus | Treatment | Groups (%) | Mutation category | Transitions | Transversions | Total |
|-------|------------|------------|-------------------|-------------|---------------|-------|
| 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 |

* 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.

* 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; P<0.05; P<0.001.

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 ED50 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
Acknowledgment

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-002318).

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