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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.

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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 *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.

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