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Ivermectin inhibits DNA polymerase UL42 of pseudorabies virus entrance into the nucleus and proliferation of the virus in *vitro* and *vivo*

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ABSTRACT

Pseudorabies virus (PRV) is an important viral pathogen of pigs that causes huge losses in pig herds worldwide. Ivermectin is a specific inhibitor of importin-α/β-dependent nuclear transport and shows antiviral potential against several RNA viruses by blocking the nuclear localization of viral proteins. Since the replication of DNA viruses is in the nucleus, ivermectin may be functional against DNA virus infections if the DNA polymerase or other important viral proteins enter the nucleus via the importin-α/β-mediated pathway. Here, we determined whether ivermectin suppresses PRV replication in hamster kidney BHK-21 cells and investigated the effect of ivermectin on the subcellular localization of the PRV UL42 protein, the accessory subunit of PRV DNA polymerase. Also, an *in vivo* anti-PRV assay was conducted in mice. Our data demonstrate that ivermectin treatment inhibits PRV infection in cells in a dose-dependent manner. Treatment of PRV-infected cells with ivermectin significantly suppressed viral DNA synthesis and progeny virus production. Ivermectin disrupted the nuclear localization of UL42 by targeting the nuclear localization signal of the protein in transfected cells. Ivermectin treatment increased the survival rates of mice infected with PRV and relieved infection as indicated by lower clinical scores and fewer gross lesions in the brain. Together, our results suggest that ivermectin may be a therapeutic or preventative agent against PRV infection.

1. Introduction

Pseudorabies virus (PRV) is the causative agent of pseudorabies (PR), which is an economically important swine disease. PRV is a member of the alpha-herpesvirinae subfamily (Klupp et al., 2004; Pomeranz et al., 2005) and the virus may cause lifelong infection in pigs by residing in the trigeminal ganglia (Hafezi et al., 2012). Vaccines are widely used to control PRV; however, vaccination has failed to provide complete protection and, in particular, the immunity induced by the vaccination does not prevent infection by the virus (Stegeman et al., 1994). Moreover, highly pathogenic PRV variants have emerged since 2012 (Tong-Qing et al., 2013) and circulate in pig farms (Wang et al., 2017; Zhou et al., 2017). Whenever the immunity of the pigs is decreased or the pigs suffer stress, PR occurs and causes varying degrees of loss. Thus, novel antiviral drugs that specifically inhibit PRV propagation are urgently needed to control the disease.

Virus DNA polymerase and RNA-depended RNA polymerase are targets of antiviral drugs currently under development. The DNA polymerase of PRV has two subunits, UL30 and UL42 (Purifoy et al., 1977). UL30 is a catalytic subunit with inherent polymerase activity and UL42 is a processivity factor that imparts increased DNA-binding specificity to the viral DNA polymerase and decreases dissociation of the primer and template without reducing the elongation rate (Weisshart et al., 1999). Decreasing UL42 expression by using RNAi significantly reduces PRV replication indicating an important role of the protein in viral proliferation (Wang et al., 2016b). A recent report found that UL42 contains an importin-α/β-mediated bipartite nuclear localization signal (NLS) that transports not only UL42 but also UL30 into the nucleus (Wang et al., 2016a). As DNA viruses replicate in the nucleus, the nuclear transport of UL42 may be a determining factor for PRV replication.

Ivermectin was identified as a broad-spectrum inhibitor of importin-α/β-mediated nuclear import (Wagstaff et al., 2012). By restraining nuclear transport of the integrase of HIV-1 and, the polymerase, non-structural protein 5, of dengue virus (DEV), ivermectin inhibits HIV-1 and DEV proliferation and thus exhibits antiviral potential (Wagstaff et al., 2012) in addition to its widely known anti-parasitic activity. Since this finding was reported, the antiviral activity of the drug has

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been investigated in many studies. Ivermectin could inhibit flavivirus replication by inhibiting the unwinding activity of the helicase NS3 (Mastrangelo et al., 2012). Furthermore, it could reduce chikungunya (Varghese et al., 2016), porcine reproductive and respiratory syndrome (Lee and Lee, 2016) virus proliferation (Barrows et al., 2016; Currie et al., 2004; Nguyen et al., 2015). As the nuclear transport of UL42 of PRV is reliant on importin-α/β-mediated nuclear import pathways (Wang et al., 2016a), ivermectin may be a candidate anti-PRV drug.

In the current study, the antiviral role of ivermectin was determined and the mechanism was studied.

2. Materials and methods

2.1. Cells and virus

BHK-21 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel), 100 μg/ml streptomycin, and 100 IU/ml penicillin, at 37 °C in 5% CO_2 atmosphere. The PRV was previously isolated from a pig farm and kept in our laboratory. The virus was propagated in BHK-21 cells and stored at −80 °C until use.

2.2. Recombinant plasmids construction

The complete open reading frame and NLS of UL42 were amplified from the genomic DNA of PRV using primers harboring restriction endonuclease sites for cloning (Table 1). The amplicons were cloned into eukaryotic expression vectors pDC315 or pDC315-N-Flag to generate recombinant plasmids pDC315-Flag-UL42 and pDC315-Flag-NLS_{UL42}.

To generate a standard curve for quantification of the number of virus copies, a part of the Glycoprotein E (GE) gene of PRV was amplified from the PRV genome and cloned into the pMD19-T Simple Vector, generating the recombinant plasmid pMD19-T-GE_1. Serial dilutions of the plasmid pMD19-T-GE_1. were used to generate a standard curve for absolute quantification of viral DNA copies. The primers used are listed in Table 1.

2.3. Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 (BestBio, China) as reported (Han et al., 2011). Briefly, cells in 96-well plates were treated with 0.5, 1, 1.5, 3 or 5 μM ivermectin (Sigma, Shanghai) dissolved in dimethyl sulfoxide (DMSO) for 24 and 48 h. Assays were performed in triplicate. Then, 10 μl CCK-8 reagent was added to the cells and incubated in the dark for 2 h at 37 °C. The OD_{450} of each well was read using a spectrophotometer (Bio-Rad). Viabilities of the cells treated with ivermectin were compared with that of negative (DMSO) control.

2.4. Analysis of cell cycle by flow cytometry

BHK-21 cells were harvested after 24 h of treatments with 2.5 μM ivermectin or DMSO. The harvested cells were then fixed with 75% ethanol and stained with MP Biomedicals, the cell cycle reagent. The cell phases were analyzed by flow cytometry (BD LSRFortessa, USA).

2.5. Titer viral TCID50

Ten-time serially diluted PRV samples in serum-free DMEM were inoculated into cells in 96-well plates. Each dilution had eight replicates. The cytopathic effect (CPE) was observed and recorded for 4 days and the viral TCID50 was calculated according to the Reed–Muench method.

2.6. Test antiviral activities of ivermectin in vitro

BHK-21 cells with 80% confluence in 12-well plates were infected with PRV at 0.01 multiplicity of infection (MOI). One hour later, the virus was removed by washing three times with phosphate buffer solution (PBS). The infected cells were treated with different concentrations of ivermectin in DMEM medium supplemented with 2% FBS. The cells (infected, infected + ivermectin, uninfected + ivermectin, and mock-treated cells) were harvested at 16, 24, 48, and 72 h post infection (hpi). Virus in cell lysates were quantified by plaque assay or testing the TCID50 or viral DNA with Q-PCR (Zanella et al., 2012). The CPE was also recorded to show virus proliferation in cells.

2.7. Western blotting

BHK-21 cells were treated with DMSO or 0.5, 1.5, and 2.5 μM ivermectin in DMSO after transfection with recombinant plasmid expressing UL42 of PRV (pDC315-UL42) or PRV infection. Cells were harvested after 48 h incubation for plasmids transfection and 24 h for PRV infection at 37 °C in an incubator with 5% CO_2 and cell nuclear proteins were extracted using a nuclear protein extraction kit following the manufacturer's instruction (BestBio). And cytoplasmic proteins were also collected for further analysis.

Proteins were separated with SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore Corporation). After blocking with 10% fat-free milk in TBS at 37 °C for 2 h, the proteins were bound with 1:3000 diluted Primary antibodies (mouse polyclonal antibody against Flag-tag (Sangon Biotech, Shanghai, China), mouse monoclonal antibody against Herpes Simplex Virus UL42 (Abcam, ab19311), mouse monoclonal antibody against β-tubulin (Sungene Biotech, Tianjin, China), and (or) Histone H3.1 (Sungene Biotech, Tianjin, China)) in TBS at 37 °C for 2 h. HRP-conjugated goat anti-mouse IgG (1:3000; Sangon Biotech, Shanghai, China) was used as the secondary antibody and protein bands were visualized using chemiluminescence luminol reagents (SuperSignal West PicoTrial Kit, Pierce).

2.8. DNA extraction and Real-time quantitative Polymerase Chain Reaction (Q-PCR)

PRV DNA was extracted from infected BHK-21 cells using a viral genome DNA extraction kit (GenStar, Beijing, China) per the manufacturer's protocol. SYBR Green-based absolute Q-PCR was used to

Table 1
Primers used in this study.

Target gene	Primer sequence (5′-3′)	Product size (bp)
pDC315-Flag-UL42-F	CGGAATTCATGTCGCTGTTCGACGACGGCC	1155
pDC315-Flag-UL42-R	CGGGATCCTTACTTATCGTCGTCATCCTTGTAATCCTTATCGTCGTCATCCTTGTAATCGAATAAATCTCCGTAGGCGTGG	
pDC315-Flag-UL42NLS-F	CGGGATCCAAGCGGCCCGCCGCCCCG	51
pDC315-Flag-UL42NLS-R	ACGCGTCGACTTACCGCGGGCGCTTGGC	
pMD19-T-GE_1-F	TCTGCGTGCTGTGCTCCC	342
pMD19-T-GE_1-R	GTCCATTCGTCACTTCCG	
GE-D-F	TCTGCGTGCTGTGCTCCC	130
GE-D-R	TCGTCGCCGTCGTAGTAG	