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Synthetic Toll-like receptor 7 ligand inhibits porcine reproductive and respiratory syndrome virus infection in primary porcine alveolar macrophages

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV), a common viral pathogen, causes huge annual economic losses to the swine industry worldwide. After triggering by specific ligands, the Toll-like receptor 7 (TLR7), a type of pattern-recognition receptor (PRR), induces antiviral cytokines production. Previously, we synthesized an adenine analog, designated SZU101, a TLR7-specific ligand. In this study, we assessed the inhibitory effect of SZU101 on PRRSV infection *in vitro*. SZU101 significantly suppressed PRRSV infection in primary porcine alveolar macrophages (PAMs) in a dose-dependent manner. Moreover, SZU101-induced inhibition involved NF-κB pathway activation in PAMs to initiate expression of TLR7-mediated cytokines and induce expression of downstream signaling IFN-stimulated genes (ISGs). Chloroquine, a TLR7 inhibitor, and BAY 11-7082, an NF-κB inhibitor, reversed both the SZU101-induced antiviral effect and induction of cytokine genes and ISGs expression. Therefore, SZU101 antiviral effects depend at least in part on TLR7-NF-κB signaling pathway. Additionally, administration of SZU101 enhanced the humoral and cell-mediated immune responses against PRRSV antigens in mice. Given these results, SZU101 holds promise as an antiviral agent and a vaccine adjuvant to prevent PRRSV infection in pigs.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an emerging viral disease causing severe reproductive failure in sows and respiratory distress in young pigs. Since its emergence in the late 1980s, PRRS has been one of the most economically costly diseases to the global swine industry (Garcia-Nicolas et al., 2014). PRRSV is a single-stranded positive-sense RNA virus classified within the *Arteriviridae* family (Neumann et al., 2005). In China, an outbreak of highly pathogenic PRRSV in 2006 caused especially great losses (Tong et al., 2007).

Many efforts to control PRRS using vaccination could not provide a sustainable protection (Jiang et al., 2009; Quan et al., 2010), possibly due to PRRSV antigenic and genomic diversity and viral immunosuppression (Kimman et al., 2009; Mateu and Diaz, 2008). Although many antiviral substances were studied (Duan et al., 2015; Hao et al., 2015; Karuppannan et al., 2012; Opriessnig et al., 2011; Yang et al., 2013), none have been used to effectively prevent PRRSV infection yet. Therefore, novel strategies to control PRRS are urgently needed.

TLRs are a family of PRRs that play critical roles in innate

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antimicrobial immune responses (Kumar et al., 2009) through direct recognition of distinct structural patterns associated with invading pathogens (Akira and Sato, 2003). Endosomal TLRs, including TLR3, TLR7, TLR8 and TLR9, participate in antiviral responses by triggering cytokines production (Kawai and Akira, 2010). Moreover, TLR agonists are known to induce intracellular signal transduction cascades and IFNs production (Zhou and Sun, 2015). TLR7 plays important roles in host immune responses against hepatitis C virus (Lee et al., 2006), hepatitis B virus (Chang and Guo, 2015), in blocking of HIV replication (Nian et al., 2012), in regulation of Japanese encephalitis virus infection (Nazmi et al., 2014) and in eliciting anti-influenza virus responses (Goff et al., 2015). Therefore, TLR7 agonists show promise as new immune-potentiating antimicrobial drugs.

In pigs, PAMs are primary target cells for PRRSV acute infection (Rossow et al., 1995) prompting use of primary culture PAMs for *in vitro* anti-PRRSV cellular immune response studies (Lee and Lee, 2012). PRRSV escapes host defenses by interfering with innate immune responses through inhibition of TLR3/7/8 expression in PAMs or dendritic cells (DCs) (Chaung et al., 2010) and by down-regulating IFNs production (Albina et al., 1998).

SZU101, an adenine analog and TLR7 ligand, has demonstrated excellent anti-tumor activity through activation of the TLR7 signaling pathway (Diao et al., 2016). Based on the structure and activity relationship studies (SAR) on the other TLR7 agonists (Bazin et al., 2015; Chan et al., 2009; Kurimoto et al., 2004, 2010) and our chemistry attempts, we realized that the adenine moiety is the core structure which is essential for the TLR7 stimulation and the modification on the phenyl ring is flexible to keep activity on TLR7. Here, SZU101's ability to inhibit PRRSV infection in PAMs *in vitro* correlated with increased mRNA levels of typical TLR7-mediated cytokines and ISGs. This antiviral effect depended at least in part upon TLR7-NF-κB signaling. Results presented here indicate SZU101 could prevent PRRSV infection.

2. Materials and methods

2.1. Cell cultures and viruses

All incubations or reactions were performed at 37 °C and 5% CO_2 in flat-bottom plates using manufacturers' instructions, unless otherwise specified. Marc-145 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS, GE Healthcare Life Sciences, USA).

PAMs were prepared from lung lavage of 8-week-old specific-pathogen-free (SPF) piglets (Landrace × Yorkshire) (Xiao et al., 2014) and maintained in Roswell Park Memorial Institute-1640 medium (RPMI-1640) with 10% heat-inactivated FBS. All animal works complied with guidelines of the Animal Care and Use Committee of Northwest A&F University after prior approval.

PRRSV strains, highly pathogenic PRRSV SD16 (HR-PRRSV/SD16) (GenBank ID: JX087437), HP-PRRSV/JXA1 (GenBank ID: EF112445), HP-PRRSV/GD-HD (GenBank ID: KP793736.1), the prototypical North American type 2 isolate VR2332 (GenBank ID: EF442771), and a low pathogenic PRRSV strain CH-1a (GenBank ID: AY032626) were propagated and titrated on Marc-145 cells and stored at −80 °C (Hao et al., 2015).

2.2. Chemicals

SZU101 (Fig. 1E) was dissolved in RPMI-1640 medium to create a 1 mg/mL stock solution. Chloroquine (CQ), a classical inhibitor of TLR7 activity, inhibits endosomal maturation to prevent proper subcellular TLR7 localization (Lee et al., 2006) (Invitrogen, San Diego, CA, USA). BAY 11-7082 inhibits NF-κB by inhibiting the phosphorylation and degradation of IκBα, which control the activation of NF-κB, followed by abolishing its biological function (Selleckchem, Houston, TX, USA).

2.3. Cell viability assay

SZU101 cytotoxicity was evaluated using the Cell Counting Kit-8 (CCK-8) (Beyotime, Nanjing, China). PAMs were cultured in 96-well plates (1×10^5/well). After cell adherence, SZU101 was added at various concentrations. After 48 h incubation, CCK-8 reagent was added (10 μL/well) and plates were incubated for 2 h. Viable cells were detected using absorbance at 450 nm. Results are a percentage of the ratio of absorbance readings of treated to untreated (100% viable) cells.

2.4. SZU101 stimulation and PRRSV infection

PAMs were seeded into 24-well plates, pretreated with serially diluted SZU101 (0, 1, 10 or 100 μg/mL) for 3 h, then infected with PRRSV at a multiplicity of infection (MOI) of 0.01. Cells and supernatants were analyzed with western blot, qRT-PCR or virus titration at various timepoints. To dismiss a direct effect of SZU101 on PRRSV infectivity, PRRSV was incubated with serially diluted SZU101 at 37 °C for 1 h then SZU101-treated virus was used to infect Marc-145 cells (MOI = 0.01) for 36 h or directly used for virus titration. Viral infectivity was detected with western blot, qRT-PCR or virus titration. To test conditioned media samples from SZU101-stimulated PAMs for anti-viral activity, PAMs were stimulated with SZU101 (100 μg/mL) for 24 h, supernatants were collected and incubated with Marc-145 cells for 18 h. SD16 (MOI = 0.01) was added, incubated for 36 h, then cells were harvested for RNA extraction and western blot.

2.5. Indirect immunofluorescence assay

PAMs after various treatments were fixed with 75% ethanol for 30 min at 4 °C. PRRSV N protein-specific monoclonal antibody (6D10) (Wang et al., 2013) served as primary antibody (1:100) and Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) (Invitrogen, CA, USA) served as secondary antibody (1:300). Stained cells were observed using confocal microscopy (Leica AF6000, Wetzlar, Germany). Control mock-infected cells provided background staining levels.

2.6. Western blot analysis

Western blotting was performed to analyze both N protein and phosphor-NF-κB p65 (pNF-κB p65) levels in PAMs post-treatments. Cell lysate proteins were separated by 12% SDS-PAGE, blotted onto polyvinylidene difluoride (PVDF) membranes, probed with 6D10 (1:2000), rabbit anti-phospho-NF-κB p65 monoclonal antibody (1:1000, Cell Signaling Technology, MA, USA) or mouse anti-α-tubulin monoclonal antibody (1:5000, Sigma-Aldrich, MO, USA) to detect viral N protein, pNF-κB p65 or α-tubulin, respectively. HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA, USA) served as secondary antibodies. Immunostained proteins were visualized using ECL Reagent (Pierce, Rockford, IL, USA).

2.7. Virus titration

Virus progeny were quantified by titration (Xiao et al., 2014). Briefly, Marc-145 cells were seeded into 96-well plates 24 h before virus infection. Virus supernatants from cell cultures were 10-fold serially diluted and 100 μL/well added (eight replicates). Seven