Antiviral Research 97 (2013) 66–73

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Inhibition of porcine reproductive and respiratory syndrome virus replication by flavaspidic acid AB

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ARTICLE INFO

Article history:
Received 11 July 2012
Revised 6 November 2012
Accepted 9 November 2012
Available online 21 November 2012

Keywords:
Porcine reproductive and respiratory syndrome virus (PRRSV)
Flavaspidic acid AB (FA-AB)
Alveolar macrophages
Therapeutic agent

ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) represents a significant challenge to the swine industry worldwide. Current control strategies against PRRSV are still inadequate and there is an urgent need for new antiviral therapies. Flavaspidic acid AB (FA-AB) is a compound derived from *Dryopteris crassirhizoma*, a traditional antiviral Chinese medicine. Here, we first identified its anti-PRRSV activity through targeting multiple stages in PRRSV infection *in vitro*. Our studies demonstrated that FA-AB could inhibit the internalization and cell-to-cell spreading of PRRSV, but not block PRRSV binding to cells. By monitoring the kinetics of PRRSV replication, we showed that FA-AB significantly suppressed PRRSV replication when treatment was initiated 24 h after virus infection. Furthermore, we confirmed that FA-AB was able to significantly induce IFN-α, IFN-β, and IL1-β expression in porcine alveolar macrophages, suggesting that induction of antiviral cytokines by FA-AB could contribute to FA-AB induced inhibition of PRRSV replication. In conclusion, we provide a foundation for the possibility to develop a new therapeutic agent to control PRRSV infection.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important viral diseases for the swine industry worldwide with characteristics of respiratory disorders and abortion in sows (Meulenberg, 2000; Rossow, 1998), leading to significant economic losses (Neumann et al., 2005). PRRS is caused by porcine reproductive and respiratory syndrome virus (PRRSV) (Collins et al., 1992; Wensvoort et al., 1991), which is an enveloped, positive-strand RNA virus belonging to the family Arteriviridae, order Nidovirales based on their similar genome and replication strategy with other members of the family (Cavanagh, 1997). The genome of PRRSV is approximately 15-kb in size (Dea et al., 1995; Mardassi et al., 1994). It has a 5′- and a 3′-untranslated region (UTR) and 10 open reading frames (ORFs 1a, 1b, 2a, 2b, and 3–7, plus the newly identified ORF5a) forming six or seven nested subgenomic viral RNAs (Conzelmann et al., 1993; Johnson et al., 2011; Meng et al., 1994; Meulenberg et al., 1993). ORFs 1a and 1b encode the non-structural proteins (Nsp) involved in processing of the viral polyproteins, genome replication, and transcription. Other ORFs encode the structural proteins including the membrane glycoproteins GP2a, GP2b, GP3, GP4, GP5 and GP5a, the matrix protein (M), and the nucleocapsid protein N (Mardassi et al., 1996; Meulenberg and Petersen-den Besten, 1996; Meulenberg et al., 1995; van Nieuwstadt et al., 1996). PRRSV has two major genotypes, the European genotype (type 1) and the North American genotype (type 2), sharing approximately 60% genome sequence homology (Forsberg, 2005; Hanada et al., 2005). Most recently, there have been devastating outbreaks of atypical PRRS in China, which is characterized by high fever, high morbidity, and high mortality. The causative agent is a highly pathogenic PRRSV (HP-PRRSV) strain with a discontinuous deletion of 30 amino acids in nonstructural protein 2 (NSP2) (Li et al., 2007; Ni et al., 2012; Zhou et al., 2008).

A substantial effort has been made to control and eradicate PRRSV infection since it was identified. However, not much progress has been made and PRRSV still remains the biggest challenge to swine industry. Present management strategies mainly focus on the prevention of infection using vaccination. Unfortunately, the two available types of PRRSV vaccines, the modified live-attenuated vaccines (MLVs) and inactivated vaccines, have certain drawbacks concerning safety (Botner et al., 1997; Nielsen et al., 2001; Scortti et al., 2006) and efficacy (Nilubol et al., 2004; Scortti et al., 2007; Zuckermann et al., 2007). Thus, there is an urgent demand for novel strategies to control PRRSV infection.

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http://dx.doi.org/10.1016/j.antiviral.2012.11.004

Previous studies have discovered a few natural compounds and compositions that have antiviral activities on PRRSV, including some glycosides, terpenoids, coumarins, isoflavones, peptolides, alkaloids, flavones, macrolides (Karuppannan et al., 2012), *N*-acetylpenicillamine (Jiang et al., 2010), sodium tanshinone IIA sulfonate (Sun et al., 2012), and morpholino oligomer (Han et al., 2009; Opriessnig et al., 2011). Opriessnig et al. showed that morpholino oligomer could inhibit PRRSV *in vivo*. However, until now there are no effective commercial drugs available to prevent PRRSV infection.

Flavaspidic acid AB (FA-AB) is a compound isolated from *Dryopteris crassirhizoma Nakai*, a semi-evergreen fern which is widely used as a traditional Chinese medicine. The rhizome of *D. crassirhizoma* commonly serves as an anti-infection agent, especially for the common cold and flu. Recently, it was used in a prescription formula to prevent SARS combined with some other Chinese herb medicines (*Astragalus*, *Atractylodes*, Red *Atractylodes*, *Pogostemon*, *Adenophora*, and *Lonicera*) (Lee et al., 2009). FA-AB structurally belongs to the family of phloroglucinol derivatives (Lee et al., 2003). Previous studies have showed that phloroglucinol derivatives possess antibacterial, antitumor, and antioxidant properties (Kapadia et al., 1996; Lee et al., 2003; Mathekga et al., 2000), and have the ability to inhibit HIV-1 reverse transcriptase (Gupta et al., 2010; Vo and Kim, 2010).

Based on the antiviral activities of *D. crassirhizoma* and phloroglucinol derivatives, we hypothesized that the FA-AB could be an antiviral ingredient of the *D. crassirhizoma*. Here, we explored the antiviral activity of FA-AB against PRRSV infection *in vitro*, and our data showed that FA-AB was able to effectively inhibit PRRSV infection through targeting multiple stages in PRRSV life cycle including internalization, cell to cell spreading, and replication.

2. Materials and methods

2.1. Cells and viruses

Marc-145 cells, a PRRSV-permissive cell line derived from MA-104 cells (Kim et al., 1993), were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS. Porcine alveolar macrophages (PAMs) were obtained from postmortem lung lavage of 8-week-old specific pathogen free (SPF) pigs, and maintained in RPMI 1640 medium with 10% FBS.

PRRSV strains, CH-1a (one of the type 2 PRRSV strain isolated in China) and Hpv (a highly pathogenic PRRSV (HP-PRRSV) isolate), were propagated in Marc-145 cells and PAMs. Virus preparations were titrated, and then stored at −80 °C. Briefly, PRRS virus was serially diluted 10-fold in complete DMEM or RPMI1640 to infect 5×10^4 Marc-145 cells or PAMs in 96-well plates. The level of PRRSV infection was determined 48 h postinfection using immunofluorescent staining for the PRRSV N protein. The viral titer is expressed as tissue culture infective dose 50% ($TCID_{50}$).

2.2. Preparation of flavaspidic acid AB

The rhizomes of *D. crassirhizoma Nakai* were collected from Liaoning province, China. The air-dried roots and rhizomes of the plant were powdered and extracted with 90% aqueous ethanol three times (1 h each time) under reflux. The ethanol extract was mixed with diatomite and eluted with hexane, chloroform, acetone, and ethanol to give four fractions. A portion of the hexane extract was subjected to silica gel column chromatography ($\varnothing 7 \times 90$ cm) with a gradient elution of hexane–acetone (100:1, 20:1, 5:1, 1:l each eluent) to give four fractions (A–D). Fraction D was applied to column Sephadex LH-20 ($\varnothing 1.7 \times 80$ cm, CHCl3-MeOH, 4:6) to get the compound, which was identified as flavaspidic acid AB by comparing spectrum data with literature (Lee et al., 2003). The farinose FA-AB was obtained and dissolved in DMSO. The amount of DMSO in each well within all experiments was adjusted to be 0.4%.

2.3. Cell viability assay

Cytotoxic effects of FA-AB were evaluated by the MTT (3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide) assay. Marc-145 cells or PAMs in a 96-well plate were cultured in 100 μl DMEM or RPMI 1640 containing 0, 10, 20, 30, 40, 50, 60, 70, or 80 μg/ml FA-AB for 48 h at 37 °C. Next, the culture medium was replaced with fresh medium containing 20 μl of 5 mg/ml MTT after washing three times with PBS, and cells were further cultured for 4 h at 37 °C. Cells were then washed carefully and 150 μl DMSO was added per well to dissolve the crystals for 10 min. The resulting absorbance of each well was recorded at 490 nm using a plate reader. The 50% cytotoxic concentrations (CC_{50}) was analyzed by GraphPad Prism (GraphPad Software, San Diego, CA).

2.4. Indirect immunofluorescence assay

The cells were fixed with methanol-acetone solution (1:1) for 10 min at 4 °C, and then were blocked with 5% goat serum in PBS for 30 min at room temperature. PRRSV N protein was detected by incubation with corresponding specific monoclonal antibody, MAb SDOW17 (1:10,000; Rural Technologies) against PRRSV N protein, and the secondary goat anti-mouse IgG (H + L) conjugated with FITC (1:200, Jackson ImmunoResearch). Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI). Immunofluorescence was observed using Leica Microsystems CMS GmbH.

2.5. Real-time reverse-transcription PCR (RT-PCR)

Total RNA was extracted from Marc-145 cells or PAMs using the TRIzol reagent. RNAs were converted to cDNA using Superscript III Reverse Transcriptase (Invitrogen). In replication assay, PRRSV RNA was detected using quantitative real-time RT-PCR with primers designed against PRRSV ORF7 (Patel et al., 2008). A plasmid containing PRRSV ORF7 sequence (Han et al., 2009) was used to generate a standard curve, and then RNA copies in all samples were calculated by comparing them with it. For the transcript levels of cytokines, relative expressions of TNF-α, IL1-β, IFN-α, and IFN-β in FA-AB-treated or non-treated PAMs with or without PRRSV (Hpv strain) infection were quantified by the $2^{-\Delta\Delta CT}$ Method (Livak and Schmittgen, 2001). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was set as a control. The primers used for real-time PCR amplification are listed in Table 1.

Table 1
List of primers for real-time PCR.

Name[a]	Sequences (50–30)[b]
ORF7-F1	AATAACAACGGCAAGCAGCA
ORF7-R1	GCACAGTATGATGCGTCGGC
IFN-α-F1	AGAGCCTCCTGCACCAGTTCT
IFN-α-R1	CTGCATGACACAGGCTTCCA
IL-1β-F1	TCTGCCCTGTACCCCAACTG
IL-1β-R1	CCCAGGAAGACGGGCTTT
IFN-β-F1	AGCACTGGCTGGAATGAAACCG
IFN-β-R1	CTCCAGGTCATCCATCTGCCCA
GAPDH-F1	CCTTCCGTGTCCCTACTGCCAAC
GAPDH-R1	GACGCCTGCTTCACCACCTTCT

[a] F1: forward primer, R1: reverse primer.
[b] Swine gene sequences were downloaded from GenBank.