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

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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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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.

2.6. Western blot analysis

The FA-AB-treated PRRSV-infected or control Marc-145 cells were lysed in a cell lysis buffer (20 mM Tris (pH7.5), 150 mM NaCl, 1% Triton X-100) containing a cocktail of proteinase inhibitors. The supernatant was harvested after centrifugation and the total protein for each sample was measured with a BCA protein assay kit (Thermo). Twenty micrograms of total protein for each sample was analyzed by 12% SDS–PAGE and transferred to nitrocellulose membranes. After blocking, the membranes were incubated for 2 h at room temperature with anti-PRRSV N protein monoclonal antibody SDOW17 (1:2,000; Rural Technologies), and anti-β-actin antibody (1:5,000; Sigma, St. Louis, MO). The membranes were then incubated with the appropriate secondary antibody for 1 h (1:5,000). The antibodies were visualized using the ECL reagent according to the manufacturer's instructions.

2.7. Antiviral assay

The antiviral effects of the FA-AB on CH-1a strain and Hpv at various concentrations were directly observed using indirect immunofluorescence 48 h post infection and the titers ($TCID_{50}$) of virus in cells treated with FA-AB at various concentrations were also determined. The 50% effective concentration (EC_{50}) was determined using a 4 parameter, nonlinear regression of dose response inhibition by plotting log (inhibitor(concentration)) vs. viral titer (variable slope) using GraphPad Prism (GraphPad Software, San Diego, CA).

2.8. PRRSV binding assay

Marc-145 cells were incubated with medium containing CH-1a at a multiplicity of infection (MOI) of 0.1 or 0.01 and FA-AB (30 μg/ml or 40 μg/ml) or DMSO (0.4% final concentration) at 4 °C for 2 h to permit binding, but prevent viral internalization. Unbound virus and FA-AB were removed and fresh medium was added. Cell cultures were then switched to 37 °C to culture for 24 h. The virus titer ($TCID_{50}$) was then measured.

2.9. PRRSV internalization assay

Marc-145 cells were incubated with medium containing CH-1a at 4 °C for 2 h to permit binding, but prevent viral internalization. The inoculums were replaced with fresh culture medium to eliminate the unbound virus particles and the temperature was shifted to 37 °C to allow virus entry to proceed. The FA-AB was then added at 0, 1, or 2 h and removed at 3 h following temperature shift (the time point when cell culture was shifted to 37 °C was set up as 0 h). The cells were carefully washed with phosphate buffered saline (PBS) three times to remove the non-internalized virus particles and FA-AB, and then continued to be

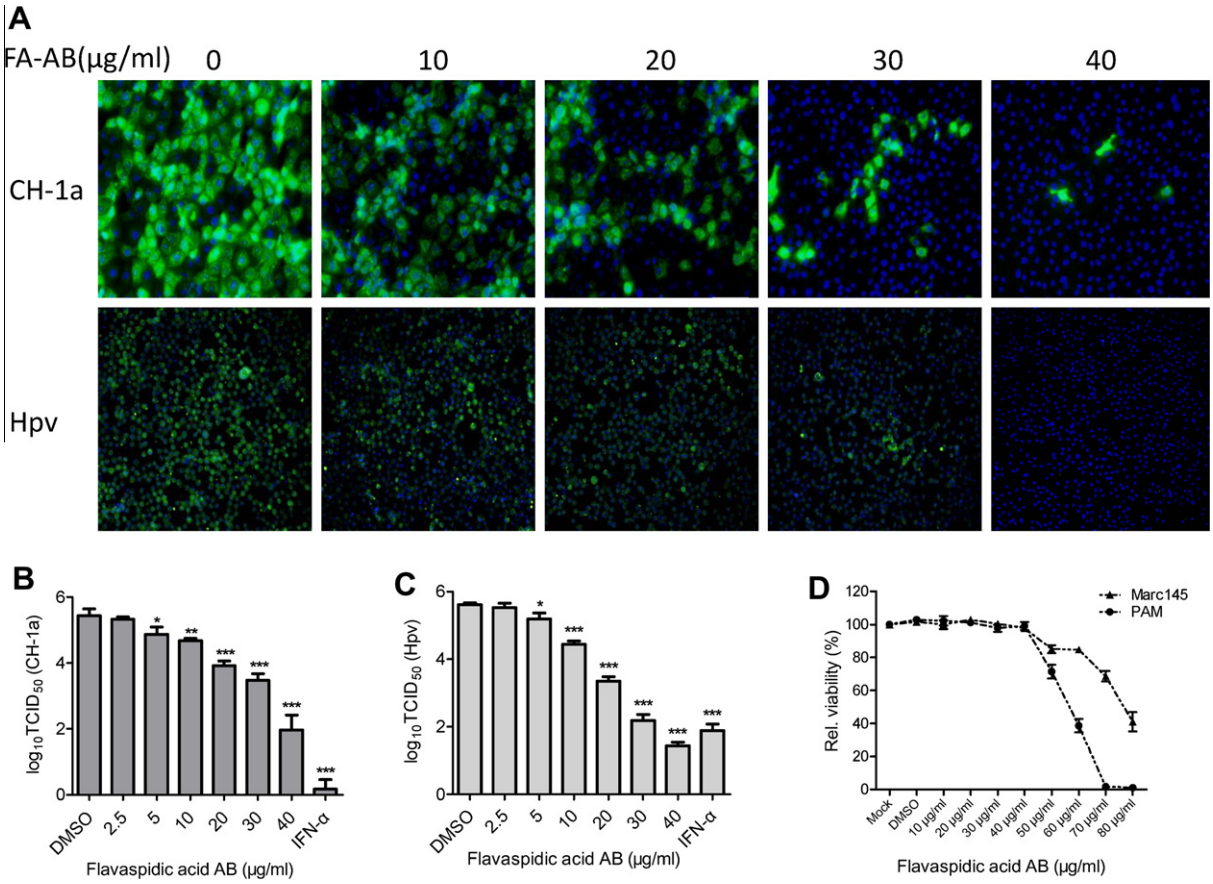

Fig. 1. Dose-dependent anti-PRRSV activity and cellular toxicity of FA-AB. (A) Anti-PRRSV activity of FA-AB was examined against the PRRSV CH-1a strain and Hpv strain in Marc-145 cells and PAMs. Cells were treated with FA-AB at indicated concentrations and simultaneously infected with PRRSV at an MOI of 0.01. Indirect immunofluorescence assay for the N protein of PRRSV was performed 48 h post-infection for CH-1a and 24 h for Hpv. Upper panels: CH-1a in Marc-145 cells. Lower panels: Hpv in PAMs. (B and C) The PRRSV titer ($TCID_{50}$) was measured after treatment with FA-AB at indicated concentrations and simultaneously infected with PRRSV at an MOI of 0.01 as above. (D) Cellular toxicity was examined in Marc-145 cells and PAMs using MTT and was expressed as relative cell viability by comparing with the viable cells in the absence of compound (set up as 100%). The data represent the mean ± standard deviation from three independent experiments. Significant differences compared with 0.4% DMSO-treated control group are denoted by $^{**}(P < 0.01)$ and $^{***}(P < 0.001)$.

cultured with fresh medium for 24 h. The virus titer ($TCID_{50}$) was then analyzed.

2.10. Cell-to-cell spreading assay

Cell-to-cell spreading was performed as previously described (Schroer and Shenk, 2008) with modifications. Briefly, Marc-145 cells were incubated with CH-1a at 37 °C for 3 h to permit viral internalization. After removing the non-internalized virus particles, cells were continued to be cultured for 40 h in medium containing various concentrations of FA-AB and neutralizing serum. Cells cultured in medium with 0.4% DMSO served as a control. Then the infected cells were observed using indirect immunofluorescent staining of the PRRSV N protein.

2.11. PRRSV replication assay

Marc-145 cells were infected with CH-1a (MOI = 0.01) for 24 h. Then the cell-free virus particles were removed and cells continued to be cultured in fresh medium containing FA-AB. Cells cultured in medium containing porcine IFN-α (provided by Dr. Wenjun Liu, Chinese Academy of Sciences, Beijing, China) served as a positive control. The viral RNA level was analyzed using real-time reverse-transcription PCR (RT-PCR) assay at 0, 12, 24, 36, 48, and 72 h after medium switching. For western analysis, CH-1a was incubated with Marc-145 cells at 37 °C for 3 h to permit entry, and then the inoculums were replaced with the medium containing various concentrations of FA-AB. Forty hours post infection, the viral N protein expression was analyzed by western blot.

2.12. Statistical analysis

All experiments were performed with at least three independent experiments. Statistical analysis was performed using GraphPad Prism Software, and differences are evaluated by *Student's t test*. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. FA-AB inhibits PRRSV infection in vitro

To explore the antiviral activity of FA-AB against PRRSV, we first examined the antiviral effects of the FA-AB on the CH-1a strain at various concentrations using indirect immunofluorescent staining 48 h post infection. As shown in Fig. 1A (upper panel), CH-1a infection was significantly inhibited by FA-AB. FA-AB induced more than 1000-fold reduction for CH-1a at the concentration of 40 μg/ml compared with the cells treated with DMSO control (Fig. 1B), and this inhibition was in a dose-dependent manner. To investigate if this anti-PRRSV activity is strain-dependent, we did the same experiments with the HP-PRRSV strain (Hpv) in PAMs. As shown in Fig. 1A (lower panel) and Fig. 1C, FA-AB showed even greater antiviral activity against Hpv infection, which could reach a 10^4-fold suppression at the concentration of 40 μg/ml, indicating that FA-AB could inhibit both traditional type 2 PRRSV strain and HP-PRRSV strain infections. FA-AB inhibited PRRSV infection with 50% effective concentration (EC_{50}) values of 3.5 μg/ml for CH-1a strain in Marc-145 cells and 4.2 μg/ml for Hpv strain in PAMs.

To rule out the possibility that nonspecific toxicity caused by FA-AB could affect PRRSV replication, we tested the cytotoxicity

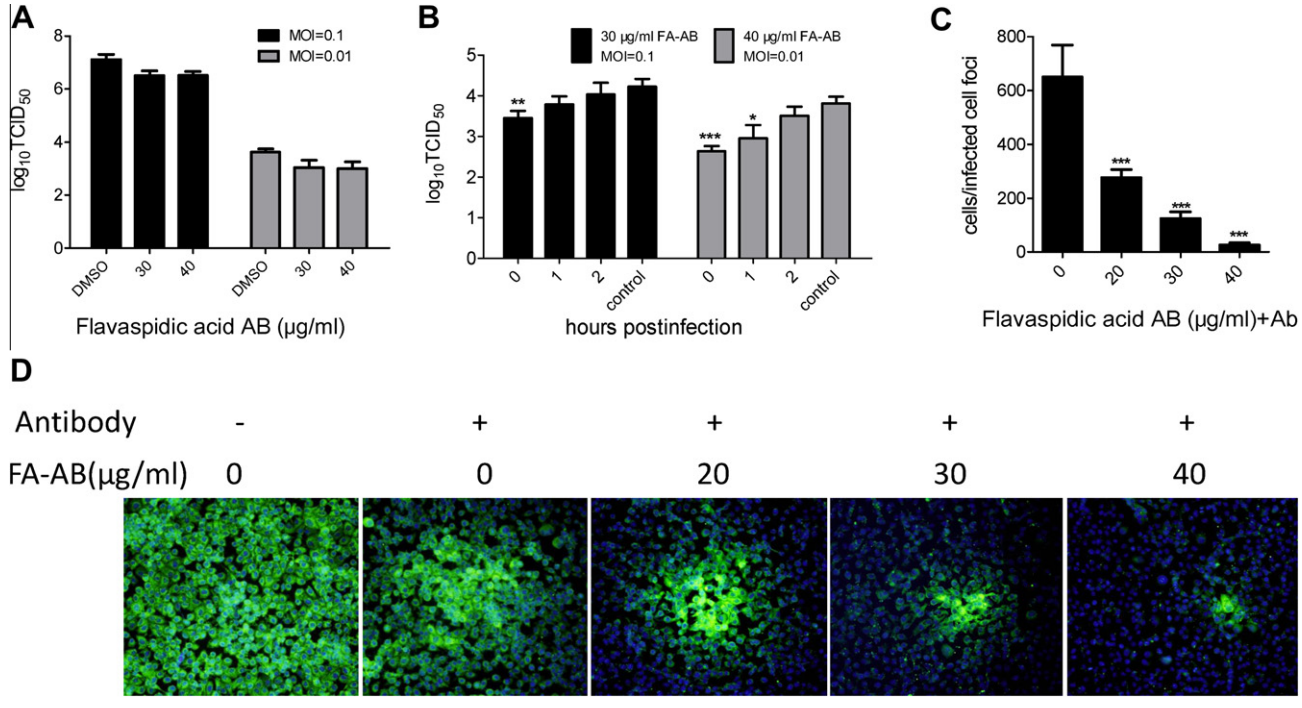

Fig. 2. Effect of FA-AB treatment on PRRSV entry and cell-to-cell spreading. (A) Viral binding assay. Marc-145 cells were incubated with CH-1a strain at an MOI of 0.1 (black bar) or 0.01 (grey bar) at 4 °C for 2 h in the presence of FA-AB. Unbound virus particles and FA-AB were removed, cells were switched to 37 °C to culture for 24 h in fresh medium, and then the virus titer ($TCID_{50}$) was measured. (B) Time-of-addition assays of viral internalization. Marc-145 cells were incubated with CH-1a strain at an MOI of 0.1 (black bar) or 0.01 (grey bar) at 4 °C for 2 h. 30 μg/ml (black bar) or 40 μg /ml (grey bar) of FA-AB was added accordingly at 0, 1, or 2 h after switching to 37 °C. Three hours later, culture medium was replaced with fresh medium and cells were continued to be cultured for 24 h. The virus titer ($TCID_{50}$) was then analyzed. (C and D) Cell-to-cell spreading assay. Marc145-cells were incubated with CH-1a strain (MOI = 0.01) for 3 h at 37 °C, and then culture medium was replaced with fresh medium containing FA-AB at indicated concentrations and neutralizing antibody (10% vol/vol). (C) PRRSV N protein expression in cells was observed using indirect immunofluorescence at 40 h pi and (D) the average number of cells in PRRSV infected foci for each group were counted. The data represent the mean ± standard deviation from three independent experiments. Significant differences compared with 0.4% DMSO-treated control group are denoted by *($P < 0.05$), **($P < 0.01$), and ***($P < 0.001$).

of FA-AB, dissolved in DMSO, at various concentrations in confluent Marc-145 cells and PAMs (Fig. 1D). Forty-eight hours following treatment, the cells cultured in medium containing 40 μg/ml FA-AB retained approximately relative viability of 100% compared with controls. The relative viability of PAMs was reduced more rapidly than Marc-145 cells when the FA-AB concentration in medium was more than 50 μg/ml. The 50% cytotoxic concentrations (CC_{50}) of FA-AB for Marc-145 cells and PAMs were 76.1 and 56.0 μg/ml, respectively, which greatly exceeded its EC_{50}.

3.2. FA-AB partially inhibits the cell entry of PRRSV

To further explore the mechanism of the inhibition of PRRSV infection by FA-AB, we determined which step(s) in the viral life cycle was inhibited by FA-AB. The process of cell entry includes early attachment and internalization. To determine whether FA-AB could inhibit PRRSV binding to Marc-145 cells, FA-AB was added in virus-cell binding assays at 4 °C, conditions in which virus binds to but does not enter cells. FA-AB had only slight effects on virus replication when added during the period of virus attachment, suggesting that FA-AB exhibited little effect on the attachment of PRRSV to the Marc-145 (Fig. 2A).

Previous studies have reported that PRRSV is internalized from the surface of Marc-145 cells within 3–6 h (Kreutz and Ackermann, 1996; Nauwynck et al., 1999). Thus, to test whether FA-AB acts at the internalization stage of infection, we examined the kinetics of FA-AB activity against PRRSV infection using time-of-addition assays (Fig. 2B). FA-AB caused approximately an eight-fold decrease of virus titers in cells infected with CH-1a at an MOI of 0.1 when FA-AB (30 μg/ml) was present in the first three hours of the virus internalization process. More significant reduction (15-fold decrease) was observed when the cells were infected with CH-1a at an MOI of 0.01 and FA-AB was at the concentration of 40 μg/ml. Taken together, these data demonstrated that FA-AB partially blocked the events in PRRSV entry that are downstream of cell attachment.

3.3. FA-AB inhibits direct cell-to-cell spreading of PRRSV

In addition to the cell-free mechanism PRRS viruses use to infect cells, viruses can also spread directly to adjacent cells without passing a cell-free stage. Thus, we next examined whether FA-AB could block the cell-to-cell spreading of PRRS virus. To rule out the possibility of the extracellular spreading of viruses, neutralizing serum was added in the culture medium. Using immunofluorescence assays, we observed a reduction of PRRSV-infected cells and the formation of discrete infected foci in the presence of neutralizing antibody compared with the controls, illustrating that the cell-free virus spreading was prevented. When FA-AB was added, the size of the isolated PRRSV-infected foci was decreased in a dose-dependent manner (Fig. 2C). At higher concentration (40 μg/ml), FA-AB could contract the foci from an area of more than six hundred infected cells to less than thirty cells (Fig. 2D). These data demonstrated that FA-AB could inhibit PRRSV infection by blocking the cell-to-cell transmission pathway.

3.4. FA-AB inhibits PRRSV replication

We next attempted to investigate the kinetics of FA-AB-mediated inhibition of PRRSV RNA production. To do this experiment, we first infected Marc-145 cells with the CH1-a strain at an MOI of 0.01. Twenty-four hours later, cell medium was replaced with fresh medium containing different concentrations of FA-AB. Then viral RNA copies in cells were quantitated using real-time PCR and virus titers in culture medium were measured at different time points. Compared with the non-drug-treated cells, the addition of FA-AB resulted in a significant reduction of viral RNA copies and this reduction reached a peak of more than 100-fold at approximately 24 h following FA-AB addition (Fig. 3A). The viral RNA level was maintained at the initial level when FA-AB was added at a concentration of 30 μg/ml, and the addition of 40 μg/ml FA-AB caused an additional decrease. The dynamic change of the virus titers in the supernatants after drug-treatment showed a similar pattern (Fig. 3B). We also tested the expression of PRRSV N protein in cells treated with FA-AB for 40 h after viral entry. As shown in Fig. 3C, FA-AB could dose-dependently inhibit the expression of viral N protein in infected cells. Thus, the FA-AB is able to inhibit PRRSV replication efficiently.

3.5. Elevated antiviral cytokine gene expression in FA-AB-treated porcine alveolar macrophages

Cytokines are able to interfere with viral infection. Thus, we postulated that FA-AB might induce antiviral cytokine expression patterns. To investigate this possibility, the expression of four cytokines including TNF-α, IL1-β, IFN-α, and IFN-β, known to be

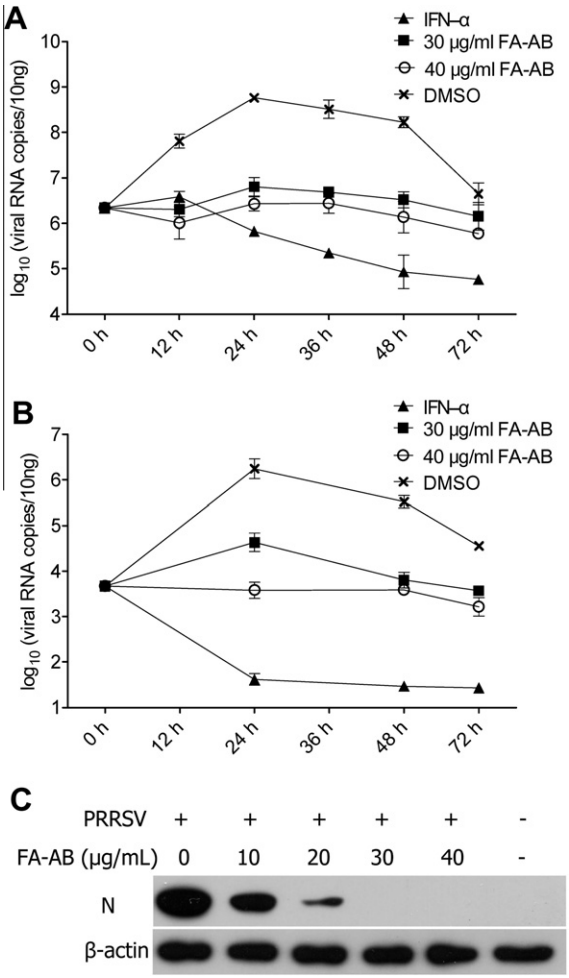

Fig. 3. FA-AB inhibited PRRSV replication. (A) The kinetics of CH-1a replication at the indicated time intervals in Marc-145 cells after treated with 30 or 40 μg/ml FA-AB, IFN-α, or 0.4% DMSO. Marc-145 cells were infected with CH-1a strain at an MOI of 0.01 for 24 h (represented as 0 h on graph), and then medium was replaced with fresh medium containing 30 or 40 μg/ml FA-AB, IFN-α, or 0.4% DMSO. The PRRSV ORF7 RNA level in cells was analyzed using real-time RT-PCR at 0, 12, 24, 36, 48, and 72 h after treatment. (B) Virus titers in supernatants at 0, 24, 48, and 72 h after treatment were measured. Data shown as mean ± standard deviationfrom three independent experiments. (C) Expression of viral N protein in FA-AB treated cells was detected by western blot. The level of viral N protein in cell lysates was analyzed 40 h post infection and analysis against β-actin served as a control.

involved in antiviral response and inflammation, were analyzed in the presence or absence of FA-AB. Porcine alveolar macrophages (PAMs) were incubated with Hpv, Hpv plus FA-AB (40 μg/ml), or FA-AB only, and real-time RT-PCR was performed to assess the relative mRNA level in PAMs after being cultured for 6 and 12 h. FA-AB treatment could elevate the levels of IFN-α (Fig. 4A), IFN-β (Fig. 4B), and IL-1β (Fig. 4C) expression in both infected and non-infected cells. The expression of IL-β and IFN-β exhibited a five-fold increase in FA-AB-treated non-infected cells. In infected cells, the elevation of IL-β expression caused by FA-AB treatment was similar to that in non-infected cells. However, in Hpv-infected cells, FA-AB induced less IFN-β mRNA expression than in non-treated cells at 12 h, which was in contrast to the results observed at 6 h. It is possible that FA-AB treatment induced IFN-β mRNA expression rapidly, reaching its peak sooner. FA-AB did not significantly induce TNF-α expression (data not shown) in PAMs. Taken together, these data suggested that FA-AB-impaired PRRSV infection could be partially due to the up-regulation of certain cytokines.

4. Discussion

D. crassirhizoma, as an effective traditional anti-infectious Chinese medicine, could play an important role in the exploration of effective antiviral drugs due to its abundant sources and pharmacological activities. Although many phloroglucinol derivatives and flavonoid glycosides have been isolated and identified from *D. crassirhizoma*, direct evidence for roles of the isolated monomers in the antiviral functions has been lacking. Here, we first demonstrated the potent antiviral activity of FA-AB, a compound of *D. crassirhizoma*. Our results showed that FA-AB could block PRRSV replication without causing cytotoxicity in Marc-145 cells or PAMs at the effective inhibition concentrations up to 40 μg/ml. The data in this study provided preliminary proof for the potential that FA-AB could be used as an antiviral agent of PRRSV.

The inhibition of PRRSV in FA-AB-treated cell cultures could result from the reduced production of virus particles in each infected cell or a failure of the virus to spread efficiently. When PRRSV infects cells, its entry process is mediated by receptor-specific endocytosis (Nauwynck et al., 1999), where the virus-cell attachment, membrane fusion, and content internalization occur sequentially. We studied the effects of FA-AB on PRRS virus attachment and internalization and found that entry of PRRSV particles that were pre-bound to cells prior to exposure to FA-AB was inhibited, suggesting that FA-AB acts on the events after virus attachment that are required for subsequent fusion and/or internalization. Therefore, we could infer that FA-AB partly inhibited the process of PRRSV entry into permissive cells, which probably is related to PRRSV utilizing different cellular receptors in each stage of entry (Van Gorp et al., 2008). However, this needs to be further investigated in the future. Moreover, viruses could spread directly to adjacent cells. For instance, measles virus infected cells transfer subviral particles by fusion-mediated cytoplasmic interconnections in order to invade uninfected cells (Firsching et al., 1999; Moll et al., 2004). Consistent with the direct cell-to-cell spreading pathway, we showed that PRRSV was able to spread in Marc-145 cells efficiently in the presence of neutralizing antibody. The addition of FA-AB substantially blocked this transmission. Thus, we suggest that FA-AB has the ability to block the invasion of PRRSV efficiently in various pathways.

Our work also provided evidences that FA-AB was able to inhibit viral RNA replication *in vitro*. However, it did not directly inhibit the viral RNA-dependent RNA polymerase (data not shown). In subsequent studies, three cytokines, IL1-β, IFN-α and IFN-β, were confirmed to be induced by FA-AB treatment. This provided a possibility that FA-AB could indirectly inhibit the PRRSV replication by regulating some antiviral cytokines. However, more studies are required to verify if the induced cytokines by FA-AB play a critical role in the FA-AB-induced inhibition of PRRSV replication. Nevertheless, our data suggested that FA-AB is not only an inhibitor

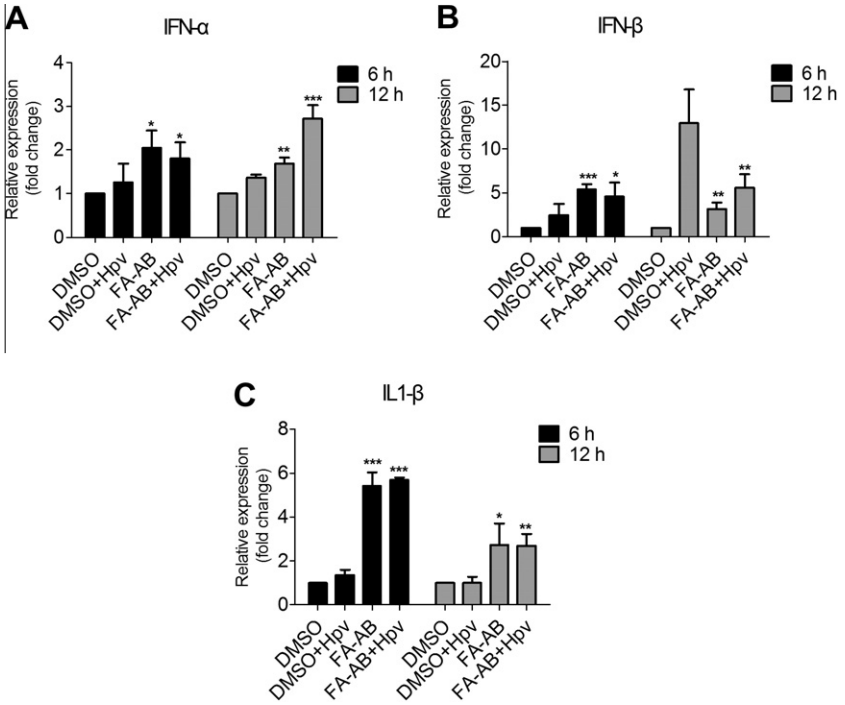

Fig. 4. Transcript levels of cytokines in PAMs. PAMs were infected with Hpv at an MOI of 0.1 in the presence or absence of FA-AB. Expressions of IFN-α (A), IFN-β (B), and IL1-β (C) were analyzed using Real-time RT-PCR at 6 and 12 h after treated with Hpv, Hpv plus FA-AB (40 μg/ml), or FA-AB only. Relative expression (fold changes) in comparison with DMSO-treated cells (set up as 1) is shown. The data represent the mean ± standard deviation from three independent experiments. Significant differences between groups treated with FA-AB and DMSO for PRRSV infected cells or uninfected cells are denoted by *($P < 0.05$), **($P < 0.01$), and ***($P < 0.001$).

acting on the viral life cycle, but also an agent probably regulating the host immune response.

Prescriptions containing *D. crassirhizoma* are able to treat many infectious diseases caused by various viruses. Thus, it is necessary to determine if the antiviral activity of FA-AB is limited to PRRSV infection. Our study showed that FA-AB also inhibited influenza A H1N1 virus infection (data not shown), suggesting that FA-AB could have a broad-spectrum antiviral activity. However, in the future, we need to investigate what are the mechanisms for FA-AB to inhibit influenza A H1N1 virus and whether FA-AB can inhibit different strains of influenza virus. Nevertheless, FA-AB has the potential to be a valuable antiviral and is worth being studied extensively in the future.

In conclusion, our work identified a new PRRSV inhibitor that acts by targeting multiple stages in PRRSV life cycle, especially the entry and replication process. Currently, novel and effective anti-PRRSV drugs are needed to complement existing strategies for PRRSV prevention and control. FA-AB, as a compound extracted from traditional Chinese medicines, has advantages in abundance and safety. It is possible that FA-AB could potentially serve as an agent for interference with PRRSV infection. However, further study *in vivo* and clinical analysis should be performed to confirm its value of inhibiting virus infection.

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