

Original article

Sodium tanshinone IIA sulfonate inhibits porcine reproductive and respiratory syndrome virus via suppressing N gene expression and blocking virus-induced apoptosis

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Background: Porcine reproductive and respiratory syndrome virus (PRRSV) causes significant economic loss in the swine industry. Currently, there is no effective way to prevent PRRSV infection. Sodium tanshinone IIA sulfonate (STS), a natural compound derived from *Salvia miltiorrhiza*, was shown to possess anti-PRRSV activity, but the underlying mechanisms remain unclear. The objective of this study was to investigate the effect of STS on PRRSV-induced cell apoptosis and PRRSV N protein expression pattern.

Methods: Relative quantification real-time PCR was used to evaluate the inhibition of STS on N gene expression. Simultaneously indirect immunofluorescence assay

(IFA) and western blot were used to assess the effect on N protein expression. Apoptosis was analysed using fluorescence microscope with an annexin V-EGFP kit. The effect of STS on caspase-3 cleaving was assessed by western blot.

Results: Our results showed that STS could inhibit viral N gene expression at both the messenger RNA stage and at the protein level in PRRSV-infected cells in a dose-dependent manner. In addition, STS could also rescue PRRSV-induced apoptosis.

Conclusions: Our data suggest that STS may serve as a base compound for developing more effective drugs against PRRSV infection.

Introduction

Porcine reproductive and respiratory syndrome (PRRS), which is caused by porcine reproductive and respiratory syndrome virus (PRRSV), emerged in the early 1990s and was considered as one of the most devastating diseases in the swine industry. PRRS is characterized by reproductive failure in pregnant sows and respiratory symptoms, particularly in young pigs [1]. PRRSV is an enveloped single-stranded, positive-sense RNA virus, and can be divided into two genotypes, European (type I) and North American (type II). The genome of PRRSV is approximately 15 kb and encodes at least 10 open reading frames (ORFs), designated ORF 1a, 1b, 2a, 2b, ORFs 3–7 and ORF5a. ORF 7 encodes a structural protein named nucleocapsid (N) protein, which is highly expressed in infected cells [2–4].

Different kinds of strategies have been developed to control PRRSV since it was identified. Vaccination

is most commonly used in industry; however, both modified live-attenuated and inactivated vaccines are insufficient to prevent the outbreak of PRRS and cannot provide complete protection [5,6]. Furthermore, vaccines have modest or often no therapeutic effect once the host is infected. In 2006, the outbreak of so-called ‘high fever’ disease in China, identified as atypical PRRS, was caused by a new PRRSV variant, which belongs to the North American PRRSV genotype. This novel variant was characterized by a 30-amino-acid deletion in non-structural protein 2 (NSP2) and spread to other provinces in China in 2007 [7,8]; therefore, developing novel strategies to control PRRSV infection is needed for the swine industry.

Previous studies demonstrated a few antiviral agents with anti-PRRSV activity, including type III interferons (IFNs), interleukin-12 (IL-12), artificial microRNAs, 12-amino-acid peptides and porcine

interleukin-29 (poIL-29) [9–13]. It was well-established that certain traditional Chinese medicines (TCMs) have strong antiviral activity [14]. A recent study showed that some natural compounds from TCMs, such as ouabain, bufalin and valinomycin, displayed potentially anti-PRRSV activities [15]. Liu *et al.* [16] reported that *Achyranthes bidentata* polysaccharide (ABPS) possessed significant effects against PRRSV *in vitro*, and sulfate modification of ABPS could enhance its activity. In addition, flavaspidic acid AB (FA-AB) derived from *Dryopteris crassirhizoma* could effectively inhibit PRRSV internalization and cell-to-cell spreading [17]. Our previous study demonstrated that sodium tanshinone IIA sulfonate (STS), the main effective component of *Salvia miltiorrhiza*, could inhibit PRRSV infection *in vitro* through direct inactivating and/or disturbing the replication of PRRSV [18]. In this study, we further investigated the anti-PRRSV mechanisms of STS through additional potential targets, including N gene/protein of PRRSV and PRRSV-induced cell apoptosis.

Methods

Cells, viruses and STS

African green monkey kidney cell line Marc-145 was obtained from China Institute of Veterinary Drug Control (Beijing, China) and were grown in DMEM supplemented with 10% fetal calf serum (FCS) and 100 IU/ml penicillin G and 100 µg/ml streptomycin at 37°C with a 5% CO₂. PRRSV vaccine (JXAI-R, number 1012001; Guangdong Dahuanong Animal Health Products Co., Ltd, Guangdong, PR China) was propagated in Marc-145 cells. Virus titres were 10^{7.5} tissue culture infective dose (TCID₅₀)/ml and 100 TCID₅₀ was used for the following experiments. Virus aliquots were stored at -80°C until use. STS was dissolved with 1% DMSO and diluted with DMEM containing 2% FCS. According to previous results [18], the maximum STS concentration used was 0.0625 mg/ml and when the concentration was lower than 0.015625 mg/ml, there was almost no anti-PRRSV activity. In this assay, in order to display dose-dependent outcomes and to ensure non-cytotoxicity, we chose three concentrations (0.0625 mg/ml, 0.03125 mg/ml and 0.015625 mg/ml) to evaluate the effect of STS on PRRSV N protein.

Relative quantification RT-PCR

Marc-145 cells seeded into 6-well plates at a density of 2×10⁵ cells/ml were grown to 100% confluence. Culture medium was then decanted and three twofold dilutions of STS were added to the wells together with PRRSV solution. After 48 h incubation, total RNA was extracted from lysates of cells by using TRIzol

reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols, followed by DNase I treatment and complementary DNA was synthesized using PrimeScript® RT Master Mix kit (TaKaRa, Dalian, China). Real-time reverse transcriptase (RT)-PCR was performed using Mx3000PTM QPCR System (Stratagene, Santa Clara, CA, USA) using SYBR® Premix Ex Taq™ Kit from TaKaRa.

Primer sequences and PCR fragments length were as follows: PRRSV N gene, forward 5'-AGAAGCC-CCATTTCCCTCTA-3' and reverse 5'-CGGATCAGACGCACAGTATG-3' (196 base pairs [bp]); β-actin, forward 5'-TCCCTGGAGAAGAGCTACGA-3' and reverse 5'-AGCACTGTGTTGGCGTACAG-3' (194 bp). The following cycling parameters were used: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 62°C for 30 s and 72°C for 30 s. The relative quantities of viral RNA were calculated using the 2^{-ΔΔC_t} method. After amplification, a melting curve and agarose gel electrophoresis were used to confirm product purity.

Indirect immunofluorescence assay

Confluent Marc-145 cells in 384-well plates were infected with three twofold dilutions of STS and a constant amount of 100 TCID₅₀ PRRSV. After 48 h post-infection, cells were fixed with pre-cooled acetone and methanol (1:1) for 30 min at -20°C. After three times washing with PBS, the fixed cells were incubated with a monoclonal antibody against PRRSV N protein for 2 h at 37°C. Unbound antibodies were washed away with PBS and sample was incubated with FITC-labelled goat anti-mouse antibody for 1 h at 37°C. Fluorescence was detected using a fluorescence microscopy (Olympus, Shinjuku-Ku, Japan).

Western blot analysis

Cells were prepared as described in section of *Relative quantification RT-PCR*. Total protein was extracted using a total protein extraction Kit (Applygen, Beijing, PR China) according to the manufacturer's instructions. Protein concentration was determined by NanoDrop spectrophotometer (ND-1000; Wilmington, DE, USA). Protein samples were subjected to SDS-PAGE and transferred onto Immobilon-PSQ PVDF transfer membrane (0.22 µm; Millipore, Bedford, MA, USA). Membrane was blocked with 5% non-fat milk in TBST for 2 h at room temperature and then incubated with antibody against PRRSV N protein antibody (1:1,000), caspase-3 (1:1,000; number 9662; Cell Signaling Technology, Danvers, MA, USA) and β-actin antibody (1:1,000; number CW0096; CWBIO, Beijing, PR China) at 4°C, overnight. Then, horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000) was incubated with membrane for 1 h

at 37°C and the protein bands were visualized using ECL (eECL Kit; Cowin Biotech Co., Ltd., Beijing, China) and exposed to autoradiography film. Band density was normalized to β -actin content.

Apoptosis determination

Apoptosis was analysed using fluorescence microscope with an annexin V-EGFP Kit (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, PR China) as previously described with modifications [19]. Briefly, EGFP-conjugated annexin V (AV) and propidium iodide (PI) were added onto each sample and then incubated for 15 min at room temperature in the dark. Only green fluorescence around the cell membrane (AV⁺PI⁻) was considered as early apoptotic cells. Late apoptotic cells were shown by green fluorescence around the cell membrane and red fluorescence in the nucleus (AV⁺PI⁺). Apoptosis of the cells was analysed by fluorescence microscope at 24, 48 and 60 h post-infection.

Statistical analysis

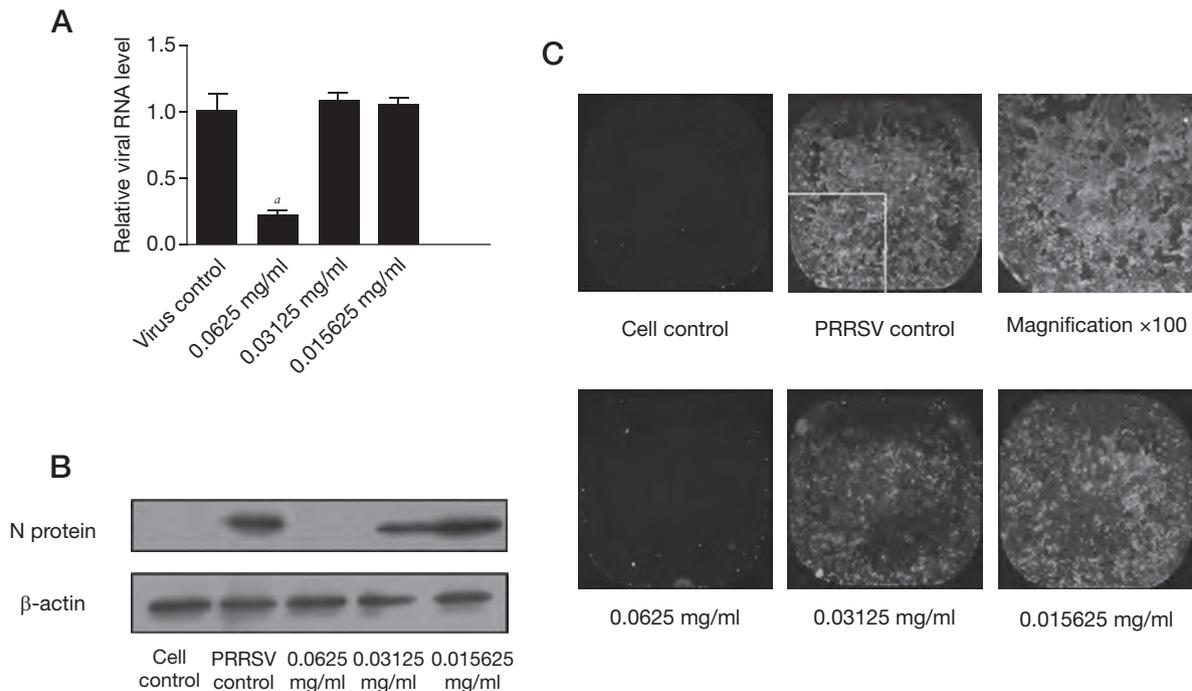
Data were analysed by one-way ANOVA implemented in GraphPad Prism 5 software. Data were expressed as mean \pm SD and $P < 0.05$ was considered to be significant.

Results

Effect of STS on N gene expression

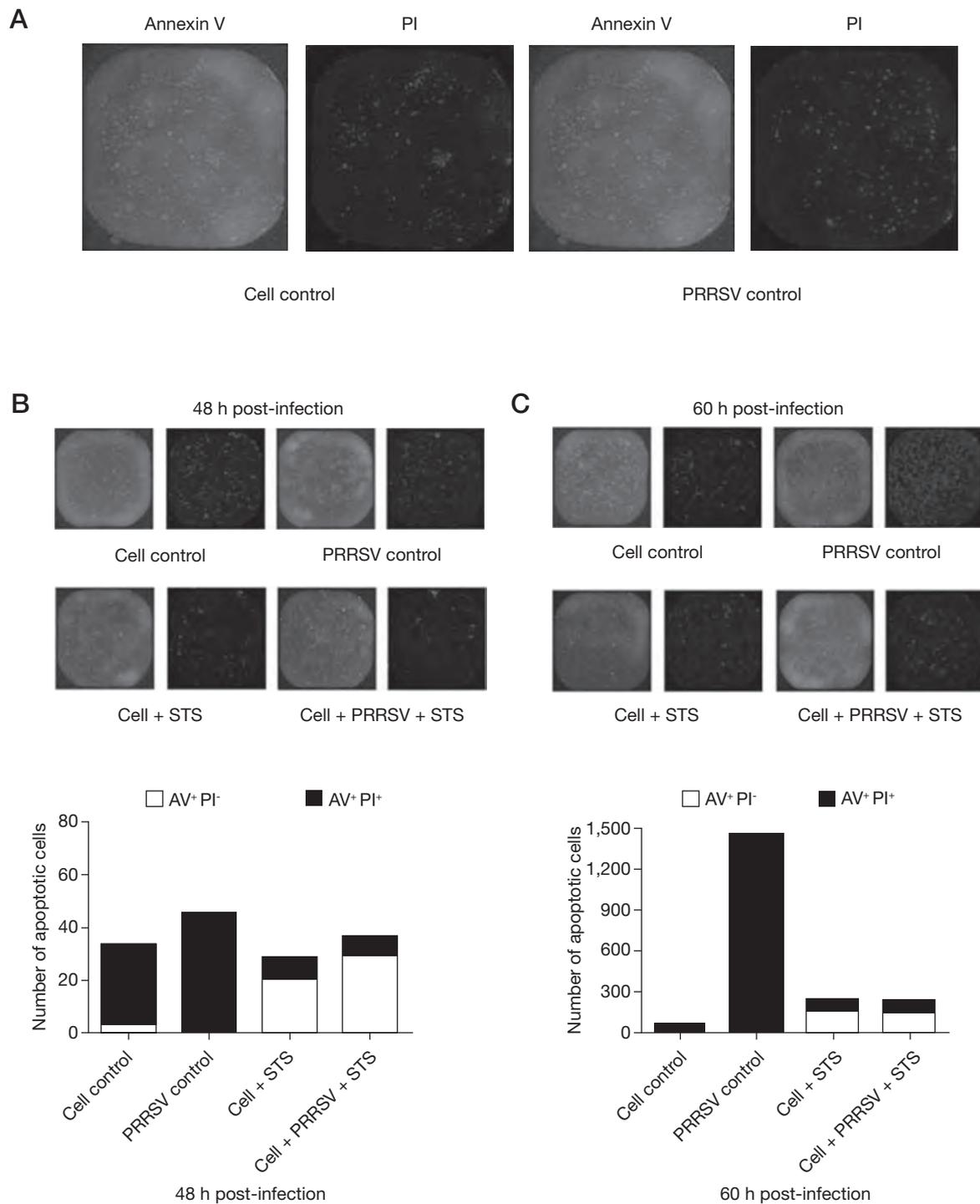
Marc-145 cells were respectively treated with three different concentrations of STS (the final concentrations of STS were 0.0625 mg/ml, 0.03125 mg/ml and 0.015625 mg/ml) and 100 TCID₅₀ PRRSV for 48 h. Relative viral RNA levels in all groups were measured by relative quantification real-time RT-PCR. As showed in Figure 1A, N mRNA level was significantly reduced when the infected cells were treated with STS (0.0625 mg/ml; Figure 1A, $P < 0.0001$), suggesting that STS might affect N gene transcription. However,

Figure 1. Effect of STS on the expression of PRRSV N gene/protein in Marc-145 cells



(A) The relative viral RNA levels in cells treated with consecutive twofold diluted concentrations of sodium tanshinone IIA sulfonate (STS) and porcine reproductive and respiratory syndrome virus (PRRSV) for 48 h were measured by relative quantification real-time PCR. Bars indicate the $2^{-\Delta\Delta Ct}$ of viral RNA in PRRSV-infected cells or STS-treated groups \pm SD from three independent repetitions. ^a $P < 0.001$. (B) Marc-145 cells in 6-well plates were infected with three twofold dilutions of STS and a constant amount of 100 tissue culture infective dose (TCID)₅₀ PRRSV for 48 h. Total protein was extracted and nucleoprotein (N) protein was analysed by western blot. (C) PRRSV N protein expression in 384-well plates at 48 h post-infection was observed by indirect immunofluorescence assay. N protein was stained with FITC (and fluoresced green). Magnification $\times 40$ except where indicated otherwise.

Figure 2. Effect of STS on PRRSV-induced apoptosis



Apoptotic cells were measured by fluorescence staining of Annexin V-EGFP Kit (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, PR China) after 24, 48 and 60 h post-infection. In early apoptotic cells (AV⁺PI⁻), only green fluorescence was found and they were around the cell membrane, whereas in late apoptotic cells (AV⁺PI⁺), green fluorescence was found around cell membrane and red fluorescence in the nucleus. Six independent wells were performed for each group. (A) Compared with cell control, porcine reproductive and respiratory syndrome virus (PRRSV)-infected cells did not show significant fluorescence of either green or red after 24 h post-infection. (B) and (C) after 48 h and 60 h co-incubation with PRRSV and sodium tanshinone IIA sulfonate (STS), the late apoptotic cells were markedly decreased compared with PRRSV control. Magnification $\times 40$. PI, propidium iodide.

the mRNA contents of N gene were same between the other two STS-treated groups (at 0.03125 and 0.015625 mg/ml) and the virus control (Figure 1A), suggesting a threshold of drug concentration for the antiviral activity of STS.

Effect of STS on N protein

Western blot and indirect immunofluorescence assay (IFA) were used to analyse the expression of N protein in all groups. When the concentration of STS was 0.0625 mg/ml, there was almost no detectable band (N protein) in western blot (Figure 1B) and undetectable green fluorescence (N protein) was observed in the IFA (Figure 1C). When the concentration of STS was decreased, the inhibition on N protein expression decreased. There was almost no inhibition at 0.015625 mg/ml.

Effect of STS on PRRSV-induced apoptosis

In present study, Annexin V-PI double staining was used to evaluate the effect of STS on PRRSV-induced apoptosis after 24, 48 and 60 h post-infection. As shown in Figure 2A, PRRSV-infected cells did not show significant fluorescence of either green or red

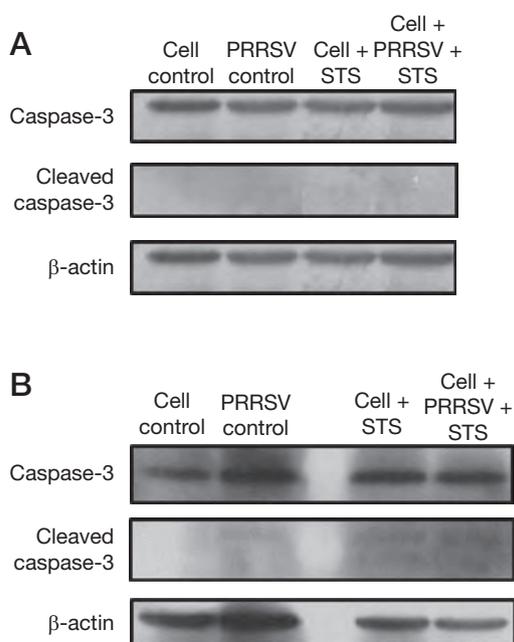
compared with cell control after 24 h post-infection. After 48 h post-infection, the number of apoptotic cell in each group was greater than at 24 h, especially at 60 h (Figure 2B and Figure 2C). However, the number of late apoptotic cells in the STS-treated group was markedly decreased compared with PRRSV control. To further confirm these observations, we analysed the caspase-3 activity using western blot at 24 and 48 h post-infection. As shown in Figure 3A, the cleaved caspase-3 was undetectable in all groups at 24 h post-infection, but both caspase-3 and its activation form were observed in all groups except for cell control group at 48 h post-infection (Figure 3B).

Discussion

Our previous studies demonstrated that STS possessed anti-PRRSV activity in Marc-145 cells model; however, the underlying mechanisms remain unclear. N protein is encoded by ORF7 and it can interact with genomic RNA during the assembly of the nucleocapsid; therefore, N protein of PRRSV is often used to assess antiviral activity of medicines [15,17]. To verify whether the N protein was an important anti-PRRSV target of STS, we firstly analysed the N gene mRNA using RT-PCR. Our results showed that STS could inhibit N gene transcription at the concentration of 0.0625 mg/ml and when the concentration of STS decreased to 0.03125 and 0.015625 mg/ml, there was no significant inhibition on N gene expression. To further investigate the effect of STS on N protein, we carried out a western blot to detect the N protein change. As shown in Figure 1B, STS could dose-dependently inhibit the expression of viral N protein in PRRSV-infected cells and the band was almost undetectable at a higher concentration (0.0625 mg/ml), which was consistent with our RT-PCR results. The same results were also confirmed by IFA. As shown in Figure 1C, there was almost no green fluorescence detected at the concentration of 0.0625 mg/ml. These observations provided clear evidence that STS, at a certain concentration (0.0625 mg/ml), could inhibit the PRRSV replication *in vitro*. In addition, when the concentration was decreased to 0.03125 mg/ml, the decreasing N protein contents and unchanging N gene mRNA expression suggest that STS might be able to interrupt biological processes after gene transcription at this concentration. The present results demonstrate that STS can significantly down-regulate N expression at both the mRNA stage and at the protein level.

N protein can suppress the production of type I IFNs, which are recognized as potent immune responsive cytokines against viral infections, both *in vitro* and *in vivo* [20]. IL-10 is a pleiotropic cytokine that is

Figure 3. Effect of STS on caspase-3 expression was detected by western blot analysis



Marc-145 cells were co-incubated with porcine reproductive and respiratory syndrome virus (PRRSV) and sodium tanshinone IIA sulfonate (STS) for 24 and 48 h. (A) At 24 h post-infection, the cleaved caspase-3 was undetectable in both the PRRSV control group and the STS treated groups. (B) At 48 h post-infection, both caspase-3 and its activation form (cleaved caspase-3) were observed in all groups except for cell control group.

involved in the persistence of viral infection, causing immunosuppression. It has been demonstrated that PRRSV N protein can up-regulate IL-10 gene expression in PAMs, which might be dependent on NF- κ B activation [21,22]. It is likely that STS attenuates the persistent infection of PRRSV, which is a key problem in eradicating PRRS [18].

PRRSV has been shown to induce apoptosis both *in vitro* and *in vivo* [23,24], and its replication was necessary for the induction of apoptosis in infected cells. In our results, PRRSV-infected cells were more stained than the cell control until 48 h and 60 h, which was consistent with the previous reports [25,26]. In addition, the number of late apoptotic cells in STS-treated group markedly decreased, although the number of early apoptotic cells in STS-treated groups (STS added to PRRSV-infected cells group and STS added to uninfected cells group) was a little increased, suggesting that STS may rescue PRRSV-induced apoptosis in the late stages of apoptosis and thus we further analysed the effect of STS on caspase-3 activity at 24 and 48 h post-infection. The cleaved caspase-3 was detected until 48 h post-infection both in the PRRSV control and STS-treated groups. These results revealed that PRRSV could induce cell apoptosis in a time-dependent manner. STS may activate caspase-3 in the early stages of apoptosis and sharply decrease the number of late apoptotic cells; therefore, STS may inactivate PRRSV before it can induce apoptosis.

In conclusion, the present study showed that STS exerted its antiviral effects via inhibition of transcription and translation of PRRSV N protein and STS could reduce PRRSV-induced cell apoptosis. We anticipate that STS can serve as a good candidate for further antiviral drug development for combating PRRSV infection.

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Disclosure statement

The authors declare no competing interests.

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