Antiviral effect of 1-Calcium phosphonate uracil against porcine reproductive and respiratory syndrome virus in vitro

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Abstract

Background: Porcine reproductive and respiratory syndrome virus (PRRSV) is an RNA virus. At present, it has been treated by various ways, including vaccination of inactivated vaccine, attenuated live vaccine, subunit vaccine, etc., but the control of PRRSV infection is still inadequate. Therefore, it is necessary to study new drugs against PRRSV infection. In this study, the antiviral effect of SR on PRRSV in vitro was studied.

Results: PRRSV was significantly inhibited in SR pretreated cells. In addition, the results also showed that SR inhibited the adhesion, entry and synthesis of PRRSV, but did not affect the release of PRRSV.

Conclusion: Our results suggest that SR may play an antiviral role in PRRSV infection in vitro, which suggests that SR may be a potential new drug to inhibit PRRSV infection.

Background

Porcine reproductive and respiratory syndrome (PRRS) has been one of the major infectious diseases threatening the healthy development of the pig industry since it was found. The clinical symptoms mainly include abortion, premature delivery, stillbirth and mummification in pregnant sows, and piglets and growing pigs with respiratory symptoms accompanied and interstitial pneumonia. PRRSV is a single strand positive RNA virus belonging to arteritis virus family, which can be divided into two genotypes, European LV strain and North American VR2332 strain[1, 2]. PRRSV can cause immunosuppression and persistent infection, which makes it difficult to prevent and control the disease[3]. Previous studies have reported that many natural compounds, microRNAs and new vaccinations can control PRRSV infection[4–6]. However, even if the current vaccination strategy is widely used, the protection against PRRSV infection is still limited. Therefore, it is necessary to develop a safe and effective antiviral strategy. 1-calcium phosphate-uracil(SR) is a derivative of uracil, which was one of the phosphorylated base drugs (Fig.1A). Its molecular formula is $C_4H_3O_5N_1P_1Ca_1$, and its molecular weight is 216.1276. This kind of drugs has the original reduction characteristic of “self-selected target”. By changing the three-dimensional structure of pathological molecules, new functional peptides, amino acids and proteins are formed, which can eliminate pathogenic pathogens. The toxicity of the body can repair and reconstruct the stress, defense, metabolism and regulation functions of the body. The current clinical research has proved that the drug has significant multiple effects, including antiviral, antibacterial toxic infection, tumor inhibition.

In this study, we investigated the antiviral effect of SR on PRRSV infection in vitro. We found that PRRSV replication were effectively inhibited by SR. At the same time, because the replication of virus genome in host cells is inhibited, SR has played an extracellular antiviral activity to PRRSV. In conclusion, SR has a high value in clinical application, which may be helpful for the antiviral treatment of PRRSV infection.

Results

The growth of PRRSV was significantly inhibited in Marc-145 treated with SR

To analyze whether SR shares the antiviral activity against PRRSV, the following experiments were carried out. First, the cytotoxicity of SR to MARC-145 cells were tested using MTT Cell Proliferation and Cytotoxicity Assay Kit. In comparison to mock and SR treatment at different concentrations had no obvious impact on the viability of MARC-145 cells by measuring the absorbance of cell cultures at 570 nm (Fig.1B). And then, we investigated the inhibitory effect of SR on PRRSV (Fig.1C).

In order to explore the antiviral function of SR, we infected Marc-145 with PRRSV DJY and SR mixture, cultured at 37 °C for two hours, washed the cells with PBS, removed the non-adsorbed virus particles, then added 2% fresh medium containing, and measured the relative expression of ORF7 mRNA by qRT-PCR at 48hpi. The results showed that compared with mock, SR significantly and continuously inhibit the replication of PRRSV (Fig.1D). And in the presence of different concentrations of SR (0, 10, 20, 40, 60, 80 and 100 μ M), compared with PRRSV infected Marc-145 cells without SR treatment, the virus titer was decreased, which also showed dose-dependent inhibition (Fig.1E).

The proliferation of PRRSV were inhibited in Marc-145 treated with SR

Marc-145 cells were pretreated with 100 μ M SR or PBS for 24 hours, and then infected with PRRSV DJY at the dose of MOI = 1. The virus titers of Marc-145 cells pretreated with SR were measured at 12, 24, 36 and 48 hpi, respectively. Compared with PBS group, the virus titer of Marc-145 cells pretreated with SR decreased to some extent (Fig. 2A). These results showed that PRRSV DJY proliferation in SR pretreated cells was affected, but in the later stage, the inhibition decreased gradually with the increase of time.

We examined whether SR can inactivate PRRSV infection. PRRSV DJY (MOI = 0.1) was mixed with gradient SR, and the mixture was incubated at room temperature for 2 hours, then infected with Marc-145 of 96 well plate, and the virus titer was determined after 48dpi. As shown in Figure 2B, compared with PBS treatment, SR treatment with concentration of 100 μ M, 10 μ M and 1 μ M had no significant effect on PRRSV titer, indicating that SR could not directly inactivate PRRSV infectivity.

PRRSV attachment and entry were significantly inhibited by SR

In order to further determine the precise steps of SR inhibition in the virus life cycle, we first evaluated the effect of SR on PRRSV adsorption. The precooled Marc-145 cells were inoculated with a mixture of PRRSV DJY and SR (100 μ M) of different MOI (MOI = 10,1,0.1,0.01), and then placed at 4 °C for another 2 hours. The cells were washed with PBS to remove the non-adsorbed virus particles, then incubated at 37 °C, and the virus titer was measured at 48 hpi. As shown in the Figure 3A, compared with PBS

treatment, SR treatment significantly inhibited the PRRSV of each infection dose, indicating that SR may inhibit the adsorption of PRRSV to the cell surface, but the effect was not obvious.

In order to further explore the effect of SR on PRRSV entry, PRRSV DJY was allowed to adsorb on precooled Marc-145 cells at 4 °C under different MOI. SR (100 µM) was then added to the virus adsorbed cells and the cells were placed at 37 °C. After incubation for 3 hours, replace the medium containing SR with fresh medium, and the cells were further cultured at 37 °C for 48 hours to determine the virus titer. The results showed that the virus titer of Marc-145 cells treated with SR decreased about 5 times under 10 to 0.01 MOI virus compared with PBS (Fig.3B). In conclusion, these results suggest that SR inhibits PRRSV from entering Marc-145 cells.

SR had significant effect on genome synthesis of PRRSV

In order to solve the problem of whether SR inhibit PRRSV genome synthesis, we analyzed the effect of SR on PRRSV genome synthesis. Marc-145 cells were infected with PRRSV DJY (MOI = 1) at 37 °C for 2 hours, and then treated with SR (100 µM) or PBS. Samples were taken at the designated time points (12h, 24h, 36h) to prepare total RNA and analyze the PRRSV genome by real-time RT-PCR. The results showed that the relative level of GP7 in Marc-145 cells treated with SR was significantly reduced compared with PBS at all the time, and showed a time gradient. At 12 hours after treatment, the relative level of PRRSV had been reduced by 10 times (Fig.3C), indicating that SR could significantly inhibit the synthesis of PRRSV genome.

In order to further study whether SR can inhibit the release of PRRSV, Marc-145 cells were infected with PRRSV DJY (MOI = 1). At 24 hpi, the inoculum was replaced with fresh medium containing SR. The virus titers in supernatant were measured 15 minutes, 30 minutes, 45 minutes and 60 minutes after medium replacement. As shown in Figure 3D, in the supernatant, compared with PBS treatment, the PRRSV titer of cells treated with SR did not decrease significantly at different time points, indicating that SR had no effect on the release of PRRSV virus particles.

Discussion

At present, most of the antiviral drugs on the market are purine derivatives or pyrimidine derivatives, such as acyclovir and valaciclovir, which are DNA polymerase inhibitors. They can inhibit the thymidine kinase and DNA polymerase encoded by the virus, so they can significantly inhibit the synthesis of DNA in infected cells, without affecting the DNA replication of non-infected cells[7]. Most uracil analogues affect protein translation by interfering with DNA / RNA synthesis. For example, as an anticancer drug, fluorouracil is used in the treatment of gastrointestinal cancer, breast cancer, rectal cancer, etc. by inhibiting thymine nucleotide synthetase, blocking the conversion of deoxypyrimidine nucleotides into thymine nucleosides, it interferes with DNA synthesis[8, 9]. All of these indicate that most of the antiviral effects of uracil occur in cells. However, our research results show that SR can also inhibit the absorption and entry of virus, which may be caused by the absorption of SR by cells in the co incubation stage of SR

and cells, so that the virus entering the cells is inhibited in the process of proliferation. There is a problem here, whether it is uracil or SR itself can't participate in the transcription of RNA, because it can't form uracil nucleotide itself, so its specific antiviral function remains to be further studied.

The latest research found that the nucleoside derivative rigid amphiphilic fusion inhibitor (Rafi), 5 - (perylene-3-ylethynyl) - arabino uridine (aury11) and 5 - (perylene-3-ylethynyl) uracil-1-acid (cm1uy11) against African swine fever virus (ASFV), Rafi stabilizes the positive curvature of the membrane through its molecular structure, thus inhibiting the fusion of the virus and the cell[10]. In addition, the researchers confirmed that Rafi. It is clear that aury11, another member of Rafis, has no effect on virus attachment, which may be due to different substituents leading to different molecular characteristics. As 1-benzoyl-3 - [(4,6-dimethyl-2-yl) methyl] uracil and 1 - (2,6-difluorobenzyl-3 - [(2,6-dimethyl-4-yl) methyl-3 - [(2,6-dimethyl-4-yl) methyl] group by a pyrenyl moiety significantly reduced the activity against HCMV[11]. Compared with nucleoside derivatives on the market, most of the antiviral drugs on the market are modified by organic substituents, which may be due to the different substituents.

At present, there is still a view that when the concentration of Ca^{2+} in cells is too high, Ca^{2+} dependent protease CaMKK β and its substrate molecules AMPK or CaMKI may be activated, which may also lead to the occurrence of autophagy[12]. Therefore, whether SR is degraded into Ca^{2+} in cells, thus inducing autophagy of cells remains to be confirmed.

Conclusion

In this study, we found that SR has obvious anti PRRSV effect, and it mainly affects the synthesis of PRRSV genome, which provides a new treatment method and new view for the treatment of PRRSV infection.

Methods

Cells, viruses and reagents

MARC-145 cells were grown in Dulbecco's modified Eagle medium (DMEM) (Fisher Scientific) supplemented with 10% FBS (Sangon Biotech, Shanghai, China). The stock of a NADC30-like PRRSV DJV and a HP-PRRSV NJ which were isolated and stored by our laboratory were used in this study. 1-Calcium phosphonate uracil was donated by Sichuan Huiling Xinkang Technology Development Co., Ltd (Sichuan Chengdu, China).

BCA Protein Assay Reagent Kit (catalogue no: C503051) and HRP-conjugated Goat Anti-Rabbit IgG (catalogue no: D110058) were purchased from Sangon Biotech (Shanghai, China). Rabbit monoclonal antibody (mAb) against PRRSV was prepared in our laboratory. Trizol reagent kit (catalogue no: 206101) was purchased from Magen (Beijing, China). TB Green® *Premix Ex Taq II* (Tli RNaseH Plus) (catalogue no: RR820Q) and [PrimeScript™ RT reagent Kit \(Perfect Real Time\)](#)(RR037A) were purchased from Takara (Beijing, China) were purchased from Takara (Beijing, China).

Cell viability assay

The impacts of SR on MARC-145 cells viability were examined using MTT Cell Proliferation and Cytotoxicity Assay Kit (MTT) (Sangon Biotech, Shanghai, China).

Seed cells at a density of 5000 cells/well for cell cytotoxicity assay in 100 μ L cell culture medium in 96-well plate. Incubate the plate for 24 hours in a CO₂ incubator before treatment. After treatment with different concentrations of SR for 48 hours. Add 10 μ L of MTT Reagent (prepared above) at a final concentration of 0.5 mg/mL to each well. Mix gently and incubate at 37°C for 4 hours in a CO₂ incubator. Aspirate the culture medium from each well carefully to prevent disruption of the cell monolayer. Add 100 μ L of Formazan Solubilization Solution into each well. Put the plate on a shaker to mix gently for 10 minutes to dissolve the formazan crystals. And then the absorbance was measured at 570 nm using a microplate reader.

Virus infection and titration

Marc-145 cells were grown on 96-well culture plate. Virus was diluted 10 times gradient (10^{-1} – 10^{-10}) and inoculated into 96-well culture plate. After 48 hours of infection, TCID₅₀ of virus was calculated by Reed-Muench method.

Indirect immunofluorescence assay (IFA)

MARC-145 cells infected with PRRSV were fixed with 4% paraformaldehyde for one night at 4°C, then blocking for 60 minutes with 5% bovine serum albumin (BSA), then treated with monoclonal antibodies against the GP4 protein of PRRSV, and then incubated with FITC-conjugated goat anti-mouse IgG for 60 minutes, and finally were observed under a fluorescence microscope (Nikon, Japan).

Real-time RT-PCR

Total RNAs were extracted from the infected MARC-145 cells using the Trizol reagent according to the manufacturer's manual. The isolated RNAs were reverse-transcribed using PrimeScrip RT reagent Kit described above. A pair of primers specific for the glycosylated membrane protein 7 (GP7) - coding region of PRRSV was used to examine PRRSV genomic RNA by the real-time PCR with TB Green Premix Ex Taq II. Real-time PCR was performed in LightCycler® 96 Real-Time PCR System (Roche, Switzerland). The obtained cDNA was pre-denatured at 94 °C for 30s, which was followed by 40 cycles of amplification at 95° C for 5 s, 60° C for 30s and 72° C for 30s with a final melting at 95° C for 5 s, 65° C for 60s and 97° C for 1s. The cellular β -actin was quantified as the internal control in MARC-145 cells. Gene-specific primer sequences were as follows: 5'- AGCCAGTCCAGAGGTAAGGG -3' (forward) and 5'- CGC CTT GGT TAA AGG CAG TC -3' (reverse) for GP7-coding region; 5'- GGG ACC TGA CCG ACT ACC TCA TG -

3¢ (forward) and 5¢- GCC ATC TCC TGC TCG AAG TCC AG –3¢ (reverse) for β -actin. The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative levels of viral genomic RNAs.

Viral attachment assay

MARC–145 cells grown on 12-well plates were pre-chilled at 4°C for 1 h and then inoculated with the mixture of PRRSV of the different MOI and SR (100 μ M), and were placed at 4° C for additional 2 h. The mixture of viruses and SR was then removed and the cell monolayers were washed three times with pre-chilled PBS before applying the fresh medium. After further incubation at 37°C for 48 h, virus titers were measured as described above.

Viral penetration assay

Monolayers of MARC–145 cells were pre-chilled at 4°C for 1 h and then infected with the viruses of the different MOI for 2 h at 4° C. The virus-containing medium was removed and the cells were washed three times with pre-chilled PBS prior to treatment with the medium containing SR (100 μ M) for 3 h at 37° C. The cells were carefully washed with PBS to remove SR and the non-internalized extracellular viruses and then were overlaid with fresh medium. After further incubation at 37° C for 48 h, virus titers were measured as described above.

Viral synthetise assay

Marc–145 cells were pre-cooled for 1h at 4°C, then infected with MOI = 1 virus at 4°C for 2h. The virus-containing medium was removed. The virus was washed with pre-cooled PBS for 3 times, and the non-internalized extracellular virus was removed. The cells were treated with SR (100 μ M) medium at 37°C for 6 hours, 12 hours, 24 hours, 36 hours and 48 hours, respectively. Fluorescence quantitative PCR was performed according to the above methods.

Viral release assay

MARC–145 cell monolayers were infected with PRRSV (MOI = 1). After 24 h post-infection (hpi), the inoculations were replaced by medium containing SR (100 μ M). At 15 min, 30 min, 45 min and 60 min after medium change, both supernatant and the infected cells were harvested separately and titrated as described above.

Statistical analysis

The data were expressed as means $\pm$ standard deviations (SD). The significant differences of among different groups were analyzed using Two-way ANOVA test of variance in the GraphPad Prism software

(version 5.0). Differences were considered statistically significant at a p value of <0.05.

Abbreviations

PRRS: Porcine reproductive and respiratory syndrome

SR:1-calcium phosphate-uracil; MOI: Multiplicity of infection

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

All authors have no competing interests to report.

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Authors' contributions

ZL and XL conceived the study. ZL and ZWX designed the experiments. XL, ZJ, XYY, RBZ, CYJ, FY, XHY and LF performed the experiments. XL, JBH and XGS wrote the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

The growth of PRRSV was inhibited by SR. Structural formula of SR (A). MTT was used to detect the toxicity of SR to Marc-145 cells(B). The inhibitory effect of SR on PRRSV was observed by IFA (C). At different time (12h, 24h, 36h, 48h) and different concentration (0, 10, 20, 40, 60, 80 and 100 μM), SR inhibited PRRSV (D and E). Data are shown as means±SD of three independent experiments (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; ns, no significant)

Figure 2

The inhibition of SR on PRRSV was not killing. Marc-145 cells were treated with 100μM SR and infected with PRRSV for 48 hours (A). PRRSV was incubated with different concentrations of SR for 2 hours to analyze the virus titer (B). The Data are shown as means±SD of three independent experiments (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; ns, no significant)

Figure 3

SR inhibits virus invasion. The different MOI PRRSV and 100 μ M SR were used to incubate for 2h, and then the non-adsorbed virus was removed, the virus titer was measured after incubation for 48h (A). The different MOI PRRSV was adsorbed on the cells, then SR (100 μ M) was added to the cells adsorbed by the virus, cultured for 3h, and SR was removed. The virus titer was measured after incubation for 48h (B). Marc-145 cells were treated with SR (100 μ M) or PBS after 2 hpi. The virus genome was measured at the specified time point (C). Marc-145 cells were treated with SR (100 μ M) or PBS after 24 hpi. The virus titer was measured at the specified time point (D). Data are shown as means $\pm$ SD of three independent experiments (* p <0.05, ** p <0.01, *** p <0.001, **** p <0.001; ns, no significant)