Antiviral Activity of Tilmicosin for Type 1 and Type 2 Porcine Reproductive And Respiratory Syndrome Virus In Cultured Porcine Alveolar Macrophages

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Abstract

Tilmicosin was investigated in a stable porcine alveolar macrophage cell line for its antiviral activity against types 1 and 2 porcine reproductive and respiratory syndrome virus (PRRSV). Tilmicosin exhibited strong antiviral effects on both genotypes of PRRSV replication in cultured porcine alveolar macrophages. The antiviral activity was more potent for type 1 PRRSV as indicated by reduction in infectious virus yield of nearly 43-fold for type 1 as compared to 14-fold for type 2. Real-time RT-PCR for detection of viral nucleocapsid gene confirmed the reduction of infectious virus titer in the presence of tilmicosin. Lydosotropide and ion-channel blocking agents efficiently inhibited PRRSV replication, suggesting that the antiviral mechanism of tilmicosin is likely associated with the alteration of endosomal pH and possibly the ion-channel activity on the viral membrane. This study indicates that tilmicosin may have potential as an early treatment or preventative measure against PRRSV infections, especially for areas where the type 1 genotype is prevalent.

Keywords: Porcine reproductive and respiratory syndrome virus (PRRSV); Arterivirus; Tilmicosin; Antivirals; Ion-channel; Nucleocapsid protein

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is considered a re-emerging disease in swine. PRRS was first recognized in the late 1980s almost simultaneously but independently in the U.S. and Germany. After its emergence, PRRS quickly spread to most pig-producing countries and has become one of the most economically important diseases to the global pork industry [1]. PRRS affects breeding herds and grower/finisher pigs, primarily manifesting as reproductive problems, increased neonatal mortality, and poor growth. Economic losses due to an acute PRRS outbreak are estimated from $250 to more than $500 per sow; and annual economic losses to the US pork producers alone are estimated to be $600 million [1]. Thus, the economic impact of PRRS is significant and imposes a substantial financial burden on swine producers.

PRRS virus (PRRSV) isolates in Europe and the U.S. present as two distinct genotypes (type 1 and 2, respectively) with only 60% sequence similarity [2,3]. Since its emergence, PRRSV has continued to evolve. A new genotype of PRRSV capable of causing clinical disease in pigs recently has been identified in the U.S. [4]. Although this new genotype is closer to type 1 PRRSV, it is genetically distinct and thus, has been designated Euro-PRRSV. In the Asian continent, a highly pathogenic strain is closer to type 1 PRRSV, it is genetically distinct and thus, has been recently identified in the U.S. [4]. Although this new genotype is closer to type 1 PRRSV, it is genetically distinct and thus, has been designated Euro-PRRSV. In the Asian continent, a highly pathogenic PRRSV appeared in China in spring 2006 [5-7] and widely spread within the country and from China to Vietnam and Russia. This newly emerged strain causes very high morbidity and mortality in comparison to the previous types and thus, has been designated as porcine high fever disease (PHFD). The PHFD outbreaks have crippled China’s pork industry and have caused multiple increases in the country’s consumer pork prices. The sudden appearance of such a highly virulent PRRSV was unexpected, and effective control measures are urgently needed to prepare for the potential emergence of a virulent PRRSV in North American swineherds.

Vaccination has been far less satisfactory than anticipated, being unable to mitigate the PRRS-associated losses. The first commercial vaccine for PRRS became available in 1994, and utilized a modified live virus (MLV), which had been attenuated through multiple passages of the virus in monkey kidney cells (Ingelvac PRRS MLV, Boehringer Ingelheim [BI] Vetmedica, Inc., St. Joseph, MO). Although the vaccine virus is able to confer appropriate levels of protective immunity for homologous virus infection, resultant heterologous protection is not adequate and thus, provides variable-to-meager levels of protection in the field. In addition, a major concern of this MLV vaccine is its safety. Shedding and spread of the vaccine virus may occur between vaccinated and non-vaccinated contact pigs as well as its transmission in the semen of vaccinated boar studs, thereby capable of spreading to and inducing disease in naive breeding herds [8-12]. Veterinarians practitioners and diagnosticians reported disease outbreaks described as swine abortion and mortality syndrome, atypical PRRS, or acute PRRS in BI MLV-vaccinated herds, many of which had been immunized multiple times with the vaccine. These clinical outbreaks, characterized by mid- or late-term abortions in breeding herds and respiratory disease in growing pigs, resulted in lesions typical of PRRSV infection. The only etiological agent identified in such herds was PRRSV that was highly homologous in the ORF1 or ORF 5 sequences to the vaccine virus used in the herd [13,14].

Viruses in general carry only a minimal number of genes and thus, for replication they must pirate the cellular machinery of infected cells. As a result, viral replication is strictly dependent upon the host...
cell metabolic pathways. The ideal therapeutically safe and effective antiviral drugs would selectively affect specific processes of the target virus with minimal side effects on normal cellular pathways. For this reason, it has been more difficult to develop virus-specific antiviral drugs in comparison to vaccines, the major focus of disease prevention, especially in veterinary medicine. Nevertheless, antiviral chemotherapy is a worthwhile consideration for ameliorating the costly effects of PRRS since a satisfactory vaccine has yet to be produced.

One potential antiviral drug is tilmicosin, a chemically modified macrolide antibiotic derivative of tylosin, which is synthesized in Streptomycetes fradiae. Tilmicosin has been developed for veterinary use as an injectable for use in cattle and sheep and as a premix feed formulation for swine [15]. It is an effective antimicrobial for Gram-positive and some Gram-negative bacteria, as well as some intracellular bacteria, such as Rodococcus spp and Mycoplasma spp. Tilmicosin has been studied to a limited extent for its antiviral effects on PRRSV. In previous studies, tilmicosin inhibited PRRSV replication in cultured porcine alveolar macrophages (PAMs) in a dose-dependent manner [16], tylvalosin, a macrolide derivative, also inhibited both European type and North American type of PRRSV in cultured cells [17]. In pigs experimentally infected with PRRSV and treated with tilmicosin as a feed additive, a reduction in the severity of lymph node hypertrophy, lung lesions and viremia was observed as compared to the non-medicated infected controls [18]. Tilmicosin reduced the disease severity in experimentally PRRSV-infected nursery pigs as measured by fewer clinical signs, better feed consumption and weight gain, and a tendency towards lower virus titers in lung and serum as compared to the non-medicated challenged pigs. More recently, tilmicosin was evaluated in sows under field conditions [19]. PRRSV serum therapy, in which animals are inoculated with viremic serum, has been an effective option to control PRRS in a breeding herd by exposing the animals to the currently circulating strain. However, this practice can result in mortalities or abortions, and therefore represents a certain level of risk. When tilmicosin was fed to gestating sows in conjunction with viremic serum inoculation, a reduction in mortality and abortion rate was observed in the treated as compared to the untreated inoculated sows. An aqueous form of tilmicosin also was evaluated in nursery pigs in a controlled environment [20]. Administering tilmicosin in the drinking water resulted in a 50% reduction in mortality, lower body temperatures, a significant increase in average daily gain and a reduction in lung lesions in the medicated as opposed to the non-treated group. It is known that tilmicosin accumulates in macrophages at high concentrations [21], the primary target cells for PRRSV replication, and thus, may explain the reduced clinical severity observed in the tilmicosin-fed pigs.

In this present study, the antiviral effects of tilmicosin were examined on both type 1 and type 2 PRRSV in cultivated PAM cells. The effects on virus yield were measured by an infectious titer assay and quantification of the viral genome. The results showed that tilmicosin inhibited virus replication and exhibited stronger antiviral effects on type 1 than on type 2 PRRSV. The ability to inhibit viral replication suggests that tilmicosin may have a therapeutic potential as a preventative measure for PRRSV infection.

Materials and Methods

Cells and viruses

African green monkey kidney (MARC-145) [22] cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with a 10% heat-inactivated fetal bovine serum (FBS, HyClone; Logan, UT) in a humidified incubator at 37°C with 5% CO₂. A stable suspension cell line of PAMs, designated as Z-mac cells (kindly provided by Dr. Zuckermann, University of Illinois, Urbana, IL), were grown in RPMI 1640 (Invitrogen; Carlsbad, CA) containing 10% FBS. The PRRSV PA8 strain [23] was used as a representative strain for type 2, while Lelystad virus (LV) was used to represent type 1 PRRSV [24].

Effective dose determination by TCID₅₀ titration assay

Approximately 1×10⁶ of Z-mac cells were seeded per well in 96-well plates. Cells were immediately treated with various concentrations of tilmicosin (Elanco Animal Health, St-Jean-sur-Richelieu, Canada) ranging from 20–80 µg/ml. Twenty-four hours later, cells were infected with 100 tissue culture infectious dose 50% end-point (TCID₅₀) of either PRRSV PA8 or LV in the presence of tilmicosin. Controls consisted of infected untreated cultures. At 18 h post-infection (p.i.), supernatants were collected and infectious virus was titrated in MARC-145 cells. The titers were recorded as TCID₅₀/ml. Infections for each concentration of tilmicosin were performed in triplicate and each experiment was repeated at least twice. Triplicate values were analyzed for variance by t-test.

Viral RNA extraction and real-time reverse transcription (RT)-PCR

Z-mac cells were infected with PRRSV in the presence or absence of tilmicosin as described above. Uninfected cells served as negative controls. Viral RNA was extracted from cell culture supernatants using QiAamp Viral RNA kit (Qiagen; Valencia, CA) according to manufacturer’s protocol. Reverse transcription (RT) reaction was carried out using M-MLV reverse transcriptase (200 U/ml, Invitrogen) in a 25 µl reaction mixture containing 12 µl RNA, 3 µl reverse primer, 5 µl 5x buffer, 1.5 µl dNTP mix (10 mM each), 2 µl DTT (10 mM), 0.5 µl RNase inhibitor and 1 µl M-MLV reverse transcriptase.

Next, real-time PCR was performed in a 25 µl total volume containing 2.5 µl cDNA template, 12.5 µl SYBR® Green PCR Master Mix (Applied Biosystems; Carlsbad, CA), and 5 µl of each primer (1 µM). Thermocycling conditions were 95°C for 2 min for 1 cycle followed by 40 cycles of 95°C for 1 s and extension at 60°C for 1 min. Oligonucleotide primer pairs for real-time PCR were designed for the amplification of the viral ORF7 (nucleocapsid protein gene) region as follows: PA8-Rtime-Fwd: 5’-AATAACACAAGCCAGAACGACGACGACG-3’ and PA8-Rtime-Rev: 5’-CCTCTGGAGCTGGTTTGGTGTA-3’ (for type 2 PRRSV PA8); LV-Rtime-Fwd: 5’-TCACCCGACAGCTGAGCTCCTCC-3’ and LV-Rtime-Rev: 5’-CACCCCGACTGGCGGATGTAG-3’ (for type 1 PRRSV LV).

Effects of chemical compounds for ion-channel inhibition and endosomal pH elevation on PRRSV

Verapamil, chloroquine, amantadine, and ammonium chloride were purchased from Sigma-Aldrich (St. Louis, MO) and their stock solutions were prepared in distilled water at concentrations of 50 µM, 20 µM, 0.4 mM, and 5 mM, respectively, and sterilized by filtration through 0.25 µm syringe filters. 1×10⁶ of Z-mac cells were seeded per well in 96-well plates and incubated for 24 h at 37°C in the presence of individual reagents, followed by infection with 100 TCID₅₀ of either PRRSV PA8 or LV in the presence of various compounds. At 18 h pi, supernatants were collected for TCID₅₀ determination and genome quantification by real-time RT-PCR as described above. Virus-infected cells in the absence of compound treatments served as controls.
**Statistical analysis**

Data were analyzed for variance and t-test. A $p$-value of $< 0.05$ was considered statistically significant.

**Results**

**Antiviral activity of tilmicosin for type 2 PRRSV**

The antiviral effects of tilmicosin were first evaluated using type 2 PRRSV in Z-mac cells. These cells, originated from porcine alveolar macrophages and established as an immortalized cell line, are fully permissive for PRRSV replication [25] and thus, served as the standard in the current study since the genetic variation would be minimal in these cells in comparison with primary PAMs prepared from different pigs for each experiment. When Z-mac cells were infected with PRRSV, cytopathic effects (CPEs) became evident at 16 h post-infection (p.i.) and most cells died by 24 h p.i. (Figure 1D). The development of CPE was faster in Z-mac cells than in MARC-145 cells which take 48 h for obvious CPE (Figure 1B). No visible difference was observed for CPE between the type 1 and type 2 PRRSV infections. Cytotoxicity visualized as cell death was observed in Z-mac cells at a high concentrations of tilmicosin (200 µg/ml) by approximately 20% decrease in the cell population as determined by manual counting using a hemacytometer (data not shown). In contrast, cytotoxicity was not evident at lower tilmicosin levels (80 µg/ml). Therefore, effective dose titration experiments were conducted using concentrations between 0-80 µg/ml. When infected with 100 TCID$_{50}$ of PRRSV PA8, the virus yield of 4.11 log$_{10}$/ml (1.29×10$^4$ TCID$_{50}$/ml) was obtained at 18 h p.i. in the absence of tilmicosin (Figure 2A). In contrast, a statistically significant ($p<0.05$) level of reduction of PRRSV was observed at 80 µg/ml; the infectious virus yield was decreased to 2.98 log$_{10}$/ml (9.55×10$^2$ TCID$_{50}$/ml), a 14-fold reduction. At less than 80 µg/ml concentration of tilmicosin, a decrease in virus yield was not statistically evident.

To confirm the reduction of virus production in the presence of tilmicosin, the relative amounts of viral genomic RNA were quantified. The viral genomic RNA was extracted from the same samples that were used for virus titrations and subjected to real-time RT-PCR for the viral nucleocapsid (N) gene of PRRSV PA8. In agreement with the results from the infective titer assay, the genomic quantification data also confirmed the anti-PRRSV activity of tilmicosin and show that tilmicosin was more potent against type 1 than type 2 PRRSV.

**Antiviral activity for type 1 PRRSV**

Since both genotypes of PRRSV circulate in swine farms in the USA and Canada, and because tilmicosin appears to possess an antiviral activity for type 2 PRRSV, this activity was further examined for type 1 PRRSV. Lelystad virus (LV) is the prototype virus of the European genotype [24] and thus, was chosen for this study. As with PA8, LV replication was inhibited by tilmicosin over a range of concentrations of tilmicosin ranging between 20 and 80 µg/ml for 24 h, after which time the cells were infected with 100 TCID$_{50}$ of either PRRSV PA8 (A and B) or LV (C and D) in the presence of the drug. At 18 h p.i, culture supernatants were collected for determination of infectious virus yields (TCID$_{50}$/ml). In parallel, viral RNA was extracted from cells in the same well and subjected to real-time RT-PCR for the N gene of PRRSV PA8 (B) or LV (D). Controls consisted of virus-infected untreated cultures. Virus titers (A and C) and relative fold changes in viral genomic RNA (B and D) are indicated. Data are shown as mean titers with standard errors (A and C) and relative amounts of RNA with standard errors (B and D). One star (*) denotes a statistically significant difference of $p<0.05$ and two stars (**) denotes a statistically significant in difference of $p<0.01$. PRRSV activity that affected synthesis of progeny genome.
Antiviral activity of various compounds on PRRSV

The biochemical nature of tilmicosin is basic due to two amine groups (pKa 7.4 and 8.5). Therefore, a possible mechanism for the antiviral activity of tilmicosin may be related to altered pH in the endosome of treated cells. In this scenario, an influx of ions from the endosome to the interior cavity of the virus is mediated through the ion-channel like pores on the viral membrane. In support of this scenario, the small envelope protein of PRRSV has been demonstrated to possess ion-channel protein-like properties [26]. Thus, experiments were conducted to examine the effects of ion-channel blockers on PRRSV infection. Verapamil, a calcium channel blocking agent, and amantadine, known as a proton channel inhibitor, were examined for their affects on PRRSV replication. In addition, two other compounds, chloroquine and ammonium chloride, both lysosomotropic agents that can increase the pH of the lysosomes and endosomes were examined. Separate cultures of Z-mac cells were incubated for 24 h with one of the various reagents, after which the cells were infected with PRRSV PA8 or LV in the presence of the compound. At 18 h p.i., culture supernatants were collected and titrated for virus production. All four compounds showed antiviral activity against type 2 PRRSV (PA8 strain) with varying degrees of potency (Figure 3A) and thus, confirmed the involvement of endosomal pH and ion channel function in the PRRSV replication. The antiviral activity of these compounds was also examined by real-time RT-PCR for relative amounts of PA8 genomic RNA (Figure 3B). These results indicate that the inhibitory effects on PRRSV were likely mediated through the elevation of endosomal pH. The antiviral effects of the lysomotropic agents and ion channel blockers also were examined on type 1 PRRSV (LV strain) for infectious virus yield (Figure 3C) and progeny genome levels (Figure 3D). In agreement with the tilmicosin data, each of the compounds contained the antiviral activities for both types of PRRSV and these activities were found to be stronger against type 1 than type 2 PRRSV.

Discussion

For macrolide antibiotics, the mechanism of action is largely unclear; one exception is erythromycin whose function is relatively well understood. It binds to the 50S ribosomal complex in bacteria and thereby inhibits protein synthesis. Unlike erythromycin, which contains only one basic amine group (pKa 7.4) in its structure, tilmicosin contains two basic amine groups (pKa 7.4 and 8.5) [27]. Tilmicosin also is highly lipophilic, which may explain its efficient uptake by macrophages by way of a favored passage through the lipid membranes of the cell. The efflux of tilmicosin from alveolar macrophages is slow, as 37% of tilmicosin is still cell-associated even after 24 h. Once it is taken up by macrophages, tilmicosin seems to localize in the lysosomes, comprising up to 85% of the intracellular drug concentration [27].

This information led us to hypothesize a possible mechanism of action of tilmicosin for inhibition of PRRSV replication in macrophages. As an initial step of infection, a virus recognizes a specific cellular receptor on the cell surface. Once bound to a cellular receptor, an enveloped virus enters the cell by either direct fusion of viral membrane with the cellular membrane or receptor-mediated endocytosis, depending on the virus. For PRRSV, several possible receptors have been reported, including CD163, hapan sulfate, vimentin, CD151 and siaoaldhesin [28-32]. PRRSV is an enveloped virus and a receptor-mediated endocytic pathway has been suggested as its means for the entry [33]. Thus, PRRSV particles are internalized via a clathrin-dependent pathway and are subsequently transported to the endosomes/lysosomes, where the uncoating of virus particles occurs [34,35]. Lysosomes, which contain proteolytic enzymes, are cellular organelles that collect foreign invaders such as bacteria, particles and worn-out organelles and digest them into fragments that may be reutilized. These proteolytic enzymes are specially processed for lysosomes in the rough endoplasmic reticulum, and are only biologically active at acidic pH. Once PRRSV particles are transported to the endosome by receptor-mediated endocytosis, acidic pH-induced conformational changes occur in the viral proteins, which subsequently triggers further uncoating of virus ultimately releasing the viral genomic RNA into the cytoplasm. If such conformational changes of the viral proteins are inhibited, uncoating will be blocked, thereby arresting further steps of PRRSV replication. Therefore, we posit that the accumulation of tilmicosin in alveolar macrophages will result in the increase of pH in the endosome due to the basic property of the compound, and as a consequence, the subsequent uncoating process will be inhibited, thereby blocking PRRSV replication. In addition, tilmicosin is reported to induce apoptosis in broncho-alveolar leukocytes and in this manner attenuate inflammation associated with the reduction in pulmonary lesions [36]. Apoptosis is a normal cellular mechanism by which infected, damaged or potentially harmful cells are removed. Increasing evidence suggests that lysosomal proteases are actively involved in apoptosis. In HeLa cells, selective lysosomal disruption resulted in apoptosis, characterized by translocation.

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provides partial protection to cell against viral infection [41]. In our less susceptible to viral infection. Hence, restriction of calcium influx that is, by disabling the channels via drug interaction, cells become the target of attack and thus, by releasing the intracellular calcium into the cytosol and increasing the cytosolic calcium concentration may contribute to the inhibition of PRRSV replication, supporting our hypothesis that the antiviral mechanism of tilmicosin depends on alteration of pH and blocking the uncoating process during entry of the virus. Overall, our study provides experimental confirmation as well as a possible mechanism of action for tilmicosin efficacy that has been empirically observed in the clinical setting. Since vaccination approaches have shown limited success, the therapeutic potential of tilmicosin as an early treatment or preventive measure for PRRSV infection should be investigated further.

**References**

live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (Invevac PRRS MLV), the parent strain of the vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. J Virol 76: 11837-11844.


