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Inhibition of porcine reproductive and respiratory syndrome virus infection in piglets by a peptide-conjugated morpholino oligomer

Tanja Opriessnig [a], Deendayal Patel [b,1], Rong Wang [b], Patrick G. Halbur [a], Xiang-Jin Meng [c], David A. Stein [d], Yan-Jin Zhang [b,e,*]

[a] Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA
[b] VA-MD Regional College of Veterinary Medicine, University of Maryland, College Park, MD 20742, USA
[c] Center for Molecular Medicine and Infectious Diseases, VA-MD Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060, USA
[d] Vaccine and Gene Therapy Institute, Oregon Health and Science University, Beaverton, OR 97006, USA
[e] Maryland Pathogen Research Institute, University of Maryland, College Park, MD 20742, USA

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) causes substantial economic losses to the swine industry in many countries, and current control strategies are inadequate. Previously, we explored the strategy of using peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) to inhibit PRRS virus (PRRSV) replication. PPMOs are nuclease-resistant and single-stranded DNA analogs containing a modified backbone conjugated to a cell-penetrating peptide and can act as antisense agents through steric blockade of complementary messenger RNA. A PPMO (designated 5UP2) targeting highly conserved sequence in the 5′-terminal region of the PRRSV genome was found to produce multi-log10 inhibition of PRRSV replication in cultured cells. To evaluate 5UP2 *in vivo*, we here administrated the PPMO to 3-week-old piglets via intranasal instillation at 24 h before, and 2 and 24 h after infection with PRRSV (strain VR2385). Blood samples were collected at 6, 10 and 14 days post-infection (dpi) for detection of PRRSV RNA and antibodies. Necropsy was performed at 14 dpi. Monitoring weight gain in all piglet groups throughout the experiment indicated that PPMO was well tolerated at the doses used. PPMO 5UP2 treatment significantly reduced PRRSV viremia at 6 dpi. On day 14, piglets receiving 5UP2 had significantly less interstitial pneumonia and lower level of anti-PRRSV antibodies than untreated piglets. In alveolar macrophages isolated at the time of necropsy, the expression of antiviral genes in PPMO-treated piglets was elevated in comparison with untreated. This study provides further data indicating that the 5UP2 PPMO can be considered a candidate component for a novel PRRS control strategy.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) continues to be the most important microbiologic challenge for the swine producing industry worldwide, causing an estimated loss of $560 million per year in the USA alone (Neumann et al., 2005). The clinical manifestations of PRRS include reproductive failure, post-weaning pneumonia, growth reduction, and increased mortality (Keffaber, 1989; Loula, 1991). The causative agent of this disease is PRRS virus (PRRSV), an enveloped, single-stranded positive-sense RNA virus of the family *Arteriviridae* (Meulenberg et al., 1993a). PRRSV was first isolated in Europe in 1991 (Wensvoort et al., 1991) and in the USA in 1992 (Benfield et al., 1992). The various strains of PRRSV each belong to one of the two PRRSV genotypes defined by genetic characteristics, European (Type 1) and North American (Type 2) (Meng et al., 1995; Meulenberg et al., 1993a). PRRSV can replicate *in vitro* in African green monkey kidney cells and cells derived thereof, including MARC-145 (Benfield et al., 1992; Kim et al., 1993; Meng et al., 1994), and in porcine pulmonary alveolar macrophages (PAMs), the primary target cells *in vivo* (Yoon et al., 1992).

PRRSV has an RNA genome of approximately 15-kb in size, which consists of a 5′ untranslated region (UTR), nine open reading frames (ORFs 1a, 1b, 2a, 2b, and 3–7) and a 3′ UTR (Meng et al., 1994; Meulenberg et al., 1993a). In PRRSV-infected cells, a set of six or seven nested subgenomic viral RNAs is formed (Conzelmann et al., 1993; Meulenberg et al., 1993b). All of the subgenomic RNAs have an identical 5′-leader sequence derived from the 5′ end of the

* Corresponding author at: Molecular Virology Laboratory, VA-MD Regional College of Veterinary Medicine, University of Maryland, 8075 Greenmead Drive, College Park, MD 20742, USA. Tel.: +1 301 314 6596; fax: +1 301 314 6855.
E-mail address: zhangyj@umd.edu (Y.-J. Zhang).
[1] Present address: Hauptman-Woodward Medical Research Institute, 700 Ellicott Street, Buffalo, NY, USA.

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genomic RNA, as well as an identical 3′-terminal sequence preceding poly-(A) tails of variable length.

Current management strategies, which focus on the prevention of infection and vaccination using commercial modified live-attenuated vaccines (MLVs) or autogenous killed vaccines, have generally been proven inadequate for long term control of PRRS. Evidence of reversion to virulence of at least one of the current MLV strains following its use in pigs (Opriessnig et al., 2002) highlights the need for novel strategies to control PRRSV transmission and disease.

Phosphorodiamidate morpholino oligomers (PMOs) are structurally similar to single-stranded DNA oligomers, and are typically synthesized to be 20–25 subunits in length. Each subunit of PMO consists of a purine or pyrimidine base joined to a novel backbone containing a morpholine ring and phosphorodiamidate linkage (Summerton, 1999; Summerton and Weller, 1997). PMOs are uncharged, water-soluble, and highly resistant to nuclease degradation (Hudziak et al., 1996; Nelson et al., 2005). PMOs are designed to bind to complementary mRNA and exert an antisense effect through steric blocking of critical regions of RNA sequence, such as a translation initiation site. To facilitate entry into cells, a PMO can be conjugated to an arginine-rich cell-penetrating peptide, to produce peptide-PMO (PPMO) (Abes et al., 2006; Moulton et al., 2004). It has been shown that PPMOs enter cells readily, and that the PMO portion of a PPMO is stable in cells and human serum for at least 24 h (Youngblood et al., 2007). The sequence-specific antiviral efficacy of PPMOs has been documented against a number of RNA viruses *in vitro* and *in vivo* (reviewed in Stein (2008)) and DNA viruses (Eide et al., 2010; Moerdyk-Schauwecker et al., 2009; Zhang et al., 2008; Zhang et al., 2007). Antisense PPMOs delivered by intranasal instillation have shown considerable antiviral efficacy against lung infections of respiratory syncytial virus (Lai et al., 2008) and Influenza A virus (Gabriel et al., 2008; Lupfer et al., 2008) in mouse models.

The study here explored the inhibition of PRRSV replication in the lungs of piglets by a PPMO designed specifically against PRRSV RNA sequence. Previous studies demonstrated potent reduction of PRRSV replication in MARC-145 and PAM cells by PPMOs designed against specific segments of the PRRSV genome (Han et al., 2009; Patel et al., 2008; Patel et al., 2009; Zhang et al., 2006). Among the PPMOs tested, 5UP2, which is complementary to an highly conserved sequence in the 5′ terminal region of the PRRSV genome, was found to be effective at inhibiting PRRSV replication in a sequence-specific and dose-dependent manner. In the study here, we evaluated the antiviral efficacy of PPMO 5UP2 in 3-week-old piglets experimentally infected with a virulent strain of PRRSV. Since transmission of PRRSV occurs primarily via the respiratory route, we used intranasal administration of both virus and PPMO. We found that PPMO 5UP2 suppressed PRRSV viremia and the development and severity of pathologic lesions in the lungs of piglets.

2. Materials and methods

2.1. Cells and virus

MARC-145 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). PRRSV strain VR2385 was grown and titered, as previously described (Patel et al., 2008).

2.2. PPMO

The 5UP2 PPMO was produced at AVI BioPharma Inc. (Corvallis, OR) as previously described (Patel et al., 2008) and was resuspended in distilled water prior to use.

2.3. Evaluation of PPMO in piglets

Twenty 3-week-old PRRSV-negative piglets weighing 5.7–8.9 kg each were randomly divided into four groups (Table 1). The piglets in groups 1–3 were inoculated with 1 ml of PRRSV VR2385 (10^5 $CCID_{50}$/ml) via intranasal instillation (I.N.), while group 4 was left uninfected. PPMO solution was administered I.N. at dosages of 0.1 and 0.5 mg/kg body weight for the piglets in groups 1 and 2, respectively. PPMO administration was carried out at 24 h pre-, 2 and 24 h post-PRRSV inoculation (hpi). Two groups of piglets, which did not receive PPMO treatment, one infected with PRRSV and the other uninfected, served as control groups.

All piglets were weighed at 0 and 14 days post-PRRSV infection (dpi). Blood samples were collected on 0, 6, 10, and 14 dpi. Serum was separated and stored at −80 °C for subsequent determination of viral RNA and anti-PRRSV antibody.

Pigs were humanely euthanized on 14 dpi by pentobarbital overdose (FATAL-PLUS, Vortech Pharmaceuticals, Ltd. Dearborn, MI). Necropsy was performed and visible macroscopic lung lesions were scored as previously described (Halbur et al., 1995; Halbur et al., 1996). Porcine alveolar macrophages were collected during necropsy as previously described (Patel et al., 2009) and frozen at −80 °C in medium containing dimethyl sulfoxide (DMSO). To evaluate lung histopathology, slices of lung tissue from each lobe were formalin-fixed, paraffin embedded, sectioned and stained with hematoxylin and eosin (HE) (Halbur et al., 1995; Halbur et al., 1996). The level of interstitial pneumonia in each fixed tissue slice was evaluated through microscopic examination. The level of interstitial pneumonia was scored ranging from 0 (absent) to 6 (severe diffuse lesions), as previously described (Halbur et al., 1995; Halbur et al., 1996). Scoring of macroscopic and microscopic lung pathology was done in a blinded fashion by two veterinary pathologists (TO, PGH).

Immunohistochemistry of paraffin-embedded blocks of lung and lymph nodes for detection of PRRSV antigen was performed as previously described by using a cocktail of two different mouse monoclonal antibodies against highly conserved epitopes on the PRRSV nucleocapsid protein, SDOW-17 at 1/5000 and SR-30 at 1/1500 (Halbur et al., 1994).

2.4. RNA isolation and real-time reverse-transcription PCR (RT-PCR)

Total RNA was isolated from serum samples or PAMs using the TRIzol® reagent (Invitrogen, Carlsbad, CA). RNA was quantified by µQuant™ Universal Microplate spectrophotometer (BioTek Instruments, Winooski, VT) and was converted to cDNA using qScript cDNA Synthesis kit (Quanta Biosciences, Gaithersburg, MD). Quantitative real-time RT-PCR was performed to detect PRRSV RNA with primers designed against PRRSV ORF7 as previously reported (Patel et al., 2008). A pCDNA3-sg7 plasmid containing PRRSV ORF7 sequence (Han et al., 2009) was used as a template to generate a standard curve, against which RNA copies in all samples were calculated.

Transcript levels for a group of cellular genes including chemokine (C–C motif) ligand 1 (CCL1), CCL2, C–X–C motif chemokine 10

Table 1
Outline of the PPMO experiment in piglets.[a]

Event	Group 1	Group 2	Group 3	Group 4
PRRSV	Y	Y	Y	N
PPMO 5UP2	Y	Y	N	N
Dosage (mg/kg)	0.1	0.5	0	0
Schedule (hpi)	-24, 2, 24	-24, 2, 24 h	N/A	N/A
Number of piglets	5	5	5	5

[a] Y: yes. N: No. N/A: not applicable.

Table 2
List of primers for real-time PCR.

Name[a]	Sequences (5′–3′)[b]
sCCL1-F1	CTGAGCTGGACATCCTGAGA
sCCL1-R1	CAACAATTGGAGGACGACAC
sCCL2-F1	CACCAGCAGCAAGTGTCCTA
sCCL2-R1	TCCAGGTGGCTTATGGAGTC
sCXCL10-F1	TTCGCTGTACCTGCATCAAG
sCXCL10-R1	CAACATGTGGGCAAGATTGA
sRNaseL-F1	GCCAGACCTAGTGGCTTCTG
sRNaseL-R1	AGAGGCCCAGAGAGTTGTGA
sOAS1-F1	CACAGCTCAGGGATTTCAGA
sOAS1-R1	TCCAACGACAGGGTTTGTAA
sMX1-F1	AGCGCAGTGACACCAGCGAC
sMX1-R1	GCCCGGTTCAGCCTGGGAAC
sOAS2-F1	AGGGGGCTCTTTCTCTTCTG
sOAS2-R1	AGGAGGCCGGTCATAGAGAT
sCCL17-F1	TGGTACCAGACCTCAGACGA
sCCL17-R1	TCTGCCCTGGAAGGTCAC
sCCL22-F1	TTACAGCTGCTCCCTGACAC
sCCL22-R1	TGGATTGGAAGGGTAAGAGG
sCXCL11-F1	AAAGCAGTGAAAGTGGCAGA
sCXCL11-R1	GCTTGCTTTGATTTGGGATT
sPKR-F1	AATTCCCCAAAGCTGAAGGT
sPKR-R1	CTGTCGGCAGTGATGAAGAA
sRPL32-F1	CTCTTCCAAGAACCGCAAAG
sRPL32-R1	GCTGAGCCACAACTGGAACT
sTNF-F1	ACCACCAACGTTTTCCTCACT
sTNF-R1	TTGATGGCAGAGAGGAGGTT

[a] F1: forward primer, R1: reverse primer.
[b] Swine gene sequences were downloaded from GenBank and a swine gene database: www.genome.iastate.edu.

(CXCL10), RNase L, 2′-5′-oligoadenylate synthetase 1 (OAS1), myxovirus (influenza virus) resistance 1 (MX1), and tumor necrosis factor α (TNF-α) in PAMs were evaluated (primers are listed in Table 2). Ribosomal protein L32 (RPL32) transcripts were also amplified and used to normalize for the amount of input RNA from PAMs. Relative transcript levels detected from the PAMs were quantified by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and shown as relative fold of change in comparison with the average of the uninfected and untreated control group.

2.5. ELISA

Antibody detection was done using the HerdChek* PRRS 2XR Virus Antibody Test Kit (IDEXX Laboratories, Inc., Westbrook, Maine) according to the manufacturer's instruction. An ELISA sample-to-positive (S/P) ratio above 0.4 was considered to be positive for the presence of anti-PRRSV antibodies.

2.6. Statistical analysis

A single factor ANOVA statistical analysis was used to evaluate significance in differences between levels of viral or cellular RNA of infected piglets receiving PPMO treatment compared to untreated. A two-tailed *P* value of smaller than 0.05 was considered significant. Mann Whitney U-test (http://elegans.swmed.edu/~leon/stats/utest.html) was also run to verify the results of *t*-tests.

3. Results

3.1. PPMO design and overview of experiments

PPMO 5UP2 was designed to complement bases 9–28 in the 5′ terminal region of the genome of PRRSV VR2385 (Patel et al., 2008). The 20-base target sequence of 5UP2 is highly conserved among strains of Type II PRRSV available in GenBank, with only a few virus strains having variation at one or two positions in the target region. PPMO 5UP2 was previously shown to have potent antiviral activity against various strains of PRRSV in both MARC-145 and PAM cell cultures (Patel et al., 2008; Patel et al., 2009). The 5UP2 likely produces inhibition of virus replication by blocking translation of ORF1a and 1b, which encode nonstructural proteins required for viral RNA synthesis and production of the structural proteins.

PRRSV infection is spread via respiratory transmission and typically results in lung lesions in piglets. We therefore administered PPMO and infected piglets with PRRSV via intranasal inoculation. PPMO doses of 3.75–4.5 mg/kg administered by intranasal administration have previously been reported as causing no ill effects to the lungs of uninfected mice (Lai et al., 2008; Lupfer et al., 2008). Three treatments of PPMO 5UP2 at doses of 0.1 and 0.5 mg/kg body weight were administered, at 24 h before, and 2 and 24 hpi. The experimental design is shown in Table 1.

3.2. PPMO 5UP2 treatment suppresses viremia in piglets

Serum samples were collected at 6, 10 and 14 dpi and PRRSV RNA levels were determined by real-time RT-PCR. At 6 dpi, piglets receiving the PPMO at doses of 0.1 or 0.5 mg/kg had significantly lower (3.7- and 12-fold reduction, respectively. $P < 0.01$) levels of PRRSV RNA than the untreated piglets (Fig. 1A). At 10 dpi, the PRRSV RNA copies in piglets receiving PPMO at doses of 0.1 and 0.5 mg/kg were 1.3- and 2.1-fold lower, respectively, than those in the untreated group (Fig. 1B), these differences were not statistically significant. Likewise there were no significant differences between groups in PRRSV RNA levels in serum samples of 14 dpi. These results indicate that PPMO treatment, administered around the time of infection, was able to suppress viremia in piglets for at least 6 days.

3.3. PPMO 5UP2 reduces PRRSV-induced pneumonia in piglets

Macroscopic and microscopic lung lesions were examined from tissues taken at necropsy at 14 dpi. Macroscopic lung lesions were mild in all three PRRSV-infected groups, with no significant differences between groups, while no lung lesions were visible in the non-infected piglets. Microscopic examination revealed that infected piglets receiving no PPMO showed severe interstitial pneumonia characterized by a thickening of alveolar septa, caused by infiltrating macrophages and lymphocytes, while those receiving 5UP2 PPMO at a dose of 0.1 mg/kg had significantly less interstitial pneumonia (Fig. 2A). However, infected piglets receiving the 0.5 mg/kg PPMO did not show microscopic lung lesion scores significantly different from the untreated group. Representative microscopic images of lung tissue sections from each treatment group are presented in Fig 2B. Immunohistochemistry of lung sections taken at 14 dpi showed PRRSV nucleocapsid protein in all PRRSV infected pigs (regardless of treatment), consistent with the result of similar viremia in all groups of these pigs at 14 dpi.

3.4. Delay in antibody production in piglets after PPMO treatment

ELISA was carried out on serum samples collected from infected piglets at 0, 6, 10 and 14 dpi, to investigate the production of anti-PRRSV antibodies. PRRSV antibodies were not detectable in the serum samples collected at 0 and 6 dpi. At 14 dpi, piglets receiving the 5UP2 PPMO at 0.5 mg/kg had an average S/P ratio under 0.4 (the cutoff value for the presence of anti-PRRSV antibodies), in contrast to the untreated group in which the average S/P ratio was 1.2 (Fig. 3). The ELISA S/P ratios for the piglets in this 0.5 mg/kg group at 10 dpi were 0.2. Although piglets receiving 0.1 mg/kg PPMO had noticeably lower S/P ratios than the untreated group at both 10 and 14 dpi, the differences were not significant. The ELISA results

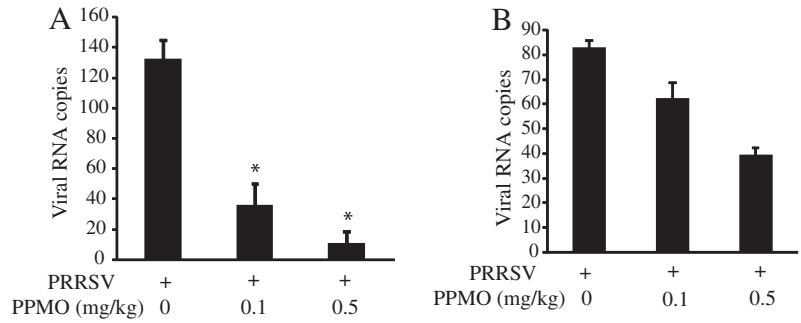

Fig. 1. PRRSV viremia detected with real-time RT-PCR. RNA was isolated from serum samples and RT-PCR was carried out with primers designed to amplify a region of PRRSV ORF7. Viral RNA copies were calculated by comparison with a plasmid-derived standard curve. (A). Average PRRSV RNA copies (×10,000) per ml on 6 dpi. Error bars show the variation among piglets in the individual groups. Significant differences between groups of PPMO-treated and untreated piglets are denoted by "*", which indicates $P < 0.01$. (B). Average PRRSV RNA copies (×1000) per ml on 10 dpi. There was no significant difference between PPMO-treated and untreated piglets.

showing that the piglets receiving PPMO at 0.5 mg/kg had lower antibody production than members of the other two groups are consistent with the viremia results.

3.5. Elevated expression of antiviral genes in porcine alveolar macrophages from PPMO-treated piglets

As PRRSV is known to interfere with the interferon-mediated antiviral response (Miller et al., 2004; Murtaugh et al., 2002) and 5UP2 treatment led to lower viremia at 6 dpi, we postulated that 5UP2-mediated inhibition of PRRSV in piglets may coincide with an elevation of the host antiviral response. Though the viremia levels in piglets in the presence or absence of 5UP2 at 14 dpi were similar, the earlier difference in viremia could have an impact on the host antiviral response. To investigate this issue, we selected a group of cellular gene products known to be involved in antiviral response and inflammation, and conducted quantitative RT-PCR to assess their expression in PAMs isolated from the piglets at 14 dpi. The expression of OAS1, RNase L, and MX1 in PAMs from piglets receiving 0.1 mg/kg PPMO 5UP2 was 3.22-, 3.27-, and 3.46-fold higher, respectively, than those from untreated piglets (Fig. 4). All of the changes were statistically significant. The expression of these three genes in PAMs from piglets of 0.5 mg/kg PPMO was not significantly different from those in untreated piglets. RNase L is an interferon-induced ribonuclease and exists in minute quantities in normal cells as a part of the body's innate immune defense (Silverman, 2007). Upon activation, RNase L destroys all RNA within the cell. OAS1 is induced by interferons and uses adenosine triphosphate in 2′-specific nucleotidyl transfer reactions to synthesize 2′,5′-oligoadenylates (2–5As), which activate latent RNase L. MX1 is also induced by interferons and is involved in host antiviral defense (Zurcher et al., 1992). Higher level expression of these antiviral genes in PAMs of piglets receiving 0.1 mg/kg PPMO treatment indicates an elevated host antiviral response.

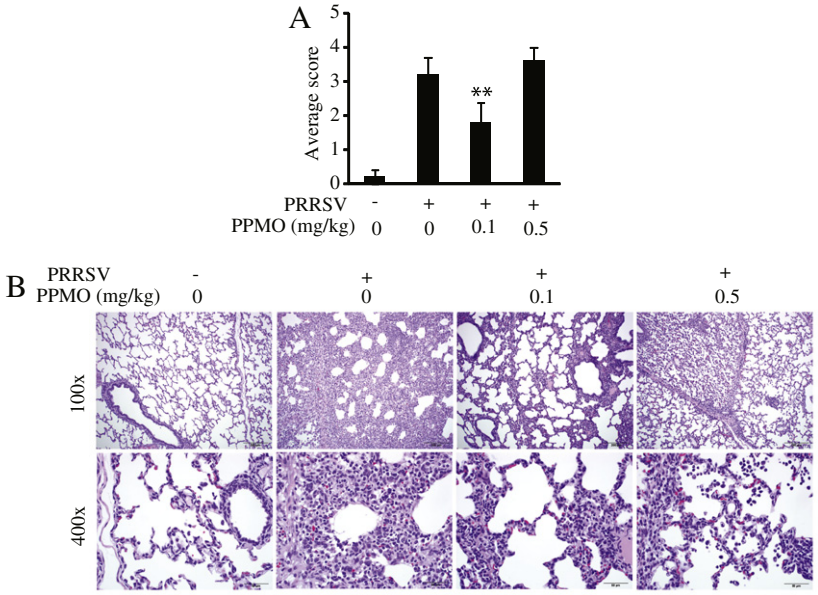

Fig. 2. Evaluation of interstitial pneumonia in PRRSV-infected piglets at 14 dpi. (A). Average scores of interstitial pneumonia in each group. Significant differences between groups of PPMO-treated and untreated piglets are denoted by "**", which indicates $P < 0.01$. Error bars show the variation among piglets in individual groups. (B). Representative histopathology images showing microscopic interstitial pneumonia observed under magnification of 100× and 400×. The left-most panel of images, from an uninfected piglet untreated with PPMO, represents a normal lung section. The second panel of images, from a PRRSV-infected piglet untreated with PPMO, shows severe interstitial pneumonia characterized by a marked thickening of the alveolar septa resulting from infiltration of macrophages, and lymphocytes. The third panel from a PRRSV-infected piglet treated with 0.1 mg/kg 5UP2 PPMO shows mild interstitial pneumonia characterized by increased numbers of macrophages and lymphocytes in alveolar septa. The fourth panel from a PRRSV-infected piglet dosed with 0.5 mg/kg 5UP2 PPMO, shows mild interstitial pneumonia. The size bars in the lower right corner of each image in the upper panel indicate 200 μm in length and the bars in the lower panel indicate 50 μm in length.

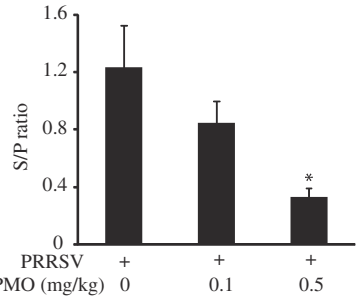

Fig. 3. Mean group ELISA S/P ratios of PRRSV antibodies in serum samples at 14 dpi. An S/P ratio above 0.4 is considered indicative for the presence of antibodies. Error bars show the variation of the S/P ratios among piglets in individual groups. Significant differences between the groups of PPMO-treated and untreated piglets are denoted by "*", which indicates $P < 0.05$.

The expression of CCL1 in PAMs from piglets receiving 0.1 mg/kg PPMO 5UP2 was 3.4-fold lower than untreated piglets. The PAMs from piglets receiving 0.5 mg/kg PPMO had CCL1 level similar to untreated piglets. Expression of CCL2 and CXCL10 was increased to more than 200- and 5-fold, respectively, in PAMs from PRRSV-infected piglets compared to non-infected piglets, but there was no significant difference among groups of PRRSV-infected piglets (data not shown). Chemokines are thought to participate in the inflammatory response by assisting in the recruitment of macrophages and lymphocytes to the site of infection (Carr et al., 1994; Miller and Krangel, 1992) and might play a role in pneumonia development in PRRSV-infected pigs. However, little change in the expression of interleukin 6, double-stranded RNA-activated protein kinase R (PKR), tumor necrosis factor-α (TNF-α), CCL17, CCL22, OAS2 and CXCL11 was detected in the PAM cells from PRRSV-infected piglets in comparison with uninfected controls

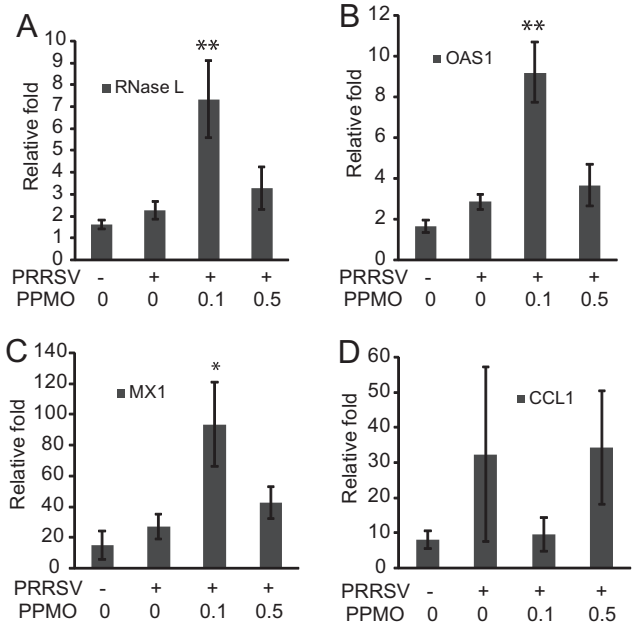

Fig. 4. Transcript levels of selected cellular genes in PAMs from piglets. Quantitative RT-PCR was conducted with primers for: RNase L (A), OAS1 (B), MX1 (C), and CCL1 (D). Average relative fold changes in comparison with PAMs from uninfected piglets are shown. Error bars show the variation among piglets in the individual groups. Significant differences between groups of PPMO-treated and untreated piglets after PRRSV infection are denoted by "*", which indicates $P < 0.05$, and "**", which indicates $P < 0.01$.

(data not shown). These results are consistent with the microscopic lesion results, reported above.

We also evaluated the levels of PRRSV genomic RNA in PAM cells isolated from the various groups of PRRSV-infected piglets at 14 dpi and found few differences (data not shown).

3.6. PPMO 5UP2 does not affect daily weight gain of piglets

To assess the effects of PPMO administration and PRRSV infection on weight gain, we weighed all piglets at 0 and 14 dpi. Average daily weight gain was 90 ± 24 and 91 ± 56 g for infected piglets receiving 0.1 and 0.5 mg/kg PPMO, respectively, and 138 ± 18 and 124 ± 32 g for the infected and uninfected piglets, respectively, receiving no PPMO. There were no significant differences between the four groups, indicating that neither PRRSV infection nor PPMO treatment affected weight gain in the piglets during the study period.

4. Discussion

This study demonstrates that intranasal delivery of PPMO 5UP2 suppressed viremia and virus-associated lung lesion development in piglets experimentally infected with PRRSV. Results from this *in vivo* study are consistent with results from previous *in vitro* work, in which a single treatment of 5UP2 protected cultured PAM cells from PRRSV-induced cell death (Patel et al., 2009).

Treatment with 5UP2 PPMO produced dose-responsive and significant reductions in viremia in the piglets at 6 dpi. However, the titer reductions at 10 dpi were not significant, indicating that PRRSV replication rebounded after initial suppression by PPMO. Perhaps if additional PPMO doses had been given at 3 or 5 dpi, viremia would have been suppressed for a longer period of time.

Infected piglets receiving 0.1 mg/kg PPMO, but not those receiving 0.5 mg/kg, had a significantly lower level of pneumonia development than untreated piglets. This result is seemingly at odds with our finding that the 0.5 mg/kg PPMO resulted in lower viremia and antibody titers than the 0.1 mg/kg dose. However, the non-dose-responsive effect of 5UP2 on lung lesion development may be due to our particular timing of sample collection for the different analyses. In VR2385-infected piglets, lung lesion development reaches a peak at around 10 dpi (Halbur et al., 1995; Halbur et al., 1996). If the infection is uncomplicated, lesions gradually resolve and are generally absent by 3–5 weeks after infection. In the 0.5 mg/kg PPMO treatment group, PRRSV replication was much reduced at 6 dpi, but increased after that, resulting in the delayed development of lung lesions. In contrast, in untreated piglets, both viral replication and lung lesion development reached a high level soon after infection. We speculate that if the necropsy had been conducted a few days earlier (e.g. 10 dpi instead of 14 dpi) or if additional PPMO doses were given at 3 or 5 dpi, a significant reduction in the severity of interstitial pneumonia lesions in the 0.5 mg/kg PPMO group would have been observed. These speculations will likely be investigated in further studies.

The transcript levels of several cellular genes in PAMs isolated from the piglets are consistent with the histopathology observations. Increased expression of OAS1, RNase L and MX1 along with lower expression of CCL1 in PAMs of piglets receiving 0.1 mg/kg PPMO is biologically congruous with a reduced development of pneumonia. The similar level of the various transcripts in PAMs from piglets receiving 0.5 mg/kg PPMO and untreated piglets is consistent with histopathology data showing a similar level of microscopic lesions between these two groups. These results also support our speculation of delayed virus replication and development of lung lesions in the 0.5 mg/kg PPMO group. The results of reduction of CCL1 in this animal test is consistent with previous re-

sults, where we noticed a significant reduction of expression of pro-inflammatory cytokine genes in 5UP2 PPMO-treated PAMs *in vitro* compared with untreated cells after PRRSV infection (Patel et al., 2009). However, in contrast to the previous *in vitro* study, here we did not detect a significant difference in expression of CCL2, CXCL10, and TNF-α between PPMO-treated and untreated PAMs after PRRSV infection *in vivo*. The discrepancy may possibly be due to the timing of PAM collection, which was at 14 days after infection in the animal test compared to only 24 h post-infection *in vitro*. In addition, this discrepancy might be smaller if the PAM collection was conducted earlier, i.e., at 6 or 10 dpi when there was lower viremia level in piglets treated with 5UP2. Future study based on the results of this experiment will address this speculation.

The serology data reflects that PPMO treatment was consistent with a reduction in anti-PRRSV antibody production, and provides an additional indication that the PPMO-mediated reduction in virus production was biologically meaningful. Despite the inconsistency of the histopathology data, the reductions in viremia, antibody production and severity of lung lesions represent encouraging data and justify further exploration of the 5UP2 PPMO as an antiviral agent against PRRSV. PPMO 5UP2 treatment did not lead to any adverse effects on piglet growth, indicating that it was well-tolerated at the doses and route of administration tested.

Novel and specific anti-PRRSV drugs are needed to complement existing strategies for PRRSV prevention and control, especially for breeding animals. Currently, PPMO is not available commercially. However, considering the high prevalence of PRRS and the economic losses it causes in the swine industry, along with the documented efficacy of PPMO in experimental systems, commercialization of PPMO appears worthy of further consideration. PPMO represents a potentially valuable agent for interference with virus spread between individual pigs during an outbreak. Although respiratory administration to individual piglets may not be commercially practical, related means of delivery, such as a directed cloud of atomized spray, may be feasible for use in swine farms. Further exploration of PPMO for PRRS control and large scale synthesis may bring the cost of PPMO to a level where it could be considered viable for swine producers. Overall, the results here suggest that 5UP2 PPMO may be a candidate antiviral to address PRRSV infection of pigs.

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References

Abes, S., Moulton, H.M., Clair, P., Prevot, P., Youngblood, D.S., Wu, R.P., Iversen, P.L., Lebleu, B., 2006. Vectorization of morpholino oligomers by the (R-Ahx-R)4 peptide allows efficient splicing correction in the absence of endosomolytic agents. J. Control. Release 116, 304–313.

Benfield, D.A., Nelson, E., Collins, J.E., Harris, L., Goyal, S.M., Bobinson, D., Christianson, W.T., Morrison, R.B., Gorcyca, D., Chladek, D., 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J. Vet. Diagn. Invest. 4, 127–133.

Carr, M.W., Roth, S.J., Luther, E., Rose, S.S., Springer, T.A., 1994. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. Proc.Natl. Acad. Sci. USA 91, 3652–3656.

Conzelmann, K.K., Visser, N., Van Woensel, P., Thiel, H.J., 1993. Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. Virology 193, 329–339.

Eide, K., Moerdyk-Schauwecker, M., Stein, D.A., Bildfell, R., Koelle, D.M., Jin, L., 2010. Reduction of herpes simplex virus type-2 replication in cell cultures and in rodent models with peptide-conjugated morpholino oligomers. Antivir. Ther. 15, 1141–1149.

Gabriel, G., Nordmann, A., Stein, D.A., Iversen, P.L., Klenk, H.D., 2008. Morpholino oligomers targeting the PB1 and NP genes enhance the survival of mice infected with highly pathogenic influenza A H7N7 virus. J. Gen. Virol. 89, 939–948.

Halbur, P.G., Andrews, J.J., Huffman, E.L., Paul, P.S., Meng, X.J., Niyo, Y., 1994. Development of a streptavidin-biotin immunoperoxidase procedure for the detection of porcine reproductive and respiratory syndrome virus antigen in porcine lung. J. Vet. Diagn. Invest. 6, 254–257.

Halbur, P.G., Paul, P.S., Frey, M.L., Landgraf, J., Eernisse, K., Meng, X.J., Lum, M.A., Andrews, J.J., Rathje, J.A., 1995. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet. Pathol. 32, 648–660.

Halbur, P.G., Paul, P.S., Meng, X.J., Lum, M.A., Andrews, J.J., Rathje, J.A., 1996. Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. J. Vet. Diagn. Invest. 8, 11–20.

Han, X., Fan, S., Patel, D., Zhang, Y.J., 2009. Enhanced inhibition of porcine reproductive and respiratory syndrome virus replication by combination of morpholino oligomers. Antiviral Res. 82, 59–66.

Hudziak, R.M., Barofsky, E., Barofsky, D.F., Weller, D.L., Huang, S.B., Weller, D.D., 1996. Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation. Antisense Nucleic Acid Drug Dev. 6, 267–272.

Keffaber, K.K., 1989. Reproductive failure of unknown etiology. Am. Assoc. Swine Pract. Newsletter 1, 1–9.

Kim, H.S., Kwang, J., Yoon, I.J., Joo, H.S., Frey, M.L., 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. Arch. Virol. 133, 477–483.

Lai, S.H., Stein, D.A., Guerrero-Plata, A., Liao, S.L., Ivanciuc, T., Hong, C., Iversen, P.L., Casola, A., Garofalo, R.P., 2008. Inhibition of respiratory syncytial virus infections with morpholino oligomers in cell cultures and in mice. Mol. Ther. 16, 1120–1128.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402–408.

Loula, T., 1991. Mystery pig disease: an update for the practitioner. Agri-Practice 12, 23–34.

Lupfer, C., Stein, D.A., Mourich, D.V., Tepper, S.E., Iversen, P.L., Pastey, M., 2008. Inhibition of influenza A H3N8 virus infections in mice by morpholino oligomers. Arch. Virol. 153, 929–937.

Meng, X.J., Paul, P.S., Halbur, P.G., 1994. Molecular cloning and nucleotide sequencing of the 3′ terminal genomic RNA of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 75, 1795–1801.

Meng, X.J., Paul, P.S., Halbur, P.G., Lum, M.A., 1995. Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the USA and Europe. Arch. Virol. 140, 745–755.

Meulenberg, J.J., Hulst, M.M., de Meijer, E.J., Moonen, P.L., den Besten, A., de Kluyver, E.P., Wensvoort, G., Moormann, R.J., 1993a. Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology 192, 62–72.

Meulenberg, J.J., de Meijer, E.J., Moormann, R.J., 1993b. Subgenomic RNAs of Lelystad virus contain a conserved leader-body junction sequence. J. Gen. Virol. 74, 1697–1701.

Miller, M.D., Krangel, M.S., 1992. The human cytokine I-309 is a monocyte chemoattractant. Proc. Natl. Acad. Sci. USA 89, 2950–2954.

Miller, L.C., Laegreid, W.W., Bono, J.L., Chitko-McKown, C.G., Fox, J.M., 2004. Interferon type I response in porcine reproductive and respiratory syndrome virus-infected MARC-145 cells. Arch. Virol. 149, 2453–2463.

Moerdyk-Schauwecker, M., Stein, D.A., Eide, K., Blouch, R.E., Bildfell, R., Iversen, P., Jin, L., 2009. Inhibition of HSV-1 ocular infection with morpholino oligomers targeting ICP0 and ICP27. Antiviral Res. 84, 131–141.

Moulton, H.M., Nelson, M.H., Hatlevig, S.A., Reddy, M.T., Iversen, P.L., 2004. Cellular uptake of antisense morpholino oligomers conjugated to arginine-rich peptides. Bioconjug. Chem. 15, 290–299.

Murtaugh, M.P., Xiao, Z., Zuckermann, F., 2002. Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. Viral Immunol. 15, 533–547.

Nelson, M.H., Stein, D.A., Kroeker, A.D., Hatlevig, S.A., Iversen, P.L., Moulton, H.M., 2005. Arginine-rich peptide conjugation to morpholino oligomers: effects on antisense activity and specificity. Bioconjug. Chem. 16, 959–966.

Neumann, E.J., Kliebenstein, J.B., Johnson, C.D., Mabry, J.W., Bush, E.J., Seitzinger, A.H., Green, A.L., Zimmerman, J.J., 2005. Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. J. Am. Vet. Med. Assoc. 227, 385–392.

Opriessnig, T., Halbur, P.G., Yoon, K.J., Pogranichniy, R.M., Harmon, K.M., Evans, R., Key, K.F., Pallares, F.J., Thomas, P., Meng, X.J., 2002. Comparison of molecular and biological characteristics of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (ingelvac PRRS MLV), the parent strain of the vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. J. Virol. 76, 11837–11844.

Patel, D., Opriessnig, T., Stein, D.A., Halbur, P.G., Meng, X.J., Iversen, P.L., Zhang, Y.J., 2008. Peptide-conjugated morpholino oligomers inhibit porcine reproductive and respiratory syndrome virus replication. Antiviral Res. 77, 95–107.

Patel, D., Stein, D.A., Zhang, Y.J., 2009. Morpholino oligomer-mediated protection of porcine pulmonary alveolar macrophages from arterivirus-induced cell death. Antivir. Ther. 14, 899–909.

Silverman, R.H., 2007. Viral encounters with 2′, 5′-oligoadenylate synthetase and RNase L during the interferon antiviral response. J. Virol. 81, 12720–12729.

Stein, D.A., 2008. Inhibition of RNA virus infections with peptide-conjugated morpholino oligomers. Curr. Pharm. Des. 14, 2619–2634.

Summerton, J., 1999. Morpholino antisense oligomers: the case for an RNase H-independent structural type. Biochim. Biophys. Acta 1489, 141–158.

Summerton, J., Weller, D., 1997. Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev. 7, 187–195.

Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., et al., 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Q. 13, 121–130.

Yoon, I.J., Joo, H.S., Christianson, W.T., Kim, H.S., Collins, J.E., Carlson, J.H., Dee, S.A., 1992. Isolation of a cytopathic virus from weak pigs on farms with a history of swine infertility and respiratory syndrome. J. Vet. Diagn. Invest. 4, 139–143.

Youngblood, D.S., Hatlevig, S.A., Hassinger, J.N., Iversen, P.L., Moulton, H.M., 2007. Stability of cell-penetrating peptide-morpholino oligomer conjugates in human serum and in cells. Bioconjug. Chem. 18, 50–60.

Zhang, Y.J., Stein, D.A., Fan, S.M., Wang, K.Y., Kroeker, A.D., Meng, X.J., Iversen, P.L., Matson, D.O., 2006. Suppression of porcine reproductive and respiratory syndrome virus replication by morpholino antisense oligomers. Vet. Microbiol. 117, 117–129.

Zhang, Y.J., Wang, K.Y., Stein, D.A., Patel, D., Watkins, R., Moulton, H.M., Iversen, P.L., Matson, D.O., 2007. Inhibition of replication and transcription activator and latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus by morpholino oligomers. Antiviral Res. 73, 12–23.

Zhang, Y.J., Bonaparte, R.S., Patel, D., Stein, D.A., Iversen, P.L., 2008. Blockade of viral interleukin-6 expression of Kaposi's sarcoma-associated herpesvirus. Mol. Cancer Ther. 7, 712–720.

Zurcher, T., Pavlovic, J., Staeheli, P., 1992. Nuclear localization of mouse Mx1 protein is necessary for inhibition of influenza virus. J. Virol. 66, 5059–5066.