

# Peptide-conjugated morpholino oligomers inhibit porcine reproductive and respiratory syndrome virus replication

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## Abstract

Porcine reproductive and respiratory syndrome (PRRS) has been devastating the global swine industry for more than a decade, and current strategies to control PRRS are inadequate. In this study we characterized the inhibition of PRRS virus (PRRSV) replication by antisense phosphorodiamidate morpholino oligomers (PMO). Of 12 peptide-conjugated PMO (PPMO), four were found to be highly effective at inhibiting PRRSV replication in cell culture in a dose-dependant and sequence-specific manner. PPMO 5UP2 and 5HP are complementary to sequence in the 5' end of the PRRSV genome, and 6P1 and 7P1 to sequence in the translation initiation regions of ORF6 and ORF7, respectively. Treatment of cells with 5UP2 or 5HP caused a 4.5 log<sub>10</sub> reduction in PRRSV yield, compared to a control PPMO. Combination of 6P1 and 7P1 led to higher level reduction than 6P1 or 7P1 alone. 5UP2, 5HP, and a combination of 6P1 and 7P1 inhibited PRRSV replication in porcine alveolar macrophages and protected the cells from PRRSV-induced cytopathic effect. Northern blot and real-time RT-PCR results demonstrated that the effective PPMO led to a reduction of PRRSV RNA level. 5UP2 and 5HP inhibited virus replication of 10 other strains of PRRSV. Results from this study suggest potential applications of PPMO for PRRS control.

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## 1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) causes an estimated US\$ 560 million in losses per year to the swine industry in the USA (Neumann et al., 2005). The clinical manifestations of PRRS includes severe reproductive failure, post-weaning pneumonia, growth reduction, and increased mortality (Keffaber, 1989; Loula, 1991). The causative agent of this disease is PRRSV, an enveloped, single-stranded and positive-sense RNA virus (J.J. Meulenber et al., 1993). PRRSV is a member of the family *Arteriviridae*, which includes equine arteritis virus (EAV), lactate dehydrogenase-elevating virus

(LDV) of mice and simian hemorrhagic fever virus (SHFV). There are two PRRSV genotypes, Europe and North America, which are defined based on antigenic and genetic characteristics (Meng et al., 1995; J.J. Meulenber et al., 1993). PRRSV was first identified in 1991 in Europe (Wensvoort et al., 1991) and in 1992 in USA (Benfield et al., 1992). PRRSV can replicate *in vitro* (Bautista et al., 1993) in MA-104, African green monkey kidney cells, and cells derived thereof, such as CL2621, MARC145, and CRL11171 (Benfield et al., 1992; Kim et al., 1993; Meng et al., 1994), and/or porcine alveolar macrophages (PAM), which are the primary target cells *in vivo* (Yoon et al., 1992).

Current management strategies and commercial live or auto-genously killed or live vaccines have been generally proven to be inadequate for long-term control of PRRS. Modified live attenuated vaccines (MLV) have been commercially available

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for years; however, there is evidence of reversion to virulence of at least one of the current vaccine strains following use in pigs (Opriessnig et al., 2002). Therefore, alternative strategies are needed for effective PRRS control. This study explored the inhibition of PRRSV propagation in cell cultures by peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) specific for PRRSV sequences.

PMO are structurally similar to single-stranded DNA in that each subunit includes a purine or pyrimidine base. In PMO each base is joined to a novel backbone consisting of one morpholine ring and phosphorodiamidate linkage per subunit (Summerton, 1999; Summerton and Weller, 1997). PMO are uncharged, water-soluble, and highly resistant to nuclease degradation (Hudziak et al., 1996). PMO bind to target mRNA by Watson–Crick base pairing and exert an antisense effect by preventing access to critical segments of RNA sequence, such as a translation initiation site, through steric blockade. This is a distinctly different process from the RNase H-dependent mechanism induced by the often-used antisense structural type phosphorothioate DNA (Summerton, 1999). A number of studies have shown that PMO can effectively and specifically block translation of target mRNA *in vitro* and *in vivo* via intravenous, intraperitoneal, subcutaneous, transdermal or oral administration (Arora et al., 2002a,b; Brent and Drapeau, 2002; Hudziak et al., 2000; Qin et al., 2000; Stein et al., 2001; Taylor et al., 1996). It has been shown that PMO conjugated to short arginine-rich cell penetrating peptides have a significantly higher efficiency of delivery into cultured cells than do non-conjugated PMO (Moulton et al., 2004; Deas et al., 2005). PPMO were found to be fairly stable in cells and human serum for at least 24 h (Youngblood et al., 2007). Sequence-specific antiviral efficacy of PPMO in cell cultures has been documented against SARS coronavirus (Neumann et al., 2005), EAV (van den Born et al., 2005), flaviviruses (Deas et al., 2005; Kinney et al., 2005), Influenza A virus (Ge et al., 2006), and Kaposi's sarcoma-associated herpesvirus (Zhang et al., 2007). Recently PPMO were also applied *in vivo* and protected animals from challenge with Ebola Virus (Enterlein et al., 2006), Coxsackievirus B3 (Yuan et al., 2006), and murine coronaviruses (Burrer et al., 2007).

PRRSV has an RNA genome approximately 15-kb in size, which consists of 5' untranslated region (UTR), nine open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, and ORFs 3 through 7) and 3' UTR (Meng et al., 1994; J.J. Meulenberg et al., 1993). Two large ORFs, 1a and 1b, together occupy over 11 kb and encode 13 non-structural proteins, which are likely involved in genome replication and transcription (J.J. Meulenberg et al., 1993). The remaining ORFs, 2a through 7 encode six membrane-associated proteins (GP2, E, GP3, GP4, GP5, and M) and nucleocapsid (N) protein (Meulenberg et al., 1995). In PRRSV-infected cells, a set of six or seven nested viral subgenomic RNA is formed (Conzelmann et al., 1993; J.J.M. Meulenberg et al., 1993). All of the subgenomic RNA have an identical 5'-leader sequence derived from the 5' end of genomic RNA, as well as an identical 3' terminal sequence preceding poly-(A) tails of variable length. The generation of co-terminal mRNA species is believed to be accomplished through a process known as dis-

continuous subgenomic mRNA synthesis (Sawicki and Sawicki, 1995; van Marle et al., 1999). The 5' end of each subgenomic mRNA of North American strains contains a common leader sequence of 190 bases (Nelsen et al., 1999), whereas in Lelystad virus, the prototype strain of the European PRRSV genotype, the leader is 221 bases (J.J. Meulenberg et al., 1993). The 3' UTR of North American and European PRRSV strains are 150 and 120 bases, respectively, excluding the poly-(A) tail, and is common to all of their subgenomic mRNA. PRRSV subgenomic RNAs are polycistronic in structure, but it is believed that only the first open reading frame (ORF) of each subgenomic RNA is translated into a viral protein (Meng et al., 1996b).

In a previous study (Zhang et al., 2006), we tested six PPMO and found one of them (5UP1), designed to target the 5' terminal region of the PRRSV genome, to be a highly effective inhibitor of PRRSV replication in a sequence-specific and dose-dependent manner. Considering the lengthy genome and sequence heterogeneity of PRRSV strains, we sought to evaluate PPMO that target other sites of the genome and that have broad reactivity against different strains of PRRSV, and also to further refine our evaluation of efficacy and PPMO mechanism of action, in the hopes of defining a potential strategy for addressing PRRSV with PPMO technology.

In this study, we evaluated a dozen PPMO targeting a variety of different sites in PRRSV genomic, subgenomic and negative-sense RNA. The peptide component of the PPMO used in this study was recently developed, and possesses improved characteristics compared to the peptide used in our previous PPMO study. We found that it was possible to obtain a greater-than-additive effect by using a combination of two PPMO. The effects of PPMO on PRRSV RNA synthesis were also assessed in an attempt to gain insight into the mechanism of PPMO-mediated inhibition of PRRSV replication. PPMO are also shown in this study to inhibit PRRSV replication in PAM cultures, the primary target cells for PRRSV infection in pigs.

## 2. Materials and methods

### 2.1. Cells and viruses

Cell line ATCC CRL11171 (Meng et al., 1996a) was maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS). PRRSV strains ATCC VR2385 (Meng et al., 1996a), Lelystad (J.J. Meulenberg et al., 1993), and NVSL97-7895 (NVSL, Ames, IA) were used to inoculate CRL11171 cells at 0.5 multiplicity of infection (MOI) for PPMO testing. Other PRRSV strains used in this study include FL-12, 11604, 16138, 16224B, 17041, 14680, 12773, and 13909 (kindly provided by Dr. Fernando Osorio, University of Nebraska-Lincoln), and Ingelvac MLV (kindly provided by Dr. Kay S. Faaberg, University of Minnesota). For virus titration, a series of 10-fold dilutions of virus were added to monolayer CRL11171 cells in a 96-well plate. The degree of cytopathic effect (CPE), characterized by cell rounding, clumping and detachment, was assessed microscopically 48 h after PRRSV inoculation, in comparison with mock-infected cells. Tissue culture infectious dose (TCID<sub>50</sub>) per milliliter was calculated based on CPE develop-

ment according to the method of Reed and Muench, as described previously (Zhang et al., 2006).

For PAM culture, broncho-alveolar lavage was collected from 4 to 5 weeks old PRRSV-negative piglets, as described previously (Shibata et al., 1997). Broncho-alveolar lavage fluid was centrifuged at  $400 \times g$  for 7 min, and cell pellets resuspended in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 10  $\mu$ g/ml streptomycin sulfate and 50  $\mu$ g/ml gentamycin. The cells were plated at a density of  $5 \times 10^5$  cells/well in 24-well cell culture plates. PAM were incubated for 24 h at 37 °C in a humidified 5% CO<sub>2</sub> incubator and washed once with plain RPMI-1640 media before virus inoculation.

## 2.2. Immunofluorescence assay (IFA)

IFA was carried out as reported previously (Zhang et al., 1998) with an N-specific monoclonal antibody SDOW17 (NVSL, Ames, IA) (Nelson et al., 1993). Specific reactions between SDOW17 and N protein were detected with goat anti-mouse IgG-fluorescein isothiocyanate (FITC) conjugate (Sigma, St Louis, MO) and observed with fluorescence microscopy.

For PAM cells, IFA was performed by fixation of PAM cells with 1% paraformaldehyde and permeabilization with 0.5% Triton X-100, followed by incubation with antibody SDOW17.

## 2.3. PPMO synthesis

PMO were synthesized at AVI BioPharma Inc. (Corvallis, OR) by methods previously described (Summerton and Weller, 1997). The peptide NH<sub>2</sub>-(RXR)<sub>4</sub>XB-COOH (where R = Arginine, X = 6-aminohexanoic acid, and B =  $\beta$ -alanine) was covalently conjugated at the 5' end of each PMO (Fig. 1A). The conjugation, purification, and analysis of PPMO were similar to the methods described elsewhere (Abes et al., 2006; Moulton et al., 2004). A random sequence PPMO (named

'CP1') having little agreement with PRRSV or primate mRNA sequences was used as control for non-sequence-specific activity of the PPMO chemistry. PPMO were dissolved in water at a concentration of 2 mM and stored at 4 °C. For experiments, PPMO were diluted to desired concentrations in DMEM.

## 2.4. PPMO treatment of CRL11171 cells

CRL11171 cells were seeded in 12-well plates at  $5 \times 10^5$  cells per well and grown overnight to near confluency. Treatment of CRL11171 cells was conducted as previously described (Zhang et al., 2006). Briefly, cells were inoculated with virus at 0.5 MOI for 2 h at 37 °C. After inoculum removal, PPMO diluted in DMEM was added to the cells and incubated for 4 h at 37 °C. DMEM medium without PPMO was included as a mock treatment control and PPMO CP1 as a negative control. After removal of PPMO, maintenance medium (DMEM supplemented with 2% FBS) was added. The cells were then incubated for 48 h at 37 °C, after which both supernatant and cells were harvested for further analysis. For experiments using PPMO combination treatments, each of the two constituent PPMO were present at equal molar concentration. For the cross strain inhibitory assay, similar procedures as described above were used, except that different PRRSV strains were used.

## 2.5. Application of PPMO to PAM culture

Macrophages were incubated in 24-well cell culture plates for 24 h prior to treatment. Cells were inoculated with PRRSV strain VR2385 at 0.5 MOI and incubated for 2 h. The cells were washed once with plain RPMI medium before addition of PPMO. The PPMO were diluted in plain RPMI medium, added to the cells and incubated for 4 h at 37 °C. The PPMO solution was removed and culture medium (RPMI supplemented with 10% FBS) was added. The cells were cultured and observed for CPE development.

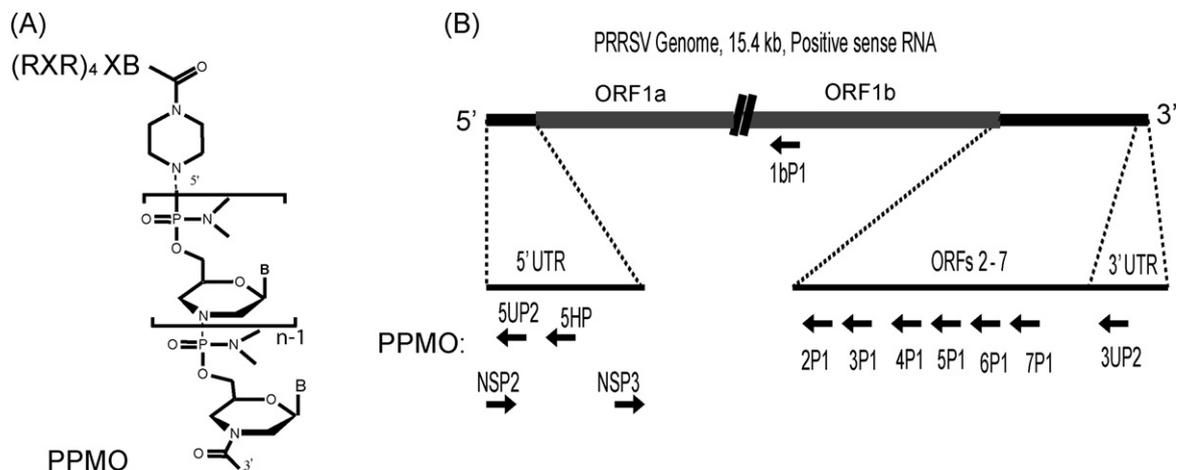


Fig. 1. Structure of PPMO and schematic illustration of PPMO target locations in the PRRSV genome. (A) PPMO structure. A morpholine ring and a phosphoramidate linkage in PMO replace the deoxyribose and phosphodiester linkage of DNA, respectively. "B" represents the bases A, G, C, or T. An arginine-rich peptide (RXR)<sub>4</sub>XB is covalently conjugated to the 5' end of each PMO. (B) Positions of PPMO target sites in PRRSV genomic RNA. The arrows indicate the 5' to 3' orientation of the PPMO in relation to the PRRSV RNA genome.

## 2.6. RNA isolation

Total RNA was isolated from PRRSV-infected CRL11171 cells by TRIzol<sup>®</sup> Reagent (Invitrogen, Carlsbad, CA). PPMO-treated CRL11171 cells (same procedure as above) were grown in 12-well cell culture plates and harvested at 24 h p.i. by direct lysis in TRIzol<sup>®</sup> Reagent after removal of culture supernatant. Cells treated with CP1 or mock-treated were included as controls. RNA was quantified by  $\mu$ Quant<sup>™</sup> Universal Microplate spectrophotometer (BioTek Instruments, Winooski, Vermont) and stored at  $-80^{\circ}\text{C}$  for further analysis.

## 2.7. Northern blot analysis

RNA samples (0.5  $\mu\text{g}$ ) were denatured at  $70^{\circ}\text{C}$  for 10 min and immediately placed on ice for 5 min. The denatured RNA was separated at 100 V for 3 h in 1% agarose gel containing formaldehyde. The separated RNA were transferred onto nylon membrane and hybridized with a DNA probe derived from PRRSV ORF7 sequence with the PCR primers SB7F3, 5'-GCGGA TCCCA AATAA CACCG GCAAG C-3' and SB7R3, 5'-CGTCT AGATG CCAGC CCATC ATGCT GAG-3'. The probe was labeled with DIG-11-dUTP by PCR DIG Probe Synthesis Kit (Roche Diagnostics, Indianapolis, IN). The blotted membrane was incubated with Anti-Digoxigenin-alkaline phosphates for 30 min, CSPD chemiluminescent substrate (Roche) for 20 min, and then exposed on X-ray film.

## 2.8. Real-time RT-PCR

For quantitative RT-PCR analysis, RNA was first treated with RNase-free DNase (Promega, Madison, WI) to remove carryover DNA from the RNA isolation procedure. Reverse transcription was carried out using SuperScript<sup>™</sup> III First-Strand Synthesis System and random hexamers (Invitrogen). Real-time PCR primers were the same as reported previously (Zhang et al., 2006). A fragment of 860 bases from the 5' end of the PRRSV genome was cloned into pcDNA3 vector, and used as template to generate standard curves for the real-time PCR, which was conducted in Chromo 4<sup>™</sup> Four-Color Real-Time System (Bio-Rad Laboratories, Hercules, CA) with iQ SYBR Green Supermix (Bio-Rad). Transcripts of  $\beta$ -actin were also amplified from the same samples in order to assure normalized quantitative RT-PCR detection of PRRSV RNA from the cells.

## 2.9. Cell-free luciferase reporter assay

Pairs of DNA oligonucleotides corresponding to PPMO target sequence in PRRSV were duplexed and subcloned upstream of luciferase coding sequence in a T7 promoter-containing reporter plasmid, pCiNeoLuc, as described previously (Zhang et al., 2006). DNA sequencing was conducted to confirm the presence of desired sequences in the resulting plasmids. CP1 was used as a negative control in the reporter assay. Each plasmid DNA was linearized downstream of the luciferase sequence. *In vitro* transcription was conducted with the T7 RiboMAX<sup>™</sup> Express Large Scale RNA Production System (Promega). *In*

*vitro* translation was carried out with Rabbit Reticulocyte Lysate Translation System (Promega). Bright-Glo<sup>™</sup> Luciferase Assay System (Promega) was used to detect luciferase yield. Luminescence signals were measured with VICTOR3<sup>™</sup> Multilabel Counter (Perkin-Elmer Life and Analytical Sciences, Wellesley, MA).

## 2.10. Cell viability assay

The viability of CRL11171 and PAM cells after PPMO treatment was determined with CellTiter-Glo<sup>®</sup> Luminescent Cell Viability Assay (Promega). Briefly, Cells were treated with PPMO under conditions similar to those described above in "PPMO treatment of CRL11171 cells". Mock-treated cells were included for comparison. CellTiter-Glo reagent was added and incubated for 10 min at room temperature. The luminescence signal was measured with VICTOR3<sup>™</sup> Multilabel Counter. Relative percentages of luminescence intensity were calculated by comparison to mock-treated controls.

## 2.11. Statistical analysis

The Student's *t*-test was used to assess the significance of differences of viral yield or RNA level between the groups of PPMO-treated cells. A two-tailed *P*-value of less than 0.05 was considered significant.

## 3. Results

### 3.1. PPMO design

PRRSV genomic sequences were retrieved from the GenBank database. Parallel sequence alignments of PRRSV strains from each of the two major genotypes show that the 5' and 3' UTRs, and the translation initiation regions of all ORFs, within each genotype, are highly conserved. These regions likely participate in essential events of the viral life cycle (Tan et al., 2001; Verheije et al., 2002), making them rational sites for anti-sense PPMO targeting. For this study, 12 PPMO were designed against PRRSV VR2385, a virulent strain of the North American genotype. PPMO sequences and target site locations in the PRRSV genome are specified in Table 1 and depicted schematically in Fig. 1B. The 5' terminal region has proven to be a productive PPMO target region in a number of positive-strand RNA viruses (Burrer et al., 2007; Deas et al., 2005; van den Born et al., 2005; Zhang et al., 2006). PPMO 5UP2 and 5HP were designed to basepair to the 5' end region of PRRSV RNA genome, in an attempt to interfere with translation of viral RNA replicases. 5HP is complementary to the hairpin loop region, which was predicted from secondary structure analysis (41). PPMO complementary to negative-sense EAV RNA inhibited EAV replication in cell culture (van den Born et al., 2005). NSP2 is complementary to the 3' end of negative-sense PRRSV RNA, and was designed to interfere with the production of positive-sense viral RNA. NSP3 is complementary to negative-sense PRRSV RNA in the transcription regulatory sequence (TRS) region, in an attempt to interfere with synthesis and/or transla-

Table 1  
PPMO<sup>a</sup> and their target sites in PRRSV RNA genome<sup>b</sup>

PPMO name	PPMO sequence (5'–3')	Nucleotide position of PPMO target in PRRSV RNA	PPMO orientation	Target region in PRRSV RNA
5UP2	CAAGGCATAGACCAACACC	9–28	Antisense	5' terminus of genomic RNA
5HP	GTTTTGGGCTGTGCCAATGG	54–74	Antisense	5' UTR of genomic RNA
NSP2	GACGTATAGGTGTGGCTCTAT	1–22	Sense	3' terminus of negative-sense RNA
NSP3	CCTTTAACCATGTCTGGGAT	180–199	Sense	TRS-region
1bP1	CAACTCGAACCCGGAGGGCATG	7982–8003	Antisense	ORF1b <sup>d</sup>
2P1 <sup>c</sup>	CCCATTTCATCTCAATTCAGGC	11749–11770	Antisense	ORF2 <sup>d</sup>
3P1	ACAGCTATTAACCATGCTG	12379–12398	Antisense	ORF3 <sup>d</sup>
4P1	GGGACGAAGCCATTCTAGGT	12922–12941	Antisense	ORF4 <sup>d</sup>
5P1	CCCAACATACTTAAACATTC	13464–13483	Antisense	ORF5 <sup>d</sup>
6P1	GGACGACTCCATTGTTCCGC	14055–14074	Antisense	ORF6 <sup>d</sup>
7P1	GGCATATTTAAACAAGGTTTACC	14560–14581	Antisense	ORF7 <sup>d</sup>
3UP2	CCGTGTGGTTCTCGCCAATTA	15070–15091	Antisense	3' UTR
CP1	GATATACACAACACCCAATT	None	None	Random sequence

<sup>a</sup> The peptide (RXR)<sub>4</sub>XB is conjugated to the 5' end of all the PMO in this study.

<sup>b</sup> PMO design was based on PRRSV VR2385 genomic RNA (GenBank accession number U03040, however, sequence of the 5' end of the genome is unpublished).

<sup>c</sup> The underlined nucleotides correspond to the AUG translation initiation codons of PRRSV ORFs.

<sup>d</sup> Translation initiation regions of PRRSV ORFs.

tion of the viral subgenomic RNA. 3UP2 is complementary to the 3' end of PRRSV RNA genome, in an attempt to interfere with negative-strand RNA synthesis. PPMO 1bP1, 2P1, 3P1, 4P1, 5P1, 6P1, and 7P1 are complementary to the translation initiation regions of ORFs 1b, 2, 3, 4, 5, 6, and 7, respectively, to attempt blocking the translation of these ORFs. PPMO 5UP1, which is complementary to nucleotide 3–23 of PRRSV 5' terminal sequence and was previously shown to be highly effective (Zhang et al., 2006) and CP1 were included as positive and negative controls, respectively.

### 3.2. Evaluation of PPMO against PRRSV in CRL11171 cells

A virulent PRRSV strain, VR2385, was used to evaluate the ability of the PPMO to inhibit PRRSV replication in CRL11171 cells. At 0.5 MOI, VR2385 reached peak replication in these cells at approximately 48 h post-inoculation. In initial experiments, PPMO were applied to monolayer cells at a final concentration of 16  $\mu$ M in DMEM and incubated for 4 h immediately after the virus inoculation period. The cells were then observed daily for CPE development and supernatants were collected at 2 days p.i. for virus titration in CRL11171 cells to determine PRRSV yield. Of the 12 PPMO tested, 5UP2 and 5HP were found to be highly effective at inhibiting PRRSV CPE development compared to CP1- or mock-treatment. The cells treated with 6P1 and 7P1 also showed less severe CPE than the controls. The other PPMO had no effect on preventing CPE development.

Titration of PRRSV in cell culture supernatants showed that treatment of CRL11171 cells with PPMO 5UP2 or 5HP caused a 4.5 log<sub>10</sub> TCID<sub>50</sub> reduction in PRRSV yield in comparison to CP1 (Fig. 2A). Treatment of the cells with 6P1 and 7P1 led to a reduction in PRRSV yield of 2 and 3 log<sub>10</sub> TCID<sub>50</sub>, respectively, compared to CP1. None of the other PPMO reduced PRRSV yield significantly. PPMO 5UP2, 5HP, 6P1, and 7P1 were selected for further characterization in this study.

Confirmation of the CPE observations and PRRSV yield titration was obtained by IFA on CRL11171 cells after virus inoculation and PPMO treatment (Fig. 2B). IFA using a monoclonal antibody against the PRRSV N-protein showed that treatment of cells with 5UP2 led to an absence of PRRSV-positive cells. In cells treated with 5HP, a few fluorescent-positive cells were observed. In cells treated with 6P1 and 7P1, some PRRSV-positive cells were observed, but far fewer than those treated with CP1 and 3UP2. These results indicate that the presence of 5UP2, 5HP, 6P1, and 7P1 inhibited PRRSV replication in VR2385-inoculated cells.

### 3.3. Inhibition of PRRSV replication in a dose-dependent manner

PPMO 5UP2, 5HP, 6P1, 7P1, and CP1 were further tested at doses between 1 and 32  $\mu$ M for inhibition of VR2385 replication in CRL11171 cells (Fig. 3). CP1 had little effect on virus replication in comparison with the mock treatment control. Each of the other four antisense PPMO reduced virus yield in a dose dependent manner (Fig. 3). Treatment of cells with 5UP2 at 4  $\mu$ M or 5HP at 8  $\mu$ M concentration led to 4.5 log<sub>10</sub> TCID<sub>50</sub> reduction in PRRSV yield compared to CP1. PPMO 6P1 or 7P1 at 32  $\mu$ M concentration led to reduction in PRRSV yield to below the detection level of this assay.

### 3.4. Cell viability assay

To assess the cytotoxicity of the PPMO used in this study, cell viability assays were conducted on CRL11171 and PAM cells treated with PPMO at 16  $\mu$ M. The experiment was repeated twice, each with three replicates. In comparison with mock treatment control, the average relative percentages of cell viability for CRL11171 cells after treatment was 94% (5UP2), 97% (5HP), 97% (6P1), 97% (7P1), and 93% (CP1). The average percentages of viable PAM cells after PPMO treatment were 93% (CP1), 94% (5UP2), 93% (5HP), and 95% (6P1 + 7P1). These results

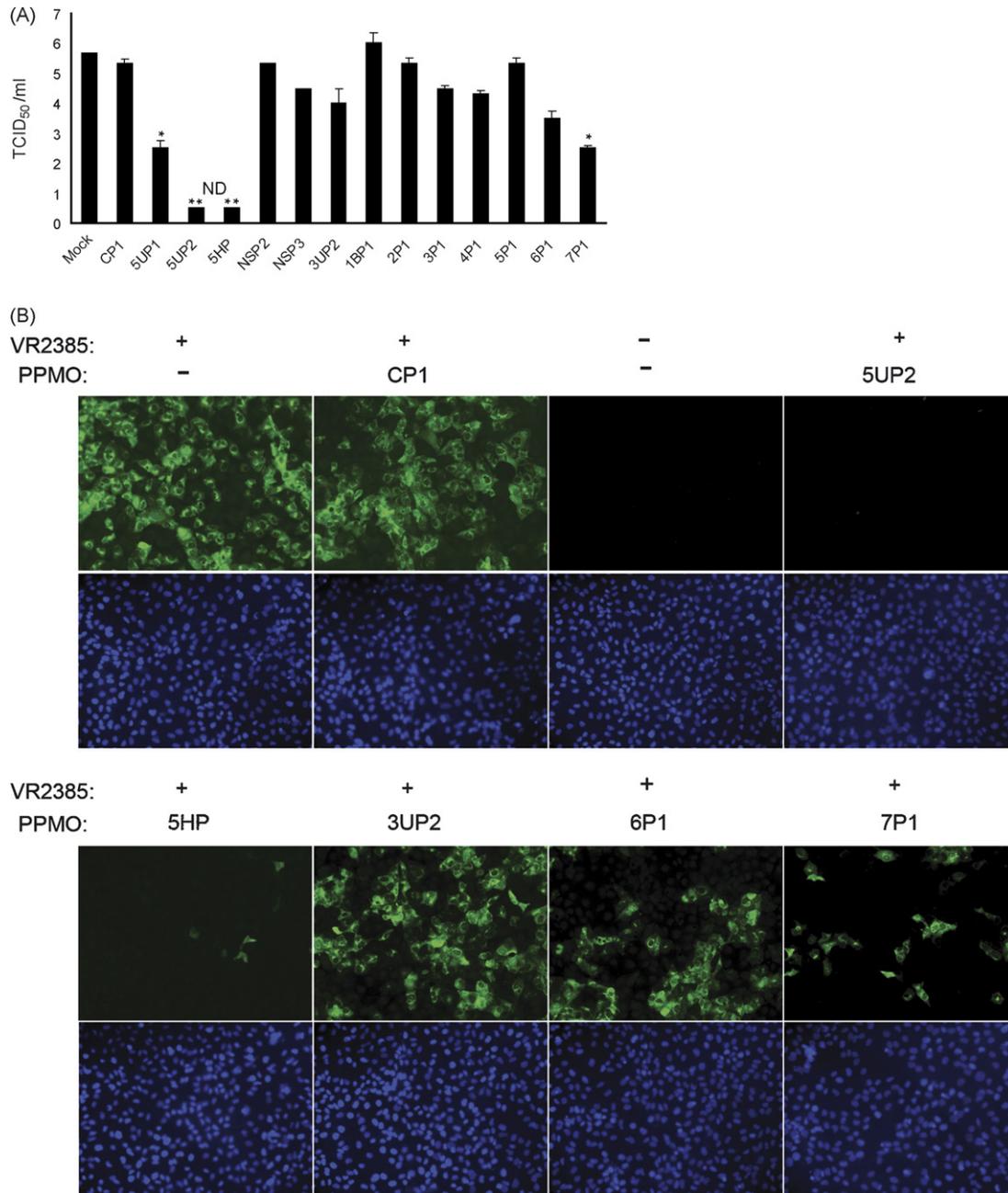


Fig. 2. PPMO-mediated inhibition of PRRSV replication in infected CRL11171 cells. (A) Titration of virus yield from CRL11171 cells after PRRSV infection and indicated PPMO treatment. Virus titers are shown as TCID<sub>50</sub> (log<sub>10</sub>/ml). “Mock” samples is from cells receiving virus inoculation but no PPMO treatment. Statistical significance of difference in viral yields between PPMO and mock treatments: \* $P < 0.05$ ; \*\* $P < 0.01$ . Cells that were treated with 16  $\mu$ M 5UP2 or 5HP had virus yields not detectable (ND) in this assay, and a bar is arbitrarily drawn to show the presence of the samples in the graph. The experiment was repeated three times and error bars are shown. (B) Immunofluorescence assay with SDOW17 monoclonal antibody. Specific fluorescence is clearly visible at 24 h post PRRSV (VR2385) infection, while no fluorescence is observed in uninfected (–) control. Treatment with PPMO 5UP2, 5HP, 6P1, and 7P1 resulted in reduction of virus replication, while 3UP2 and CP1 did not appear to have any effect under identical conditions. The images below the green fluorescence images were taken with a DAPI filter to show the total number of living cells.

demonstrated that PPMO generated little cytotoxicity in both CRL11171 and PAM cells, further indicating that the suppression of virus replication observed in the antiviral experiments above was due to sequence-specific effects.

In an attempt to derive a “Selectivity Index” (SI) (the ratio of the concentration of drug causing 50% cytotoxicity [CC<sub>50</sub>] divided by the concentration of drug causing a 50% inhibition of

viral production) relevant to the various conditions of this study, we sought to determine the CC<sub>50</sub> for PPMO 5UP2, 5HP, 6P1, and 7P1. CRL11171 cells were treated for 4 h with concentrations of each PPMO from 10 to 120  $\mu$ M. Cell viability was determined at 48 h after the treatment period. The CC<sub>50</sub> for 5UP2, 5HP, 6P1, and 7P1 are 60, 120, 90, and 120  $\mu$ M, respectively. Based on a 50% inhibition of viral production of approximately 1, 2.1,

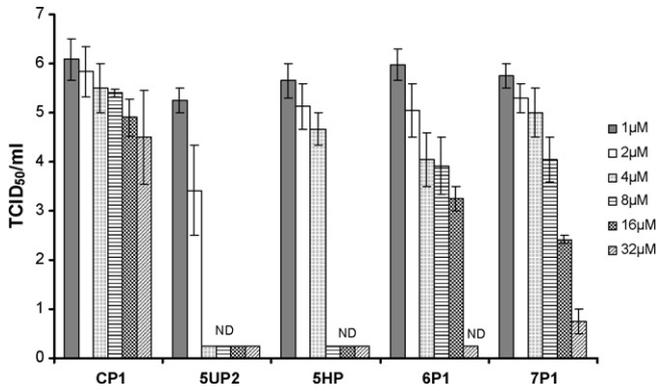


Fig. 3. Dose-dependent inhibition of PRRSV replication. Virus yield in cell culture is reduced concomitantly with increasing concentrations of 5UP2, 5HP, 6P1, and 7P1. Cells receiving some of the treatments had virus yields not detectable (ND) in this assay, and a bar is arbitrarily drawn to show the sample presence in the graph. The dose-dependent inhibition of PRRSV replication by 5UP2, 5HP, 6P1, and 7P1 is significantly different from CP1 ( $P < 0.05$ ). The experiment was repeated three times and error bars are shown.

1.5, and 2  $\mu$ M, respectively, for these four PPMO (Fig. 3), we conclude that the SI for these antisense PPMO is 60, 57, 60, and 60 under these conditions.

3.5. Combinatory effect of PPMO

To investigate the effect of treatment with PPMO combinations on PRRSV replication, PPMO were paired and tested at equal molar concentrations, for example, a combination at 4  $\mu$ M final concentration consisted of each PPMO being present at 2  $\mu$ M. The PPMO 5UP2 was not included in this test since it was already established as effective at 2  $\mu$ M. The PPMO that target regions in PRRSV thought to be involved in transcription events were tested in pairs: 5HP + 6P1, 5HP + 7P1, 3UP2 + NSP2, 3UP2 + NSP3. Other pairs of PPMO targeting the translation initiation regions of ORFs 2 to 7 were also tested: 2P1 + 3P1, 3P1 + 4P1, 5P1 + 6P1, and 6P1 + 7P1. The results of virus titration showed that only the combination of 6P1

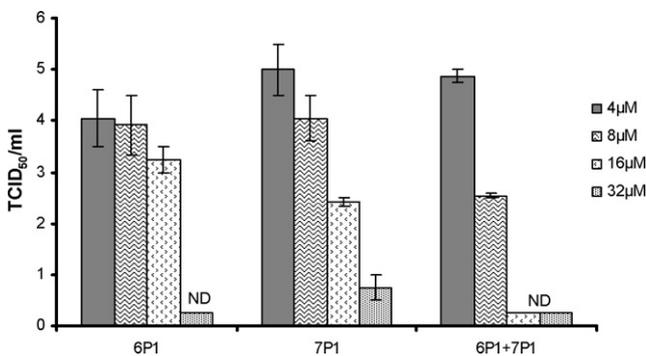


Fig. 4. Combinatory inhibition assay of PPMO. Two PPMO were added at equal molar concentration to cells after PRRSV inoculation. The combination treatment of 6P1 and 7P1 led to significant lower virus yield ( $P = 0.003$ ) than did either PPMO alone. Cells that were treated with 16 or 32  $\mu$ M 6P1 + 7P1 had virus yields not detectable (ND) in this assay, and a bar is arbitrarily drawn to show the presence of the samples in the graph.

and 7P1 (6P1 + 7P1) caused significant reduction ( $P = 0.003$ ) in PRRSV yield in comparison with a constituent individual PPMO (Fig. 4). None of the other PPMO combinations showed significantly higher inhibitory activity than that of a constituent individual PPMO.

3.6. Effect of PPMO treatment on PRRSV RNA synthesis

To determine PPMO effect on PRRSV RNA synthesis, CRL11171 cells were inoculated with VR2385, treated with PPMO and harvested at 24 h p.i. for RNA isolation. PPMO 5UP2, 5HP, NSP2, NSP3, and 3UP2 were included in this experiment as they were designed to block PRRSV RNA synthesis. The combination of PPMO 6P1 + 7P1 was also used to see if it had any effect on PRRSV RNA synthesis. PPMO 6P1, 7P1, and CP1 were included, for comparison. In Northern blot analysis, at least seven distinct RNA species were detected in the mock-treated sample, consistent with what would be expected from using a probe that detects ORF7, and indicating that all PRRSV genomic and subgenomic RNA containing ORF7 sequence was

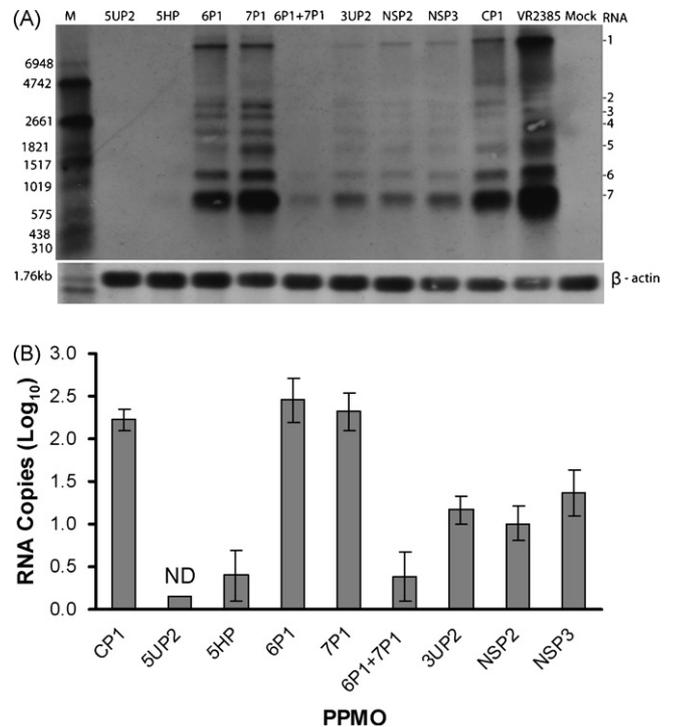


Fig. 5. PPMO inhibition of PRRSV RNA synthesis. (A) Northern blot analysis with probe from PRRSV ORF7. CRL11171 cells were inoculated with VR2385 at 0.5 MOI and treated with PPMO at 16  $\mu$ M. The cells were harvested at 24 h p.i. No detectable RNA is seen after treatment with 5UP2 or 5HP. RNA molecular weight markers are on the left. PRRSV RNA transcript designations are indicated on the right. As a loading control, the transcript of  $\beta$ -actin was detected and is shown in the lower panel. (B) Quantitation of PRRSV RNA by real-time RT-PCR with primers designed to amplify a region of ORF1a. Treatment of the cells with PPMO 5UP2, 5HP, 6P1 + 7P1, 3UP2, NSP2 and NSP3 at 16  $\mu$ M led to significant reduction of PRRSV genomic RNA level ( $P < 0.05$ ). The cells were harvested at 24 h p.i. and total RNA was isolated for real-time RT-PCR. Cells treated with 5UP2 had no PRRSV RNA detectable (ND) in this assay and a bar is arbitrarily drawn to show the sample in the graph. The PRRSV RNA copy numbers in each sample was calculated in comparison with a standard curve, after normalization with  $\beta$ -actin transcript levels (see Section 2.8).

detected. PRRSV RNA in samples treated with 5UP2 or 5HP was below the detection level (Fig. 5A). PPMO 6P1 or 7P1 had no effect on viral RNA level, but the combination of 6P1 and 7P1 led to significant reduction of viral RNA level (Fig. 5A). PPMO 3UP2, NSP2, and NSP3 also apparently led to a modest reduction of viral transcription in comparison with CP1. The Northern blot shows that the, control PPMO, CP1, produced a small yet noticeable effect on PRRSV RNA level compared to mock-treatment. However, the slight non-specific inhibition caused by CP1 was far less than the high level of inhibition produced by several of the antisense PPMO.

Real-time RT-PCR with primers specific for PRRSV ORF1a was also used to evaluate RNA levels. Only PRRSV genomic RNA could be detected with the primers. In cells treated with 5UP2, PRRSV RNA was not detectable (Fig. 5B). In cells treated with 5HP or 6P1 + 7P1, PRRSV genomic RNA was reduced by 67- and 69-fold, respectively, compared to CP1-treated cells. The presence of 3UP2, NSP2, and NSP3 also led to 11-, 17-, and 7-fold reduction of PRRSV genomic RNA in comparison to CP1-treated cells. Treatment with either 6P1 or 7P1 did not reduce PRRSV RNA. These results are consistent with the Northern blot analysis above.

### 3.7. Sequence-specific inhibition of target mRNA translation in a cell-free reporter assay

To further characterize the sequence-specificity of the PMO-mediated inhibition of PRRSV replication, we conducted a cell-free luciferase reporter assay. The target sequences of PPMO 5UP2, 5HP, 6P1, and 7P1 were subcloned upstream of luciferase in a reporter expression plasmid. In this assay, PPMO binding to its RNA target typically results in inhibition of translation of the downstream luciferase coding sequence. Effects of PPMO treatment on target RNA translation were assessed by measurement of luciferase production. Incremental concentrations of PPMO were added to *in vitro* translation reactions to determine PPMO inhibitory effect on translation. As shown in Fig. 6, 100 nM or higher concentration of 5UP2, 7P1, 5HP, and 6P1 reduced luciferase yield by more than 90%, while CP1 had no or little effect on translation of corresponding RNA. PPMO 6P1 was slightly less effective than the other three PPMO in this test. The results of this assay indicate that the antisense PPMO specifically bound complementary RNA and thereby inhibited the translation of luciferase.

### 3.8. Inhibitory effect of PPMO against heterologous PRRSV strains

The PPMO in this study were designed against the VR2385 strain of PRRSV. To determine the efficacy of the PPMO against eleven other PRRSV strains, NVSL97-7895, FL-12, 16244B, 16138, 11604, 17041, 14680, 12773, 13909, MLV, and Lelystad, cross strain inhibition assays were conducted. The Lelystad strain is a prototype of the European genotype. The rest are strains of the North American PRRSV genotype. PPMO 5UP2, 5HP, and the combination of 6P1 + 7P1 were tested against

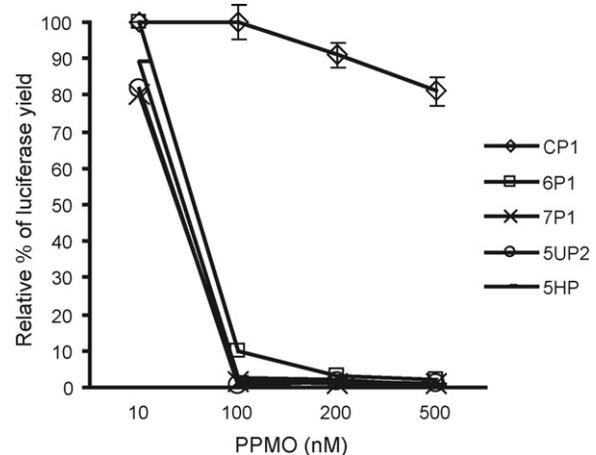


Fig. 6. Inhibition of target RNA translation by PPMO in a cell-free luciferase reporter assay. Relative percentages of luciferase level were calculated in comparison with signal of control PPMO sample at 10 nM. The level of luciferase production decreased in response to increasing concentrations of PPMO 5UP2, 5HP, 6P1, and 7P1, while CP1 has little effect on luciferase RNA translation.

each of the PRRSV strains. Virus inoculation and PPMO treatment were conducted in the same manner as described above for VR2385. Virus titration results showed that 5UP2 and 5HP effectively suppressed replication in all strains except Lelystad (Fig. 7A). The 6P1 + 7P1 combination led to a noticeable reduction in virus titer of a majority of the 10 North American isolates, including NVSL, 16244B, 14680, 12773, 13909, and MLV. Replication of Lelystad was not inhibited by any of the PPMO tested.

Sequence alignment of the PPMO target sites in the PRRSV strains showed that Lelystad had low sequence identity with the other strains (Fig. 7B). PRRSV NVSL, 11604, 14680, and 16244B have one nucleotide mismatch with VR2385 in the 3' end of the 5UP2 target site. In the 5HP target site, the only mismatch found was in NVSL, which has a single nucleotide difference in the middle of the PPMO target region. In the 6P1 target site, NVSL and 11604 have two mismatches, Lelystad has five mismatches and two deletions, and all others have one mismatch. In the 7P1 target site, one mismatch occurs in all strains except Lelystad, which has five mismatches. The sequence alignment results indicate that nucleotide mismatches in the PRRSV target sites of 6P1 and 7P1 are likely responsible for the low level of inhibition produced by these two PPMO.

### 3.9. Inhibitory effect of PPMO in PAM culture

Alveolar macrophages are the primary target cells for PRRSV infection in pigs (Yoon et al., 1992). Evaluation of PPMO effect on virus replication in PAM has relevance to the biology of a natural infection. To facilitate adaptation to laboratory culturing, PAM cells were pre-incubated for 24 h before their inoculation with PRRSV. After virus inoculation, PAM were treated with PPMO 5UP2, 5HP, 6P1 + 7P1, or CP1, and then observed at 24 h p.i. for CPE development. The PAM that were treated with

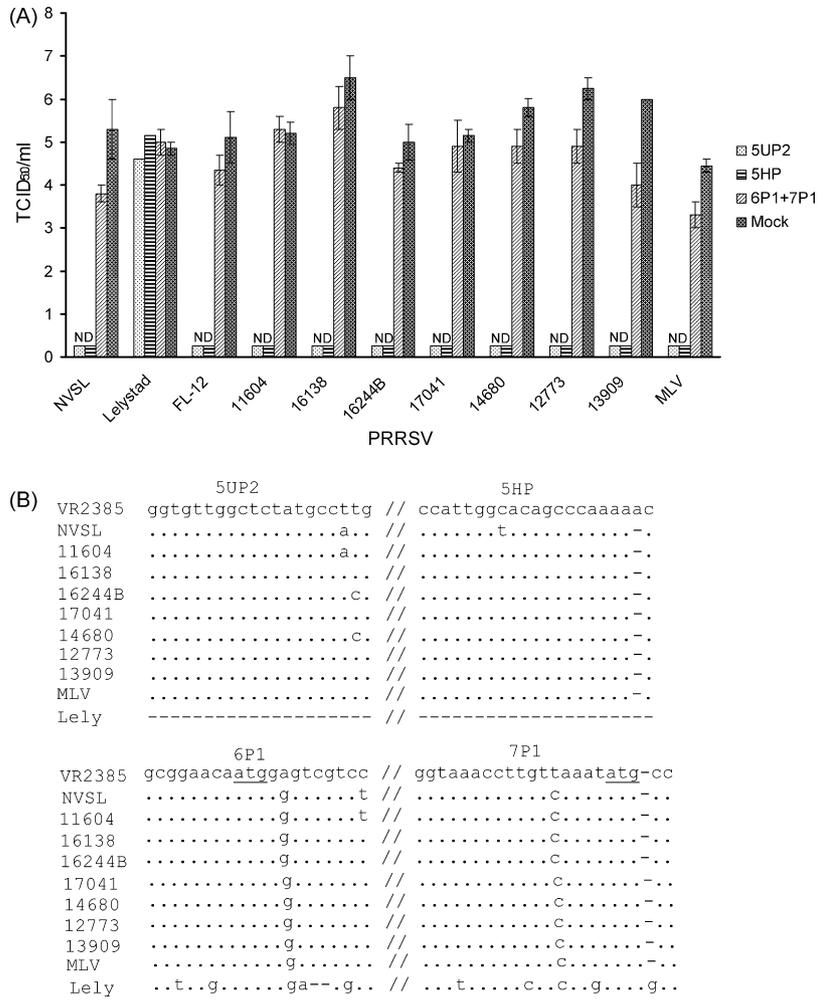


Fig. 7. Cross strain inhibition assay. (A) Virus yield titration shows inhibition of ten North American PRRSV strains by PPMO 5UP2 and 5HP. Lelystad is a prototype of European PRRSV genotype. All other strains are within the North American PRRSV genotype. “Mock” denotes virus infected cells receiving no PPMO. Treatment of cells with PPMO 5UP2 or 5HP led to suppression of PRRSV replication of all North American strains, producing virus yields not detectable (ND) in this assay. Bars are arbitrarily drawn for those samples with no detectable virus (“ND”) to show the presence of samples in the graph. (B) Sequence analysis identifies nucleotide mismatches between PPMO and their complementary target sites in PRRSV RNA. PRRSV strain names are listed in the first column. The sequence of strain VR2385 is used as the reference sequence, as the PPMO were designed against it. PPMO names are listed above the blocks of PRRSV sequences. “Lely” stands for Lelystad strain, which has little similarity in the 5’UTR to other strains, as indicated by symbol “-” in the alignment of 5UP2 and 5HP target sequences. For all other sequences, only nucleotides differing from reference sequence are shown, and identical nucleotides are indicated with “.”. Missing nucleotides are indicated with “-”. The initiation codons ATG of ORFs 6 and 7 are underlined. GenBank accession numbers for PRRSV strains in the alignment are listed in parenthesis: NVSL 97-7895 (AY545985), Lelystad (M96262), FL-12 (derived from infectious clone of AY545985), 16244B (AF046869), 11604 (EF523345), Ingelvac MLV (EF484033), and 16138 (EF523346). Nucleotide sequences for the other strains in the figure are unpublished.

PPMO 5UP2, 5HP, or combination of 6P1 + 7P1 showed no or little CPE development, while the CP1- or mock-treated control cells suffered severe CPE (Fig. 8A). This result indicates that PPMO treatment protected the cells from PRRSV-induced CPE development, and also that PRRSV replicates rapidly in PAM.

To investigate the PPMO-mediated protection of PAM from CPE development, we treated of PAM with 5UP2 twice, 48 h apart. No CPE was observed in the cells at 7 days p.i. (data not shown), indicating that PPMO treatment protected PAM from CPE for at least 1 week after PRRSV inoculation.

IFA was conducted with PAM cells after PRRSV inoculation and PPMO treatment. The cells were fixed at 12 h p.i. to mini-

mize cell death in mock-treated control. Results showed that the approximate percentages of PRRSV-positive cells were 2% with 5UP2 treatment, 8% with 5HP, 8% with 6P1 + 7P1, and 20% in mock-treated control (Fig. 8B). The results demonstrated that treatment with antisense PPMO reduced PRRSV infection in PAM cells.

PRRSV yield in PAM culture was titrated in CRL11171 cells. Treatment of PAM with PPMO 5UP2, 5HP, and 6P1 + 7P1 led to 32-, 5-, and 5-fold reduction in virus yield, respectively, compared to mock-treated control (Fig. 8C). The reduction in virus titer in PAM was considerably less than had been observed in CRL11171 cells after similar PPMO treatment.

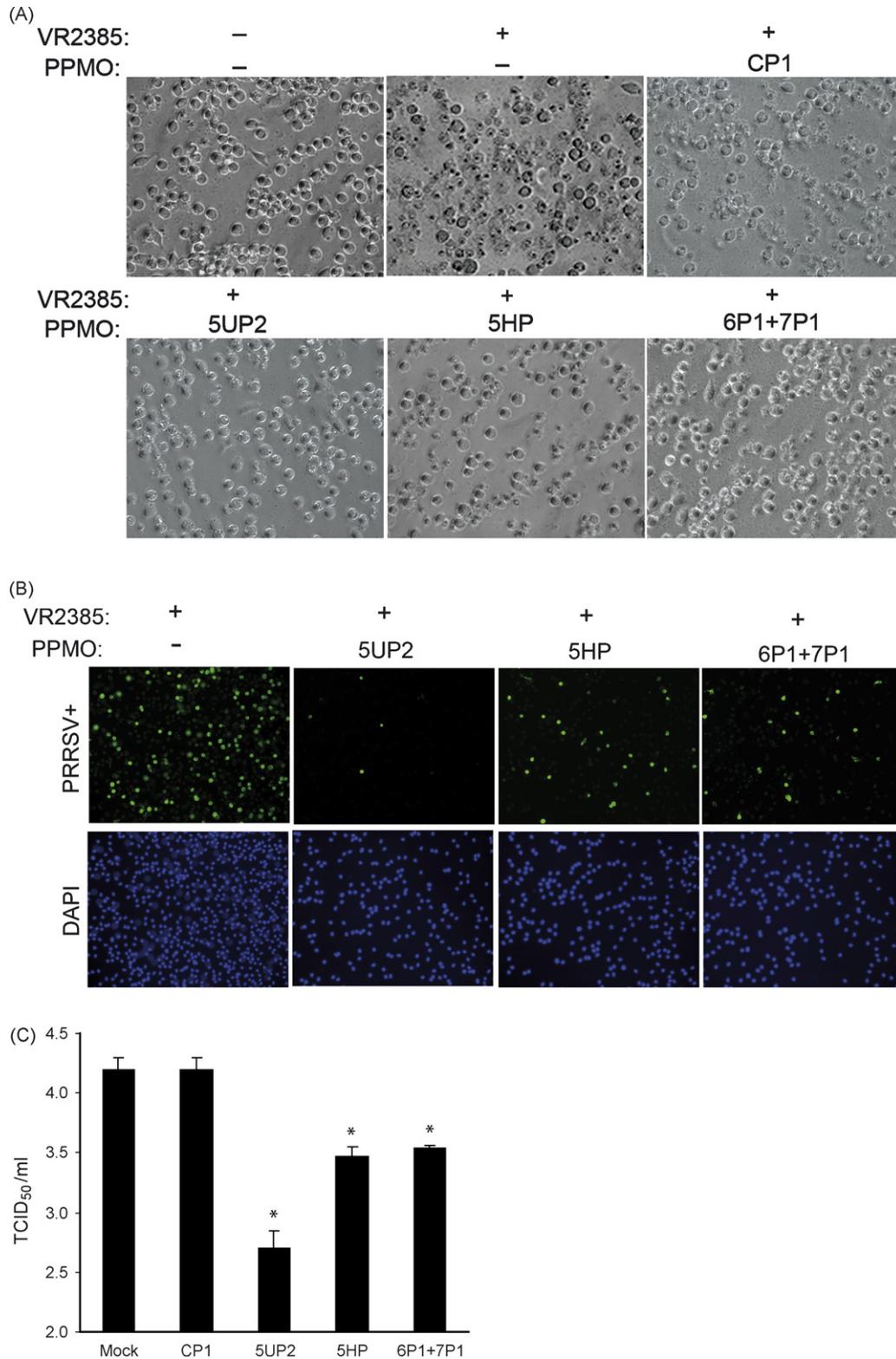


Fig. 8. PPMO-mediated suppression of PRRSV replication in porcine alveolar macrophages. (A) Protection of PAM from PRRSV CPE development. Infection of PAM with VR2385 led to severe CPE development (see +VR2385, -PPMO) in comparison with uninfected control cells (-VR2385, -PPMO). Application of PPMO 5UP2, 5HP, or a combination of 6P1 + 7P1 at 16  $\mu$ M resulted in inhibition of CPE development, while CP1 did not. (B) Reduced PRRSV infection in PAM detected by IFA with SDOW17 antibody. Specific fluorescence is clearly visible at 12 h after VR2385 inoculation. Treatment with PPMO 5UP2, 5HP, 6P1 + 7P1 resulted in reduction of virus replication, while CP1 did not. The images below the green fluorescence images were taken with a DAPI filter to show the total number of living cells. (C) Titration of virus yield from PAM cells after PRRSV infection and PPMO treatment. Virus titers are shown as TCID<sub>50</sub> (log<sub>10</sub>/ml). “Mock” sample is virus inoculation with no PPMO treatment. Statistical significance of the difference in viral yield compared to mock-treatment \* $P < 0.05$ . The experiment was repeated three times and error bars are shown.

#### 4. Discussion

Employing morpholino oligomers to inhibit RNA virus replication has become an appealing prospect after reports of the successful knockdown of cellular proteins via inhibition of pre-mRNA splicing and/or translation of mRNAs (Heasman, 2002). In RNA virus genomes, there are a variety of regions for PMO targeting. In our previous study, six PPMO, designed to target the 5' and 3' UTRs, the TRS-region of genomic RNA, and the 3' end of negative-sense PRRSV RNA, were evaluated (Zhang et al., 2006). Only one of those PPMO, 5UP1, targeting the 5' terminal region of the genome, was found to be effective at inhibiting PRRSV replication. In the present study, 12 PPMO were designed to target a wider variety of sites within the PRRSV genome, including the 5' and 3' UTRs, translation initiation regions of ORFs 2 through 7, and negative-sense PRRSV RNA. Of the PPMO tested here, 5UP2 was found to be the most highly effective PPMO, followed by 5HP, 6P1, and 7P1.

In this study, PPMO 5UP2 appeared more effective than 5UP1 did in our previous study (Zhang et al., 2006). The target sites for 5UP1 and 5UP2 are located in the 5' terminal region of the PRRSV RNA genome and overlap by 15 nucleotides. We note, however, that comparing results between the two studies is complicated by the fact that the first study employed PPMO in which the peptide component was R<sub>9</sub>F<sub>2</sub>R<sub>4</sub> (P4), while in the present study we utilized the more recently developed (RXR)<sub>4</sub> (P7) peptide. PPMO 5HP complements a hairpin sequence in the 5' UTR, and was also quite effective, reducing PRRSV yield by 4.5 log<sub>10</sub> (Fig. 2A). A PPMO targeting the corresponding region in EAV was likewise found to be highly effective (van den Born et al., 2005).

It has previously been reported that a combination of PMO compounds can outperform a single agent. This was shown *in vivo* with non-conjugated PMO against Ebola virus (Warfield et al., 2006), and the authors of that study suggested that slowing virus replication may allow sufficient time for development of antiviral immune responses and viral clearance. Likewise Ge et al. showed that a combination of PPMO produced greater efficacy than either PPMO alone against Influenza A virus in cell culture (Ge et al., 2006). Our results showed that treatment with a combination of 6P1 and 7P1 led to significantly higher PRRSV inhibition than did either of the two PPMO alone. However, many other PPMO combinations in this study failed to outperform their constituent single agents.

To elucidate the mechanism of PMO-mediated inhibition, we conducted Northern blot and RT-PCR analyses (Fig. 5). Treatment of cells with 5UP2 or 5HP reduced PRRSV RNA to the level of below detection, indicating inhibition of PRRSV RNA synthesis, likely accomplished through blocking translation of the replicase encoded by ORF1a/b. Treatment with a combination of 6P1 and 7P1 also produced a reduction in PRRSV RNA level, probably due to the blockage of the translation of the transcripts for ORFs 6 and 7. Based on their target locations in the 3' end of PRRSV genome, we speculate that the combination of 6P1 and 7P1 may have reduced PRRSV genome replication by interfering with assembly or progression of the replicase complex.

Our results clearly show a reduction of PRRSV RNA level in cells treated with 5UP2 or 5HP. A PPMO-associated reduction in PRRSV RNA production was also observed by RT-PCR in our previous study (Zhang et al., 2006). Conversely, similar experiments against EAV showed no significant change in the level of any viral RNA transcripts, as assessed by gel hybridization analysis (van den Born et al., 2005). This seeming discrepancy may have resulted from differing experimental conditions. For RNA isolation, cells were harvested at 24 h p.i. in the PRRSV, but 36 h in the EAV experiments (van den Born et al., 2005). There likely are some differences in the replication mechanics between PRRSV and EAV, since PPMO designed to target the respective TRS-regions were reported to be effective against EAV (van den Born et al., 2005), yet ineffective against PRRSV (Zhang et al., 2006).

Among the 12 PPMO tested in this study, only 4 were found to be significantly efficacious. The reasons for the ineffectiveness of the other PPMO are unclear, but could include inaccessibility of PRRSV target sequence, or, that successful PPMO/target RNA duplexing did not affect PRRSV replication.

PPMO 5UP2, 5HP, and combination of 6P1 + 7P1 effectively inhibited PRRSV-induced CPE development in primary PAM, the major target cell type of PRRSV infection in host animals. IFA confirmed the PPMO inhibition of virus replication. These results indicate that PPMO may indeed have potential to control, or reduce the severity of, PRRSV infection in pigs. It was unexpected that PPMO-mediated reduction of virus yield in PAM was less than that observed in CRL11171 cells. This difference but may be because PRRSV has a higher replication rate in PAM than it does in CRL11171 cells, and the PPMO were unable to significantly interfere with the more rapid viral replication. Alternatively, differential uptake of PPMO by CRL11171 and PAM cultures may have had an effect. However, virus replication in PAM after treatment with 5UP2 did not result in cytopathic effect, and the cells were healthy for at least 1 week after virus inoculation. Further study is being undertaken to further investigate PPMO-mediated protection of primary cells.

PPMO 5UP2 and 5HP inhibited replication of ten North American PRRSV strains in our cross-strain inhibition assay. Sequence alignment (Fig. 7B) showed that the region targeted by these PPMO contains highly conserved sequences. In addition, analysis of PRRSV sequences from the GenBank indicates that the complementary sequence of 5UP2 and 5HP are highly conserved across North American PRRSV strains. Thus, these two antisense PPMO may be suitable for application against most North American PRRSV strains. The broad inhibition by 5UP2 and 5HP also further confirms that the 5' UTR of PRRSV is a highly productive target region for PPMO, and that this region likely plays an important role in arterivirus replication. A one base mismatch between 6P1 or 7P1 PPMO and their respective target sites in ten North American PRRSV strains may explain the low activity observed. (Fig. 7A and B). It is noteworthy that all 10 strains had identical sequences in both the 6P1 and 7P1 target sites. Thus, a minor sequence modification of PPMO targeting these regions may lead to improved efficacy.

A cell viability assay of PPMO-treated CRL11171 cells and PAM detected no cytotoxicity, indicating that the suppression of PRRSV replication observed in the antiviral assays was due to PPMO-specific inhibition of PRRSV molecular events. The absence of PPMO-induced cytotoxicity at effective antiviral concentrations is an important attribute of these compounds, when considering potential *in vivo* applications.

The prevalence of PRRSV and financial losses associated with PRRSV infection in swine herds is high, and current strategies to control PRRS, including the use of commercial and autogenous vaccines, are inconsistent and generally less than adequate (Meng, 2000; Opriessnig et al., 2002). Specific anti-PRRSV drugs would be useful to complement other strategies of PRRSV prevention and control. Under the conditions of this study, four PPMO showed potent and specific anti-PRRSV activity in cell culture, and can be considered potential drug candidates for use in PRRSV control. Further investigation into the pharmacokinetic, toxicological and antiviral properties of these PPMO *in vivo* is warranted.

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