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# Enhanced inhibition of porcine reproductive and respiratory syndrome virus replication by combination of morpholino oligomers

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## ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) has caused heavy economic losses in the swine industry worldwide and current strategies to control PRRS are inadequate. Previous studies have shown that peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO) can be an effective antiviral against the PRRS virus (PRRSV). PPMO is structurally similar to DNA with modified backbone and is resistant to nuclease. This study was designed to examine increasing inhibitory effect of PPMO combination. Two pairs of PPMOs were identified to have enhanced suppression of PRRSV replication in cell culture, while individual constituents did not work under the same testing conditions. PPMO 5UP1 that is complementary to 5' terminus of PRRSV genome was paired with 4P1 or 7P1 that are complementary to sequence in the translation initiation regions of ORFs 4 and 7, respectively. The PPMO combination also inhibited replication of heterologous strains in the North American PRRSV genotype. Treatment of the cells with the combinations reduced PRRSV RNA and protein levels. In cell-free or cell-based luciferase reporter assays, the PPMO combination suppressed target mRNA translation more effectively than individual constituents, indicating that the suppression was due to their antisense effect. These results suggest potential application of these PPMO combinations for PRRS control.

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## 1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is recognized as one of the most economically important diseases in the swine industry worldwide. In the United States, PRRS alone costs an estimated \$560 million in losses per year to the swine industry (Neumann et al., 2005). The etiologic agent of PRRS was first isolated on porcine alveolar macrophages in Europe in 1991 and this isolate was designated as Lelystad virus (LV) (Wensvoort et al., 1991). In 1992, a similar virus was isolated on an MA104-derived cell line, CL 2621, in the U.S. and the isolate was ATCC VR-2332 (Collins et al., 1992). The most recent outbreaks occurred in Vietnam and China (Normile, 2007). PRRSV is classified as a member of the family *Arteriviridae* (Meulenbergh et al., 1993).

Genomic sequence analyses of the PRRSV isolates from different regions worldwide indicate the existence of two distinct genotypes: Type I European (the prototype is LV) and Type II North American (the prototype is VR-2332) (Meng et al., 1995). North American and European isolates were found to have approximately 67% homology at the nucleotide sequence level. The genome of PRRSV is about

15 kb in length. It has a cap structure at its 5' end and a poly(A) tail at its 3' end (Meng et al., 1994; Meulenbergh et al., 1998). The genome contains nine ORFs and the genome organization starts with the 5' untranslated region (UTR), followed by nine open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, and ORFs 3–7) and 3' UTR. ORFs 1a and 1b are located at the 5' end of the genome and comprise about 80% of the genome. ORF1a and ORF1b encode viral replicase as a polyprotein, which is proteolytically processed to 12 mature non-structural proteins (NSPs) by virus-encoded proteases (Fang et al., 2004).

Current management strategies and commercial or autogenous live or inactivated vaccines are inadequate for PRRS control. PRRS remains the most economically important disease for swine industry though a commercial vaccine has been available for more than a decade. The high mutation rate of PRRSV replication, the high prevalence of PRRSV infection, and the lack of a broadly protective vaccine suggest the need to explore alternative strategies for PRRS control. We have demonstrated that phosphorodiamidate morpholino oligomer (PMO) can be effective antiviral against PRRSV (Patel et al., 2008; Zhang et al., 2006). Several PMOs targeted at the 5' terminal region of PRRSV genome have been shown to be effective in inhibiting PRRSV replication in cells in a sequence-specific and dose-dependent manner.

PMO is structurally similar to single-stranded DNA in that each subunit includes a purine or pyrimidine base. However, in PMO, each base is joined to a novel backbone consisting of one morpho-

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line ring and phosphorodiamidate linkage per subunit (Summerton and Weller, 1997). PMO is uncharged, water-soluble, and highly resistant to nuclease degradation. PMO binds to target mRNA by Watson–Crick base pairing and exerts 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). PMO that are conjugated with short arginine-rich cell penetrating peptides has displayed a higher efficiency in delivery into cultured cells (Moulton and Moulton, 2004). This peptide-conjugated PMO (PPMO) has been found to be fairly stable in cells and human serum for at least 24 h (Youngblood et al., 2007). The sequence-specific antiviral efficacy of peptide-conjugated PMO compounds has been documented against a number of viruses in animal models, including PPMO against Ebola virus (Enterlein et al., 2006; Warfield et al., 2006), Coxsackievirus B3 (Yuan et al., 2006), murine coronavirus (Burrer et al., 2007), Dengue virus (Stein et al., 2008), and West Nile virus (Deas et al., 2007; Deas et al., 2005).

In this study, we characterized the effect of PPMO combination on inhibition of PRRSV replication *in vitro*. We have found enhanced inhibition of PRRSV replication by combination of two PPMOs. Individual PPMO did not inhibit PRRSV replication at the concentrations tested. Further work showed sequence-specific blockage in target mRNA translation.

## 2. Materials and methods

### 2.1. Cells and viruses

Cell line CRL11171 was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The PRRSV VR2385 was used in the study (Meng et al., 1996) to inoculate CRL11171 cells at 0.5 multiplicity of infection (MOI) for PPMO testing. Other PRRSV strains used in this study include LV (Wensvoort et al., 1991), 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, National Animal Disease Center). Virus titers were determined from the median tissue culture infectious dose (TCID<sub>50</sub>) according to the method of Reed and Muench as described previously (Zhang et al., 2006).

### 2.2. Indirect immunofluorescence assay (IFA)

IFA was carried out as reported previously with an N-specific monoclonal antibody EF11 (Zhang et al., 1998). Specific reactions between EF11 and N protein were detected with goat anti-mouse IgG-fluorescein isothiocyanate (FITC) conjugate (Sigma, St Louis, MO) and observed under fluorescence microscopy.

### 2.3. PPMO treatment of cells

PPMO was synthesized at AVI BioPharma Inc. (Corvallis, OR) and PPMO treatment of CRL11171 cells was performed in 12-well plates as previously described (Patel et al., 2008; Zhang et al., 2006). Briefly, cells were inoculated with virus at 0.5 MOI for 2 h. PPMO was diluted to the desired concentrations in plain DMEM and added to the PRRSV-infected cells after inoculum removal. PPMO CP1 that contains irrelevant random sequences was also included as a negative control. After 4-h incubation, the PPMO solution was removed. The cells were rinsed with DMEM and maintenance medium (DMEM supplemented with 2% FBS) was added. The cells were then incubated at 37 °C for 48 h. At 48 h postinfection (p.i.), both supernatant and cells were harvested for further analysis.

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

Total RNA was extracted from PRRSV-infected cells by TRIzol<sup>®</sup> Reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). The RNA was quantified in  $\mu$ Quant<sup>™</sup> Universal Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). Quantitative real-time RT-PCR was done as described previously (Patel et al., 2008; Zhang et al., 2006). A fragment of 860 nucleotides (nt) from the 5' end of the PRRSV genome was cloned into a pcDNA3 vector, and used as template to generate standard curve. The SYBR Green real-time PCR was performed on Chromo 4<sup>™</sup> Four-Color Real-Time System (Bio-Rad). Transcript of  $\beta$ -actin was also detected as an internal reference for normalization.

### 2.5. Plasmid construction and luciferase reporter assay

The 312 nt at 5' UTR of subgenomic RNA7 was PCR-amplified from PRRSV cDNA with forward primer 5UF1 (5'CTAGC TAGCG ACGTA TAGGT GTTGG CTC3') and reverse primer SB7-R4 (5'ACGCC TCGAC CTTCT GCTGC TTGCC GGTG3'). The PCR product was digested with *NheI* and *Sall* and subcloned into a luciferase reporter vector PciNeoLuc, as described previously (Zhang et al., 2006). This cloning placed the PRRSV 5' UTR upstream of the luciferase coding sequence. DNA sequencing was performed to confirm the presence of the desired sequence in the resulting plasmid, PciNeoLuc-5UTR. The plasmid DNA was linearized downstream of the luciferase gene with *NotI*. *In vitro* transcription was conducted with T7 RiboMAX<sup>™</sup> Express Large Scale RNA Production System (Promega) according to the manufacturer's instructions. *In vitro* translations were carried out by rabbit Reticulocyte Lysate Translation System (Promega) with 2  $\mu$ g transcribed RNA. A series of dilutions of 1, 5, 50 and 100 nM of PPMO 5UP1, 7P1, and CP1 were tested. Combinations of 5UP1 at 1 or 5 nM with 7P1 or CP1 at 5, 50 and 100 nM were compared to the mock-treatment control (no PPMO). A control without RNA and PPMO was also included in the translation assay. The luciferase yield under the absence or presence of the PPMOs was measured by the Bright-Glo<sup>™</sup> Luciferase Assay System (Promega) with VICTOR3<sup>™</sup> Multilabel Counter (PerkinElmer Life and Analytical Science, Wellesley, MA). Luminescence signal in test samples was expressed as relative percentage of that in the mock-treated control reaction.

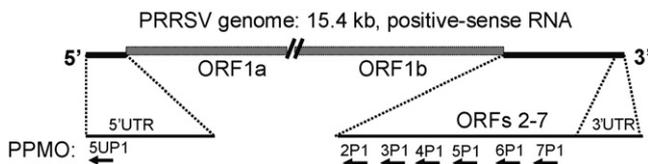
### 2.6. Statistical analysis

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

## 3. Results

### 3.1. Enhanced inhibition of PRRSV yield in cell culture by PPMO combination

PPMO 5UP1 was found to be effective in inhibiting PRRSV yield in cell culture (Zhang et al., 2006). Treatment of CRL11171 cells with 5UP1 at the final concentration of 2  $\mu$ M resulted in a moderate (about 1.0 log<sub>10</sub>) reduction in PRRSV yield. PPMO 5UP1 was designed to complement the 5' terminal region of PRRSV genome in an attempt to block translation of viral RNA replicase (Fig. 1). To evaluate the effect of PPMO combination on inhibiting PRRSV replication, the PPMO 5UP1 was paired with each of the following individual PPMO at different concentrations: 2P1, 3P1, 4P1, 5P1, 6P1, and 7P1. The 2P1, 3P1, 4P1, 5P1, 6P1, and 7P1 were designed to be complementary to the translation initiation regions of PRRSV ORFs 2–7, respectively, in order to inhibit the translation of these ORFs



**Fig. 1.** Schematic illustration of PPMO target sequence locations in PRRSV genome. The arrows indicate the 5'–3' orientation of the PPMOs in relation to the PRRSV RNA genome.

(Fig. 1). All of these PPMO sequences are complementary to the genomic sequence of VR2385, a virulent strain of North American genotype (Patel et al., 2008; Zhang et al., 2006). A moderate reduction of PRRSV replication was also observed for the cells treated with PPMO 6P1 or 7P1 at a relatively high concentration, but the PPMO 2P1, 3P1, 4P1, and 5P1 were found to have no effect (Patel et al., 2008).

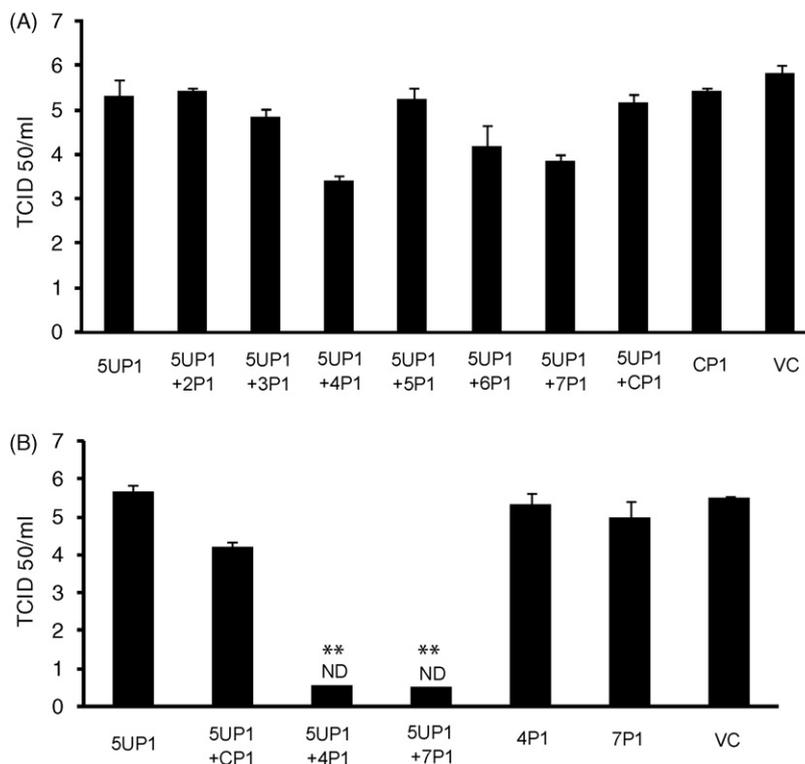
In our initial test, the 5UP1 at the concentration of 2  $\mu\text{M}$  was paired with 16  $\mu\text{M}$  of each of the other PPMOs. Compared to the control group, treatment of CRL11171 cells with 2  $\mu\text{M}$  5UP1 alone reduced the PRRSV yield for 1.0  $\log_{10}$  TCID<sub>50</sub>/ml, which is in agreement with the previous observation (Zhang et al., 2006). In contrast, treatment of CRL11171 cells with a combination of 2  $\mu\text{M}$  5UP1 and 16  $\mu\text{M}$  of the other PPMOs against PRRSV resulted in complete inhibition of PRRSV yield, which was below detection level (data not shown). To further assess the effect of PPMO combination, the 5UP1 concentration was reduced from 2 to 0.5  $\mu\text{M}$  and the other PPMOs were reduced to 8  $\mu\text{M}$ . Treatment with 5UP1 alone at the concentration of 0.5  $\mu\text{M}$  did not reduce virus yield compared to virus control or mock treatment (Fig. 2A). Among all of the PPMO pairs tested, a combination of 0.5  $\mu\text{M}$  5UP1 and 8  $\mu\text{M}$  4P1, 6P1, or 7P1 showed more enhanced inhibition effects on PRRSV replication than other

PPMO pairs (Fig. 2A). CRL11171 cells treated with 0.5  $\mu\text{M}$  5UP1 in conjunction with 8  $\mu\text{M}$  of 4P1, 6P1, or 7P1 caused a 1 to 2  $\log_{10}$  reduction in PRRSV yield in comparison to the pair of 5UP1 + CP1 control. Among all the pairs tested, the 5UP1 + 4P1 and 5UP1 + 7P1 led to the biggest reduction in PRRSV yield in multiple repeats and were selected for further characterization.

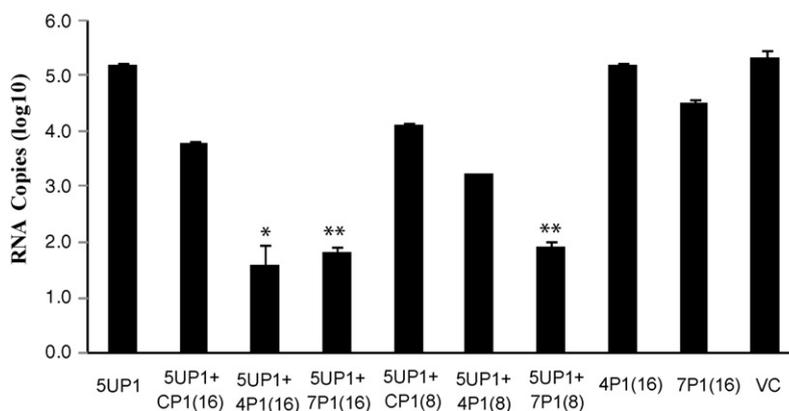
Treatment with 16  $\mu\text{M}$  of 4P1 or 7P1 in combination with 0.5  $\mu\text{M}$  of 5UP1 strongly inhibited PRRSV replication and virus yields were below detection level, while each individual PPMO alone did not show detectable effect at the same concentrations tested (Fig. 2B). This experiment was repeated three times and similar results were observed each time. These results demonstrated that combination of 5UP1 (0.5  $\mu\text{M}$ ) with 4P1 or 7P1 (8 or 16  $\mu\text{M}$ ) was more effective in inhibiting PRRSV replication than a constituent individual PPMO of the pairs. It was noticed that the 5UP1 + CP1 combination also led to a little reduction in virus yield. However, this reduction is insignificant in comparison with that by the combination of 5UP1 with 4P1 or 7P1.

### 3.2. Effect of combination PPMO treatment on PRRSV RNA synthesis

PPMO 5UP1 was designed to bind to the 5' terminal 21 nt of PRRSV genome. This region is believed to be critical in the pre-initiation of translation of viral genomic RNA (Van Den Born et al., 2004). Previous work at this lab has demonstrated that treatment of CRL11171 cells with 16  $\mu\text{M}$  5UP1 led to a significant reduction in PRRSV minus-strand RNA synthesis (Zhang et al., 2006). To assess the effect of PPMO combination on PRRSV RNA synthesis, CRL11171 cells were treated with the two pairs of PPMO combinations, 5UP1 + 4P1 and 5UP1 + 7P1 and harvested at 40 h p.i. for RNA isolation. The 5UP1 PPMO was used at the concentration of 0.5  $\mu\text{M}$ ,



**Fig. 2.** Enhanced inhibition of PRRSV replication in infected CRL11171 cells by PPMO combination. (A) Titration of virus yields from CRL11171 cells after PRRSV infection and indicated PPMO treatment. Virus titers are shown as TCID<sub>50</sub> (log<sub>10</sub>/ml). PPMO 5UP1 was used at 0.5  $\mu\text{M}$ , and the other PPMOs used at 8  $\mu\text{M}$ . VC indicates cells with virus infection but no PPMO treatment. (B) Virus yields from cells treated with combination of 5UP1 at 0.5  $\mu\text{M}$  and the other PPMOs used at 16  $\mu\text{M}$ . Cells that were treated with PPMO combination 5UP1 + 4P1 and 5UP1 + 7P1 had virus yield below detection level (ND), and a bar is arbitrarily drawn to show the samples in the graph. The significance of difference in viral yields between the treatments is indicated by  $**P < 0.01$ . Error bars indicate variations.



**Fig. 3.** Effect of PPMO combination on PRRSV RNA synthesis detected by real-time RT-PCR. The 5UP1 was used at 0.5  $\mu$ M and the concentrations of the other PPMOs are indicated in parenthesis. The significant difference in viral RNA copies between the treatments is indicated by \* $P < 0.05$  and \*\* $P < 0.01$ . The PRRSV RNA copy numbers were calculated based on a standard curve after normalization with transcript of  $\beta$ -actin.

while 4P1 or 7P1 was tested at two different concentrations of 8 and 16  $\mu$ M. Real-time RT-PCR was performed to assess the levels of PRRSV genomic RNA. PPMO 5UP1 at the concentration of 0.5  $\mu$ M had no effect on viral RNA level, consistent with virus yield titration. PPMO 4P1 or 7P1 at the concentration of 16  $\mu$ M also did not lead to much reduction of viral RNA level when compared to that in mock-treatment control (Fig. 3). The combination of 5UP1 (0.5  $\mu$ M) with 4P1 (16  $\mu$ M) or 7P1 (8 or 16  $\mu$ M) led to over 1000-fold (3 log<sub>10</sub>) reduction of viral RNA level (Fig. 3). Moderate reduction in viral RNA level was observed when the combination of 5UP1 (0.5  $\mu$ M) with 4P1 (8  $\mu$ M) was used. The results indicate the treatment with PPMO combination had enhanced effect on the inhibition of PRRSV RNA synthesis. It was noted that the 5UP1 and CP1 combination also slightly reduced the viral RNA level compared to the mock treatment, which is consistent with viral yield reduction shown in Fig. 2B. However, this reduction in viral RNA level was much smaller than that by combination of 5UP1 with 7P1 or 4P1.

### 3.3. Effect of PPMO combination treatment on PRRSV protein level

The effect of treatment with PPMO combination on PRRSV protein translation was examined by IFA using a monoclonal antibody EF11 against N protein (Zhang et al., 1998). Treatment of CRL11171 cells with combination of 5UP1 (0.5  $\mu$ M) with 4P1 or 7P1 (16  $\mu$ M) led to an absence of PRRSV-positive cells (Fig. 4). In cells treated with combination of 5UP1 with the control PPMO CP1, the percentage of fluorescent-positive cells was similar to the cells treated with 5UP1 alone. In cells treated with 7P1 or 4P1 alone, PRRSV-positive cells were also observed. These results indicate that the combination of 5UP1 with 4P1 or 7P1 inhibited N protein expression in VR2385-inoculated cells.

In this experiment, the PPMO 4P1, 7P1 and CP1 were tested at 16  $\mu$ M. The 5UP1 was used at 0.5  $\mu$ M. Cell viability assay previously conducted in our lab has demonstrated that PPMO at the concentration of 16  $\mu$ M did not have detectable cytotoxicity in CRL11171 cells. Cells treated with 16  $\mu$ M 5UP1 or 7P1 had viability similar to the cells treated with CP1 and mock-treatment control (Patel et al., 2008; Zhang et al., 2006). The results indicate that inhibition of the N protein expression observed in this study was due to the PPMO's sequence-specific effects.

### 3.4. Sequence-specific inhibition of target mRNA translation

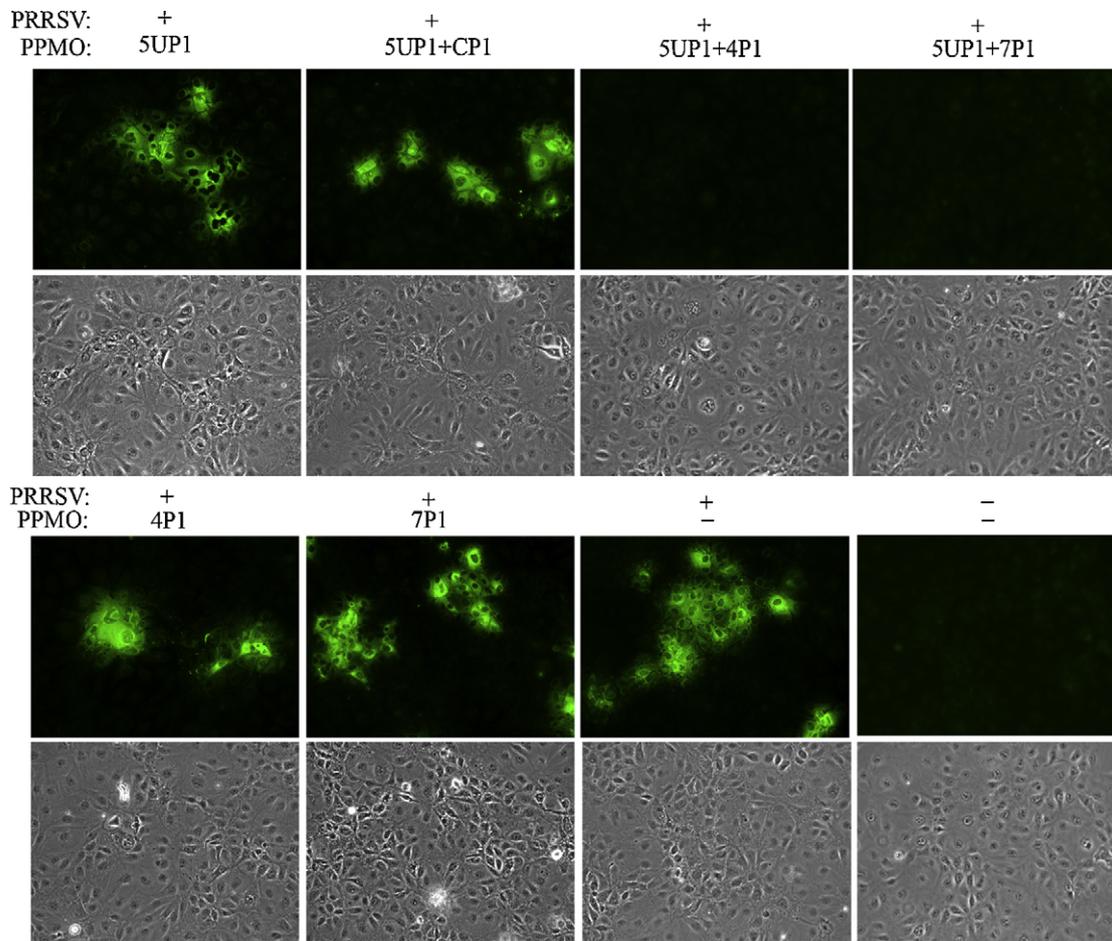
To further characterize the effect of PPMO combination, a cell-free luciferase reporter assay was conducted. The 312-nt sequence of PRRSV 5'UTR region from subgenomic RNA7 was cloned upstream of a luciferase reporter gene in pCiNeoLuc vector. In this

assay, PPMO binding to its target RNA was expected to inhibit its translation, leading to lower luciferase yield. Cell-free transcription and translation were conducted to assess the luciferase yield in the presence and absence of PPMO. In this test, the cellular uptake of PPMO was avoided and the direct effect of PPMO combination on target mRNA translation was determined. Different concentrations of PPMOs were used to determine dose-responsive reduction of luciferase yield. The 5UP1 at the concentrations of 5 and 50 nM resulted in the reduction of luciferase yield to 26% and 11%, respectively, compared to the mock-treatment control (Fig. 5A). The 7P1 at the concentrations of 5 and 50 nM resulted in the reduction of luciferase yield to 55% and 36%, respectively. When 1 nM 5UP1 was combined with 5 and 50 nM 7P1, the luciferase production was down to 44% and 32%, respectively. When 5 nM 5UP1 was combined with 5 and 50 nM 7P1, the luciferase production was down to 12% and 9%, respectively, which were far lower than that of each of these two PPMOs alone. When 5UP1 was combined with CP1, the luciferase yields were similar to the values of 5UP1 alone. The result demonstrated that the combination PPMO's enhanced effect on inhibition of target mRNA translation was sequence-specific.

Since the plasmid pCiNeoLuc-5UTR contains 5'UTR sequence of subgenomic RNA7, which has the target sequence of PPMO 5UP1 and 7P1, this plasmid was also used to assess the effect of PPMO combination on target RNA translation in transfected CRL11171 cells. The luciferase production was quantitatively measured and compared between the cells treated with PPMOs and the mock-treatment control. The combination of 5UP1 (0.5  $\mu$ M) + 7P1 (16  $\mu$ M) resulted in about 30% reduction in luciferase yield in comparison with treatment of 5UP1 (0.5  $\mu$ M) alone (Fig. 5B). The 7P1 at 16  $\mu$ M did not have detectable effect in this assay in comparison with mock-treatment control. The combination of 5UP1 (0.5  $\mu$ M) + CP1 (16  $\mu$ M) did not reduce the luciferase yield compared with 5UP1 (0.5  $\mu$ M) alone. This quantitative result further confirmed that combination of 5UP1 (0.5  $\mu$ M) + 7P1 (16  $\mu$ M) had synergistic inhibitory effect on the translation of target gene, while 5UP1 + CP1 has no such effect.

### 3.5. Inhibitory effect of PPMO combination against heterologous PRRSV strains

The PPMOs in this study were designed on the basis of the sequence of VR2385, a strain of North American genotype. To determine the efficacy of PPMO combination against other PRRSV isolates, cross-strain inhibition assay was conducted. PRRSV strains FL-12, 16244B, 16138, 11604, 17041, 14680, 12773, 13909, Ingelvac MLV, and Lelystad were used in this test. The Lelystad strain is the prototype of the European PRRSV genotype. The other strains used



**Fig. 4.** Immunofluorescence assay with N-specific antibody. Cells were infected with PRRSV and treated with PPMOs as indicated. IFA was conducted at 24 h p.i. PPMO combination of 5UP1 0.5  $\mu\text{M}$  with 4P1 or 7P1 at 16  $\mu\text{M}$  resulted in inhibition of PRRSV replication below detection level. The images below the green fluorescence images were taken under phase contrast from the same field to show total number of cells.

in this test belong to the North American genotype. Virus titration results showed that the combinations of 5UP1 + 4P1 or 5UP1 + 7P1 effectively inhibited PRRSV replication of all strains except Lelystad virus (Fig. 6A). Treatment of PRRSV-infected cells with PPMO combinations of 5UP1 at 0.5  $\mu\text{M}$  with 4P1 or 7P1 at 16  $\mu\text{M}$  led to virus yield below detection level in this assay. In contrast, replication of the Lelystad virus was not inhibited by any of the two combinations.

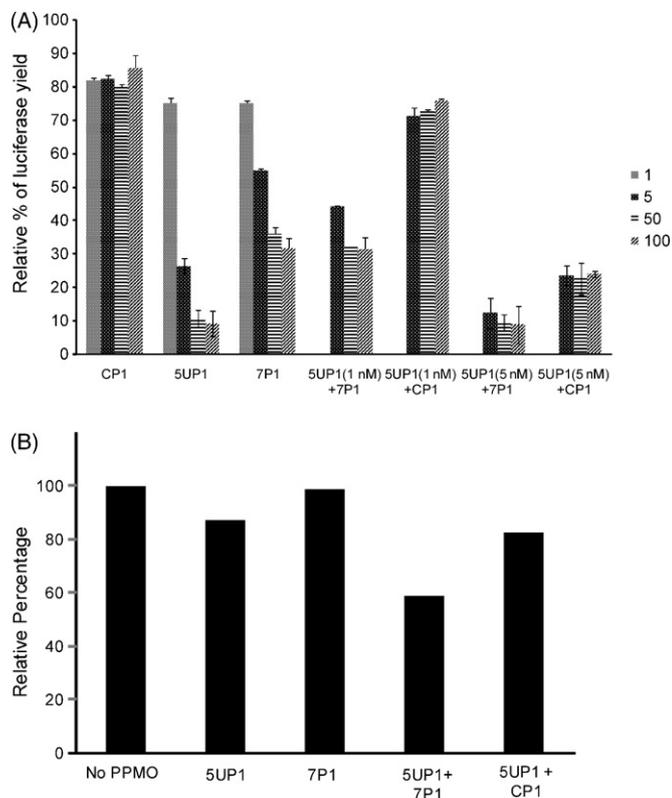
Sequence alignment of the PPMO target sites in the PRRSV strains showed that Lelystad virus has low sequence identity with the other strains. In comparison with VR2385, Lelystad has little similarity in the 5UP1 complementary sequence, 5 nt mismatch in the 4P1 complementary sequence, and 5 nt mismatch in the 7P1 complementary sequence (Fig. 6B). PRRSV 16244B has 4 nt mismatch with VR2385 at 5' end of the 5UP1 target site. All other strains have identical sequence with VR2385 in 5UP1 target site. In the 4P1 target site, only 1 nt mismatch was found in 14680, FL-12, and 11604 among all the North American PRRSV strains tested. In the 7P1 target site, one mismatch occurs in all strains except Lelystad. The results of cross-strain inhibition assay further confirmed the sequence-specific inhibition of PPMO on the PRRSV replication and indicates the potential application of the PPMO combination in the field against prevalent heterologous PRRSV isolates.

#### 4. Discussion

The high prevalence of PRRS and the limited efficacy of both inactivated and modified live attenuated vaccines have shown the

need to search for alternative control strategies. We have demonstrated enhanced specific inhibition of PRRSV replication by PPMO combination in this study. The 5' terminal UTR of PRRSV genome was found to contain the most productive target sites for inhibition of virus replication with PPMO and no cytotoxicity was detected at the concentrations used (Patel et al., 2008; Zhang et al., 2006). PPMO 5UP1 targeting the 5' terminus of PRRSV genome was found to be highly effective in inhibiting the PRRSV replication through interfering with the translation of the viral genomic RNA to PRRSV replicase. In this project, combination of 5UP1 with 4P1 or 7P1 led to effective inhibition of PRRSV replication in cell culture.

In our test of PPMO combinations, one of the PPMOs in the pairs was 5UP1, and the other one was from PPMOs against translation initiation regions of ORFs 2–7. PPMO 5UP1 was tested at 2 and 0.5  $\mu\text{M}$ , respectively. To our surprise, the combination of 5UP1 at 2  $\mu\text{M}$  with all the other PPMOs tested completely inhibited VR2385 replication to below detection level, while 5UP1 alone at 2  $\mu\text{M}$  had a little effect. This result suggests the enhanced effect of combination of two PPMOs. PPMO 5UP1 at the concentration of 0.5  $\mu\text{M}$  was paired with 8 or 16  $\mu\text{M}$  of the other PPMOs. Of the total six pairs in combination, two pairs, 5UP1 + 4P1 and 5UP1 + 7P1 showed effective inhibition of PRRSV replication. But individual PPMOs alone at the concentrations tested did not have inhibitory effect. Virus titration clearly demonstrated that 5UP1 combined with 4P1 or 7P1 was effective in a dose-responsive manner in inhibiting PRRSV production in infected cells. It is not clear whether the presence of 4P1 or 7P1 enhanced the blocking effect of 5UP1 or the PPMO pairs had the combinatory effect of blocking both PPMOs complemen-

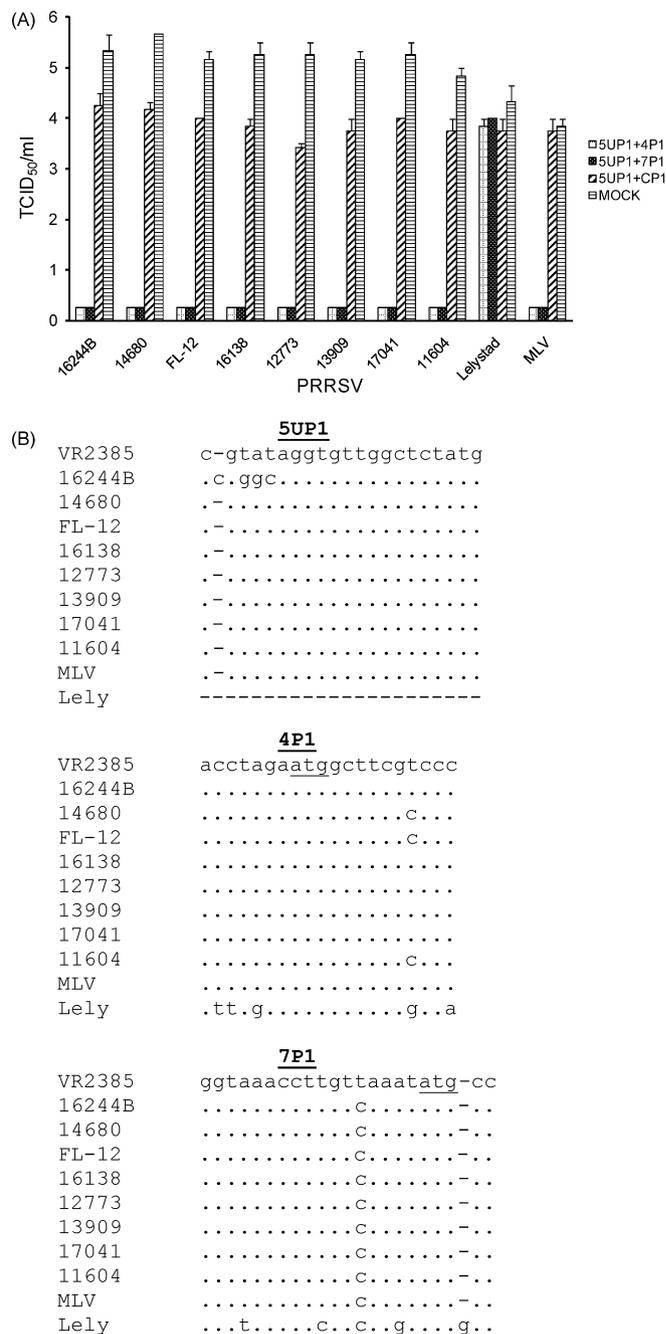


**Fig. 5.** Inhibition of target RNA translation by PPMOs in luciferase reporter assay. (A) Cell-free luciferase reporter assay. Relative percentages of inhibition were calculated in comparison with signal from mock-treatment control. PPMO concentrations of 1, 5, 50, and 100 nM were used in this assay and shown in different bars. PPMO 5UP1 at 1 and 5 nM was combined with different concentrations of 7P1 or CP1. Error bars show variation between three replicates. (B) Luciferase reporter assay in transfected cells. PciNeoluc-5UTR plasmid was transfected into CRL11171 cells and luciferase yield was measured. Relative percentages of luciferase yield were calculated in comparison with signal of none PPMO control. Combination of 5UP1 0.5  $\mu$ M + 7P1 16  $\mu$ M shows enhanced inhibitory effect, while 5UP1 + CP1 has no such effect.

tary sites. It is likely that the combinatory effect of blocking both target sites was responsible for the reduction of PRRSV replication since combination of 5UP1 with CP1 or the other PRRSV PPMOs did not have the enhanced effect. The reason that the other pairs were less effective is not clear, but could be due to inaccessibility of PRRSV target sequence or successful PPMO/target-RNA hybrid did not affect PRRSV replication. It is also speculated that GP<sub>4</sub> and N proteins have essential roles in PRRSV replication. However, we do not have data to support this speculation since GP<sub>4</sub> or N gene expression was not monitored and their roles in PRRSV replication are not well understood.

Our results are consistent with previous reports that PPMO combination yielded stronger inhibition than individual PPMOs (Ge et al., 2006; Patel et al., 2008). Other pairs of PPMOs, such as 2P1 + 3P1, 3P1 + 4P1, etc. were tested and no increasing inhibition was detected (Patel et al., 2008). But our study tested the combinations of PPMO targeting 5' terminus paired with others in complementary to translation initiation regions of ORFs 2–7 of PRRSV. Their effect on viral RNA synthesis and protein translation and potential mechanisms are investigated.

Treatment of cells with the combination of 5UP1 with 4P1 or 7P1 led to a significant reduction of PRRSV genomic RNA level, indicating inhibition of PRRSV RNA synthesis, which is likely accomplished through blocking translation of the replicase encoded by ORF1a/1b. The RNA level from the cells treated with the 5UP1 at 0.5  $\mu$ M with 4P1 or 7P1 at 16  $\mu$ M was about 2 log<sub>10</sub>, which was unexpected since the virus yield was below detection level. The possible reasons are



**Fig. 6.** Cross-strain inhibition assay. (A) Virus yield titration shows inhibition of nine North American PRRSV strains by PPMO combination of 5UP1 + 4P1 or 5UP1 + 7P1. Lelystad is a prototype of European PRRSV genotype. All other strains are North American PRRSV genotype. “Mock” is virus infection control without PPMO. Treatment of the cells with the two PPMO combinations led to suppression of PRRSV replication of all North American strains, which had virus yields not detectable in this assay, and bars are arbitrarily drawn to show the samples in the graph. (B) Sequence alignment of complementary sequences of 5UP1, 4P1, and 7P1, respectively, to show nucleotide mismatches between PRRSV strains. PRRSV strain names are listed in the first column. “Lely” stands for Lelystad strain, which has little similarity in the 5' UTR to other strains, as indicated by “-” in the alignment of 5UP1 target sequence. PPMO names are listed above the sequence. VR2385 sequence is used as the reference sequence, as the PPMOs were designed against it. For all other sequences, only nucleotides differing from the reference sequence are shown, and identical nucleotides are indicated as “.”. Missing nucleotides are indicated as “-”. The initiation codon ATG of ORFs 4 and 7 are underlined. GenBank accession numbers for PRRSV strains in the alignment are listed in parenthesis: 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.

that some replicase had already been produced at the time of PPMO treatment or that there was no complete virion production in the cells. The first speculation might be the case since the virus RNA translation initiates right after entry into the cells. It is expected that pretreatment of the cells with the PPMOs before virus inoculation would reduce the virus RNA translation. But our previous results showed that treatment post-virus inoculation led to stronger reduction of virus yield (Zhang et al., 2006). The second speculation is more plausible since the virus titration assay did not detect PRRSV from the cells treated with the two PPMO pairs. In addition to the inhibition at RNA level, the PPMO combination also led to inhibition of viral N protein expression. This result is consistent with the reduction of PRRSV genomic RNA level. The reduction of N protein in cells treated with 5UP1 + 4P1 indicates that PRRSV subgenomic RNA synthesis was reduced since 4P1 does not bind to subgenomic RNA7 and inhibit its translation.

To further elucidate the mechanism of the inhibition by PPMO combination, a cell-free luciferase translation assay was performed in the presence or absence of the PPMO combination. This assay avoids PPMO delivery issues in cell culture and directly tests the PPMO combinatory effect. For this assay, only the fragment of PRRSV 5'UTR region (312 nt) from subgenomic RNA7 was cloned into a luciferase reporter plasmid, as a proof of principle. Binding of PPMO pair to their RNA target sites inhibits the translation of target mRNA that encodes luciferase. Our results indicated that 5UP1 + 7P1 displayed enhanced effect in inhibition of translation of luciferase reporter mRNA in a sequence-specific and dose-responsive manner. The 5UP1 + 7P1 combination showed much lower luciferase yield than either of the two PPMOs alone at the concentration tested, while 5UP1 + CP1 combination had no effect. The results of the cell-free assay indicate that both 5UP1 and 7P1 complementary sites were blocked since both individual PPMOs and the combination showed dose-responsive inhibitory effect.

To examine the effect of PPMO combination on target mRNA translation in cells, a cell culture-based translation assay was also performed. Transient transfection of the luciferase reporter plasmid, which contains the PRRSV 5'UTR region upstream of the luciferase sequence, into the CRL11171 cells resulted in expression of luciferase. Similar enhanced inhibition on the translation of target RNA was confirmed by transfection of the luciferase reporter in the presence of the PPMO combination. The cell-based translation assay further verified the combinatory effect of 5UP1 and 7P1.

These two PPMO pairs were also shown to have an inhibitory effect against nine other North American PRRSV strains in our cross-strain inhibition assay, but had no inhibitory effect on the European Lelystad strain. Sequence alignment showed that 5'UTR region in PRRSV genome is quite conserved across strains in the same genotype. However, the European strain is quite different from the North American strains and shares only 40% sequence identity in 5'UTR and 67% in ORFs 2–7 with North American strains. This result further proved that the PPMO works in a sequence-specific manner, which is quite a good advantage of PPMO as an antiviral compound compared to small chemical drugs. These results indicate that the PPMO combination has beneficial implications to protect pigs against infection by heterologous PRRSV isolates.

As the most important swine disease, PRRS causes enormous economic losses in the swine industry. Due to its easy transmission and high variability of PRRSV, and the lack of a broadly protective vaccine, the control of this disease is difficult. Current vaccines available have limited effect, especially when used against the heterologous PRRSV strains, which are commonly found in outbreaks. PPMO combinations of 5UP1 + 4P1 and 5UP1 + 7P1 inhibited replication of 10 North American PRRSV strains in our cross-strain inhibition assay. Sequence alignment showed that PRRSV sequences targeted by these PPMOs are highly conserved. Analysis of PRRSV sequences from the GenBank indicates that the comple-

mentary sequences of 5UP1 and 7P1 are highly conserved across North American PRRSV strains (Patel et al., 2008). Thus, these two pairs of PPMOs have the potential for application against most North American PRRSV strains. It would be a plausible advantage to use PPMO combinations rather than a single PPMO in consideration of heterologous PRRSV strains in the field and potential benefit to have less chance in generation of escape mutant virus. Further investigation into the pharmacokinetic, toxicological, and antiviral properties of these PPMO combinations *in vivo* against heterologous PRRSV strains is warranted.

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