RESEARCH REPORT
SWINE HEALTH

Title: Development of a New Generation of Antisense Antiviral Drug against PRRSV – **NPB #06-168**

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Industry Summary:

This project has explored a novel class of antiviral drugs to suppress PRRSV replication. These drugs were designed on the basis of PRRSV genomic sequences. Their specific binding to the virus genome is expected to interfere with virus replication. Such compounds were found to be effective in inhibition of PRRSV replication in cell culture, including porcine primary alveolar macrophages, in a dose-responsive manner. Addition of these compounds to cells with experimental PRRSV inoculation reduced the virus yield to less than 0.01% in comparison to the cells of mock treatment control. Combination of two such compounds led to more effective inhibition than individual one. These compounds effectively inhibited virus replication of heterologous strains from North America. Treatment of the primary alveolar macrophages with one such compound protected the cells from PRRSV-induced cell death. In animal test with piglets, administration of one such compound reduced lung lesion and viremia from PRRSV infection. These results indicate that the antiviral compounds are potential anti-PRRSV drugs to complement other strategies to control PRRS.

For further questions on this project, please contact me at zhangyj@umd.edu.

Scientific Abstract:

The prevalence of PRRSV infection in swine herds is high and currently no effective strategies are available to control the infection. The current vaccines are unable to control PRRSV, and in some cases, even posed problems due to reversion of the modified live PRRSV vaccine to the pathogenic phenotype. Specific anti-PRRSV drugs are needed to complement other strategies in PRRS prevention and control. In this project, we proposed to continue our exploration of a new generation of molecular antiviral compounds, Phosphorodiamidate Morpholino Oligomer (PMO). PMOs are analogs of short DNA oligonucleotides with modified chemical structures, resulting in highly specific binding to target sequences and stable presence in the host. The PMOs are designed to specifically bind to PRRSV genomic RNA and block PRRSV replication. Two pairs of PMOs were identified to have enhanced suppression of PRRSV replication in cell culture, while individual constituent did not work under the same testing conditions. PMO 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 PMO combination also inhibited replication of heterologous PRRSV strains in the North American genotype. We also examined in

detail PMO-mediated inhibition of PRRSV replication in primary cultures of porcine pulmonary alveolar macrophages (PAM). Treatment of PAM with PMO 5UP2 resulted in protection from PRRSV-induced cell death for at least seven days, and produced no elevation in caspase activity. The virus titer in PAM was reduced by 99% in comparison with controls. In a preliminary test of in piglets, intranasal delivery of PMO 5UP2 reduced microscopic lung lesion that was induced by PRRSV infection. The piglets after PMO treatment had similar average daily weight gain to other control groups, indicating that the PMO compound had no detectable toxicity. These results indicate that the antiviral compounds are potential anti-PRRSV drugs to complement other strategies to control PRRS.

Introduction:

The prevalence of PRRSV infection in swine herds is high and currently no effective strategies are available to control the infection. The current vaccines are unable to control PRRSV, and in some cases, even pose problems due to reversion of the MLV PRRSV vaccine to the pathogenic phenotype. Specific anti-PRRSV drugs are needed to complement other strategies in PRRS prevention and control. This project intends to develop a novel class of antivirals, antisense PMOs, to truncate PRRSV infection and help PRRSV elimination. The PMO compounds are analogs of short DNA oligonucleotides with modified chemical structure, resulting in high specific binding to target sequences and complete resistance to degradation in the host. The compounds are designed on the basis of PRRSV genomic sequences with a goal to block PRRSV replication. Application of the specific anti-PRRSV drugs could yield significant economic benefits to swine industry, especially for breeding farms, by preventing or eliminating PRRSV infection.

Objectives:

1. To determine combinatory cross-strain inhibitory effect of the PMO compounds against heterogeneous PRRSV strains

2. To test dosage and delivery route of selected anti-PRRSV compounds in pigs

3. To test safety, efficacy and pharmacokinetics of anti-PRRSV PMOs in pigs with experimental infection

Materials & Methods:

PMO synthesis: PMOs were synthesized at AVI BioPharma Inc. (Corvallis, OR). The peptide NH-$(RXR)_4$XB-COOH (where R=Arginine, X =6-aminohexanoic acid, and B=beta-alanine) was covalently conjugated at the 5' end of each PMO. The conjugation, purification, and analysis of $(RXR)_4$XB-PMO (P7-PMO) compounds (referred to simply as 'PMO' in the remainder of this report) were similar to the methods described elsewhere (5, 7, 10). A random sequence PMO (named 'CP1') having little agreement with PRRSV or primate mRNA sequences was also utilized to control for non-sequence-specific activity of the P7-PMO chemistry.

Cells and virus: ATCC CRL11171 cells and a virulent PRRSV strain VR2385 were used in this study. Porcine alveolar macrophages (PAM) cells were isolated by infiltrating of alveolar lavage from 3-4-week-old healthy pigs. For virus titration, 10-fold series of dilutions were made and tested on the monolayer CRL11171 cells. Cytopathic effect (CPE) was observed and recorded at 48 h post inoculation in CRL11171 cells. For PAM, CPE was observed and recorded at 24 h and 48 h post-infection. Tissue culture infectious dose ($TCID_{50}$) was calculated according to the method of Reed and Munch. Other PRRSV strains used in this study include LV, 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).

PMO treatment: The cells were plated in 12-well cell culture plates and grown overnight. VR2385 virus was diluted and inoculated to the cells at 0.5 multiplicity of infection (MOI). After 2 h incubation, the

virus was removed and the monolayer cells were rinsed with plain cell culture medium DMEM. PMO were diluted in plain DMEM and added to the monolayer cells. After gentle mixing, the cells were incubated at 37^{o}C for 4 h. After 4 h incubation, the PMO solution was removed from each well of the plate and maintenance cell culture medium was added. The cells were further incubated at 37°C for 48 h. CPE was observed and recorded. Cell culture supernatant was harvested for titration of virus yield. PAM cells were plated in 24-well plates at a density of 5X10^{5} cells/well in this experiment.

Caspase detection and cell viability assay: The assays for activities of caspase-3, and -7 were performed with Caspase-Glo 3/7 Assay System (a single kit to detect activities of both caspases concurrently) as per the manufacturer's instructions (Promega, Madison, WI). Briefly, PAM cells were cultured in 96-well plates for 24 or 72 h prior to virus inoculation. At 24 h post virus inoculation, Caspase-Glo reagent was added to the cells at 1:1 ratio and the luminescence signal was then detected with VICTOR3^{TM} Multilabel Counter (Perkin-Elmer Life and Analytical Sciences, Wellesley, MA).

PAM viability was determined with CellTiter-Glo Cell Viability Assay (Promega). Briefly, CellTiter-Glo reagent was added to cells and incubated for 10 minutes at room temperature. The luminescence signal was measured with Multilabel Counter. Relative percentages of luminescence intensity were calculated by comparison to mock-infected and mock-treated controls.

Animal test: Two animal tests were conducted. In the first test, three-week old SPF piglets were randomly divided into 6 groups with 6 pigs in each group (Table 1). The administration of PMO 5UP2 and virus followed the design listed in Table 1. The piglets in all groups except group 6 were inoculated with 1 ml of PRRSV VR2385 (10^5 $TCID_{50}$/ml) via intra-nasal route (I.N.). The PMO 5UP2 was given one dose via I.N. route every day, for a total of 3 doses at -24, 2, 24 h post virus inoculation. For monitoring virus shedding, 2 pigs were added on day 4 to each group as contact controls.

Table 1. PMO 5UP2 test in piglets (1)

Event	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Total
VR2385 10^5 $TCID_{50}$/pig	Yes	Yes	Yes	No	Yes	No	
PMO	5UP2	5UP2	5UP2	5UP2	None	None	
PMO dosage (mg/kg)	0.1	0.5	1.0	1.0	None	None	
PMO delivery route	I.N.	I.N.	I.N.	I.N.			
PMO inoculation schedule	-24, 2, 24 h p.i.	-24, 2, 24 h p.i.	-24, 2, 24 h p.i.	-24, 2, 24 h p.i.			
ELISA on day 14	Yes	Yes	Yes	Yes	Yes	Yes	
Piglet numbers	6	6	6	6	6	6	36
Contact control piglets	2	2	2	2	2	0	10
Necropsy on day 14 p.i.	Yes	Yes	Yes	Yes	Yes	Yes	

Rectal temperatures were recorded daily for 14 days. Blood samples were collected for antibody detection by IDEXX ELISA kit. Necropsies were performed on day 14 post virus inoculation. The severity of gross lung lesions were scored as reported previously (2, 3). Samples of lung and lymph node were collected during necropsy for histological examination, scoring and immunohistochemistry. Evaluation of gross and microscopic lesions was done in a blinded fashion.

In the second animal test, three-week old SPF piglets were randomly divided into 4 groups with 5 in each group (Table 2). The administration of PMO and virus followed the design listed in Table 2.

PMO and virus administration, blood collection, necropsy, ELISA and lung lesion scoring were done similarly as described above for the first test.

Table 2. PMO test in piglets (2)

Event	Group 1	Group 2	Group 3	Group 4	Total
VR2385 10^5 $TCID_{50}$/piglet	Yes	Yes	Yes	No	
PMO	5UP2	5UP2	None	None	
PMO dosage (mg/kg)	0.1	0.5	None	None	
PMO delivery route	I.N.	I.N.			
PMO inoculation schedule	-24, 2, 24 h p.i.	-24, 2, 24 h p.i.			
ELISA on serum of day 14	Yes	Yes	Yes	Yes	
Piglet numbers	5	5	5	5	20
Contact control piglets	2	2	2	0	6
Necropsy at day 14 p.i.	Yes	Yes	Yes	Yes	

RNA isolation and real-time RT-PCR (**reverse transcription-PCR):** Total RNA was extracted from 250 µl pig serum samples by TRIzol® LS Reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). The RNA was quantified using µQuant™ Universal Microplate spectrophotometer (BioTek Instruments, Winooski, Vermont). Quantitative real-time RT-PCR was done as described previously (Zhang et al. 2006; Patel et al. 2008). PRRSV ORF7 was cloned into pcDNA3 vector, and used as template to generate standard curves. The SYBR Green real-time PCR was performed on Chromo 4™ Four-Color Real-Time System (Bio-Rad). PRRSV RNA copies were calculated based on standard curve and shown as $\log_{10}$. Relative percentages of PRRSV RNA copies in comparison with PRRSV/no PMO group were also shown.

Statistical analysis: The significance of differences of viral yield or other parameters between the groups of PMO-treated cells was assessed by Student *t*-test. A two tailed *P*-value of less than 0.05 was considered significant.

Results:

Objective I. To determine combinatory cross-strain inhibitory effect of the PMO compounds against heterogeneous PRRSV strains

1. Determining combinatory effect. PMO 5UP1 was found to be effective in inhibiting PRRSV replication in cell culture (10). Treatment of CRL11171 cells with 5UP1 at the final concentration of 2 µM resulted in a moderate (about 1.0 $\log_{10}$) reduction in PRRSV yield. PMO 5UP1 was designed to complement the 5' terminal region of PRRSV genome in an attempt to block translation of viral RNA replicase. To evaluate the effect of PMO combination on inhibiting PRRSV replication, the PMO 5UP1 was paired with each of the following individual PMO: 2P1, 3P1, 4P1, 5P1, 6P1, and 7P1 at different concentrations. The 2P1, 3P1, 4P1, 5P1, 6P1, and 7P1 were designed to be complementary to the translation initiation regions of PRRSV ORFs 2-7, respectively, to inhibit the

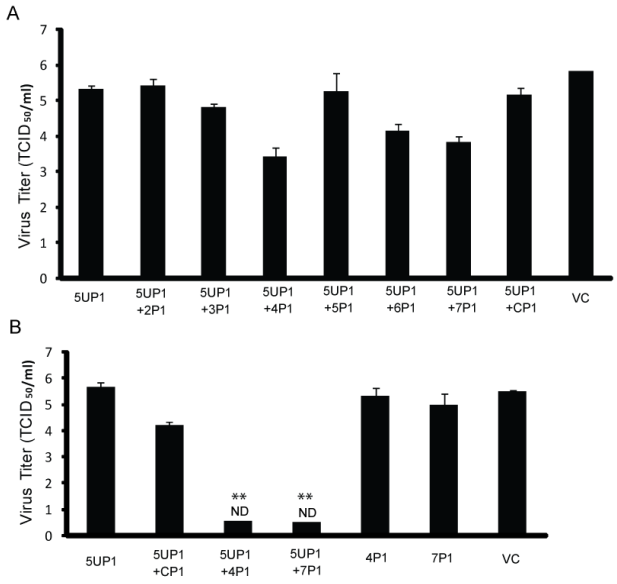

Fig. 1. Enhanced inhibition of PRRSV replication in infected CRL11171 cells by PMO combination. A. Titration of virus yield from CRL11171 cells after PPRSV infection and indicated PMO treatment. Virus titer is shown as $TCID_{50}$ ($\log_{10}$/ml). PMO 5UP1 was used at 0.5 µM, and other PMO used at 8 µM. "VC" indicates cells with virus infection but no PMO treatment. B. Virus yields from cells treated with combination of 5UP1 at 0.5 µM and other PMO used at 16 µM. Cells that were treated with PMO 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.

translation of these ORFs. All these PMO sequences are complementary to the genomic sequence of VR2385, a virulent strain of North American genotype (6, 10). A moderate reduction of PRRSV replication was also observed for the cells treated with PMO 6P1 or 7P1 at a relatively high concentration, but the PMO 2P1, 3P1, 4P1, and 5P1 were found to have no effect (6).

In our initial test, the 5UP1 at the concentration of 2 μM was paired with 16 μM of each of the other PMO. Compared to the control group, treatment of CRL11171 cells with 2 μM 5UP1 alone reduced the PRRSV yield for 1.0 $\log_{10}$ $TCID_{50}$/ml, which is in agreement with the previous observation (10). In contrast, treatment of CRL11171 cells with a combination of 2 μM 5UP1 and 16 μM of other PMO against PRRSV resulted in complete inhibition of PRRSV replication, and the virus yield was below detection level (data not shown). To further assess the effect of PMO combination, the 5UP1 concentration was reduced from 2 to 0.5 μM and the other PMO were reduced to 8 μM. Treatment with 5UP1 alone at the concentration of 0.5 μM did not reduce virus yield compared to virus control or mock treatment (Fig. 1A). Among all these PMO pairs tested, a combination of 0.5 μM 5UP1 and 8 μM 4P1, 6P1, or 7P1 showed more enhanced inhibition effects on PPRSV replication than other PMO pairs (Fig. 1A). CRL11171 cells treated with 0.5 μM 5UP1 in conjunction with 8 μM 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 μM of 4P1 or 7P1 in combination with 0.5 μM of 5UP1 strongly inhibited PRRSV replication and virus yields were below detection level, while each individual PMO alone did not show detectable effect at the same concentrations tested (Fig. 1B). This experiment was repeated three times and similar results were observed each time. These results demonstrated that combination of 5UP1 (0.5 μM) with 4P1 or 7P1 (8 or 16 μM) was more effective in inhibiting PRRSV replication than a constituent individual PMO of the pairs.

2. Inhibitory effect of PMO against heterologous PRRSV strains.

To determine the efficacy of PMO 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. Other strains belong to 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. 2). Treatment of PRRSV-infected cells with PMO combinations of 5UP1 at 0.5 μM with 4P1 or 7P1 at 16 μM 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. It is not surprising that the PMO has little effect on Lelystad virus because Lelystad virus has low sequence identity with the other strains. In comparison with VR2385, Lelystad has 13 nt mismatch in the 5UP1 complementary sequence, 5 nt mismatch in the 4P1 complementary sequence, and 5 nt mismatch in the 7P1 complementary sequence. The results of cross strain inhibition assay further confirmed

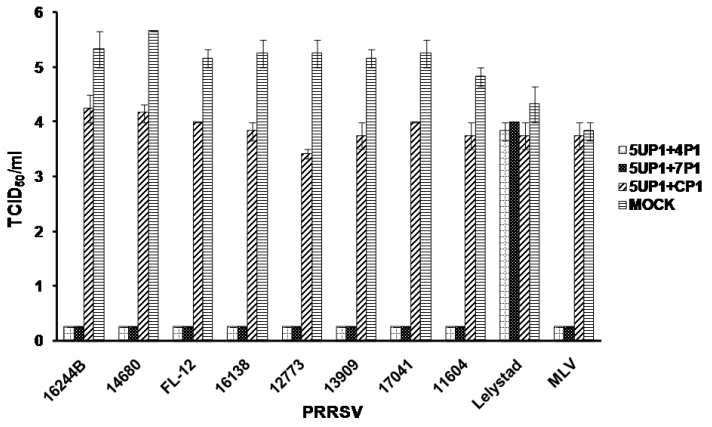

Fig. 2. Cross strain inhibition assay. Virus yield titration shows inhibition of nine North American PRRSV strains by PMO combination of 5UP1+4P1 or 5UP1+7P1. Lelystad is a prototype of European PRRSV genotype. All other strains are North American PRRSV genotype. "Mock" is virus infection control without PMO. Treatment of the cells with the two PMO 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.

the sequence-specific inhibition of PMO on the PPRSV replication and indicates the potential application of the PMO combination in the field against prevalent heterologous PRRSV isolates.

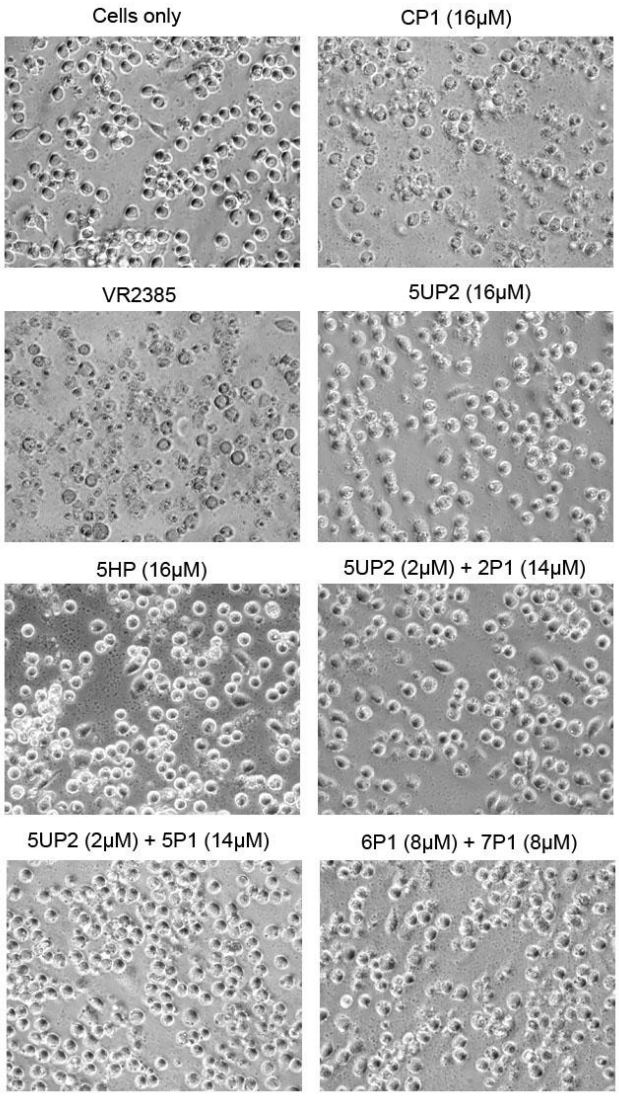

Fig. 3. PMO-mediated suppression of PRRSV replication in porcine alveolar macrophages. Inoculation of the cells with VR2385 leads to severe CPE development (VR2385) in comparison with uninfected control cells (cells only). Note the difference between cells treated with CP1 and cells with other PMO.

3. PMO mediated suppression of PRRSV replication in PAM:

Porcine alveolar macrophages are the primary target cells for PRRSV infection in pigs and evaluation of PMO effect on virus replication in PAM has relevance to the biology of a natural infection. We demonstrated that treatment with PMO 5UP2, 5HP, or combination of, 5UP2+2P1, 5UP2+5P1, and 6P1 + 7P1 protected PAM from PRRSV-induced CPE and death. No CPE was visible for one week in PAM treated with PMO 5UP2, indicating that PMO treatment protected PAM from PRRSV CPE for at least 1 week after PRRSV inoculation (Fig.3). At 4 days post-infection, PRRSV re-inoculation of the PAM that were treated with PMO 5UP2 did not lead to PRRSV replication. Cell viability assay of the PAM showed that the cells were live and had similar viability to non-infected control cells (Fig. 4). IFA and PRRSV yield titration were conducted and result was consistent with the CPE observation. Culture supernatant was collected every day from PRRSV-infected PAM and titrated on CRL11171 cells for PRRSV yield. Treatment with PMO 5UP2 reduced virus yield by over 99% in comparison with PRRSV-infection control (Fig. 5). After fourth day post infection, the virus yield in PMO-treated well was under detection level. These results clearly showed that the PMO treatment provided protection of the cells from PRRSV infection. To further explore the mechanism of the protection, we conducted assays to determine the apoptosis pathway after PRRSV infection of PAM.

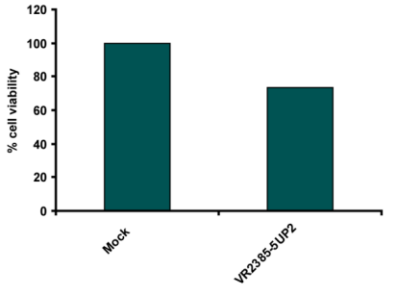

Fig.4. Cell viability assay of PAM that were infected with PRRSV and treated with PMO 5UP2. Non-infected and non-treated PAM was included as control. The viability was shown as percentage of control cells.

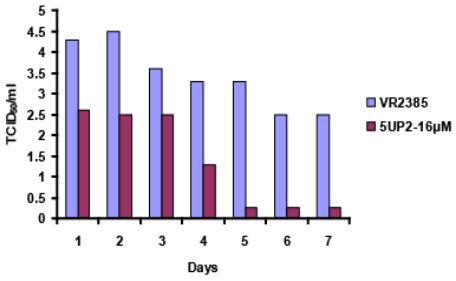

Fig. 5. Titration of virus yield from PAM cells after PRRSV infection and PMO treatment. "VR2385" sample is PAM that was infected with virus but no PMO treatment.

4. Protection of PAM from PRRSV-induced apoptosis.

It was reported that PRRSV induces apoptosis through a mitochondria-mediated pathway in MARC-145 cells (4). However, the detail mechanism of apoptosis in PAM, relevant to the biology of a natural host, is largely unknown. We tested the caspase 3/7 level in PAM post-PRRSV infection using Caspase 3/7-glo kit (Promega). The treatment of PAM with PMO 5UP2 protects cells from PRRSV induced apoptosis. Results showed that caspase 3/7 level increased significantly in PAM post infection (Fig. 6A). The PAM of 72-h pre-incubation had higher caspase level than 24-h pre-incubation. PRRSV-inoculated PAM showed approximately 3.5-fold higher caspase level in 24 h pre-incubated PAM and approximately 6-fold higher in 72 h pre-incubated PAM in comparison to mock-treated control. The presence of PMO 5UP2 prevented the elevation of caspase level in PAM. We also demonstrated that the caspase elevation was due to the active virus replication since UV-inactivated virion or 5UP2 alone did not affect the caspase level (Fig. 6B).

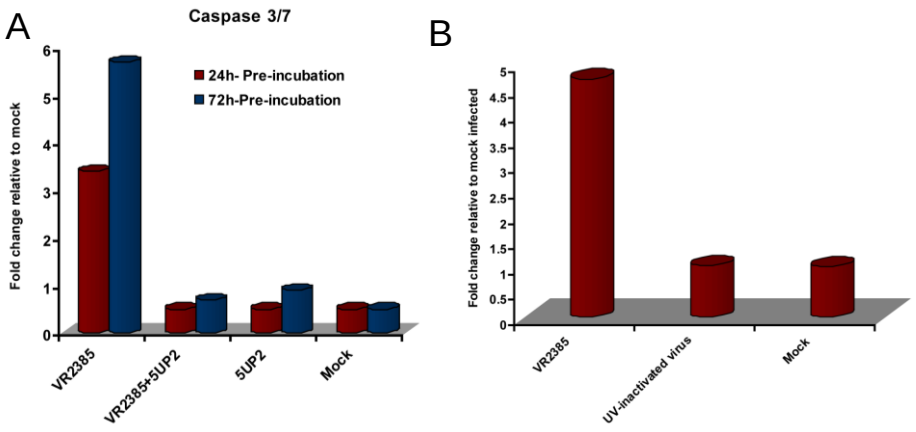

Fig. 6. Protection of PAM from PRRSV-induced apoptosis. (A) Detection of caspase (3/7) level in PRRSV-inoculated PAM and effect of PMO 5UP2 treatment. PAM were pre-incubated for 24 h or 72 h before PRRSV infection. (B) Detection of caspase (3/7) level in PAM inoculated with live or UV-inactivated virus. UV-inactivated virus didn't lead to elevation of caspases.

These data clearly demonstrated that PMO treatment protected PAM from PRRSV-induced apoptosis. PRRSV infection of PAM leads to elevation of caspase 3 and 7, while the presence of PMO prevented the elevation.

Objective 2. To test dosage and delivery route of anti-PRRSV PMOs in piglets

We have tested dosage and delivery of PMO 5UP2 in piglets that were experimentally infected with PRRSV. PMO 5UP2 was selected due to its exceptional performance in cell culture. Two animal tests were conducted.

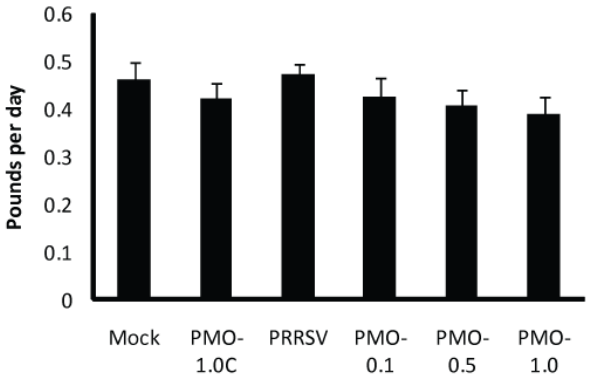

Fig. 7. Average daily weight gain of pigs in PMO test. Mock group is normal pigs without virus infection or PMO treatment. Group "PMO-1.0C" is the control group of PMO treatment at dose of 1.0 mg/kg body weight without PRRSV infection. Group "PRRSV" is virus infection control without PMO treatment. The rest groups were infected with PRRSV and treated with PMO at 0.1, 0.5, or 1.0 mg/kg body weight.

1. *Animal test 1*. Experimental design of the first animal test is illustrated in Table 1. Three doses of PMO 5UP2 at 0.1, 0.5, and 1.0 mg/kg body weight were tested. The intranasal delivery route was selected since PRRSV causes a respiratory disease in young pigs and the virus challenge is through intranasal route. The PMO was given at -24, 2, and 24 h post PRRSV inoculation. A control group without PRRSV challenge was included to test the PMO toxicity in piglet. A virus-infection control group and a normal piglet group were also included for comparison.

The piglets were weighed before the start of the experiment and again before necropsy. Average daily weight gain was calculated to assess the effect of the PMO treatment and PRRSV infection. There is no significant difference (p = 0.46) between the groups of treatment and controls (Fig. 7), indicating that

the PMO treatment did not have adverse effect on pig growth. The average daily weight gain in groups of PMO-treated pigs was a little lower than "Mock" and "PRRSV" groups, which could be because of the additional stress during intranasal administrations of PMO solution in initial three consecutive days. But the difference between groups was not statistically different. The PMO dose at 1.0 mg/kg was well tolerated in pigs and higher doses can be tried in future.

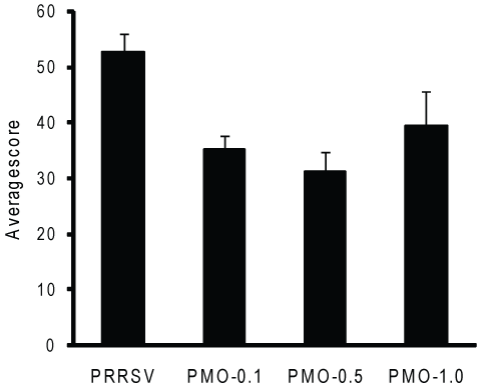

Fig.8. Average gross lung lesion score in PRRSV-infected pigs. The higher the score, the worse the lung lesion. A scale of 0-6 score was assigned to different parts of lung.

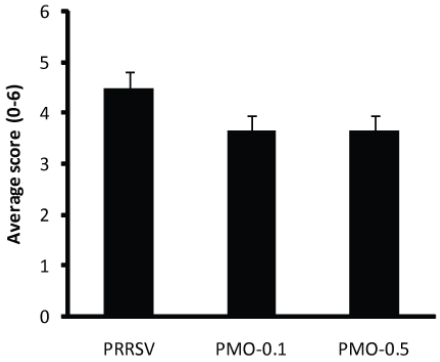

Fig. 9. Average microscopic lesion score. There is significant difference between PRRSV and PMO groups (P=0.02).

Gross lung lesion was scored during necropsy to assess the pneumonia status. There was significant difference between groups of PRRSV-infected pigs (p = 0.009) (Fig. 8). All PMO-treated groups had lower average gross lung lesion score than mock-treatment control "PRRSV", indicating PMO-mediated protection against PRRSV infection.

The lung tissue was further examined under microscope for interstitial pneumonia. This was scored ranging from 0 (=absent) to 6 (=severe diffuse). The groups of PMO 0.1 and 0.5 mg/kg had lower average score of interstitial pneumonia than group "PRRSV" and the difference was significant (p = 0.02) (Fig. 9), indicating that the PMO treatment led to certain level protection. The group of PMO 1.0 mg/kg did not have significant difference from group "PRRSV".

ELISA was done and PRRSV antibodies were detected in pigs from all PRRSV-infected groups. No significant difference in titer or trend between groups could be seen, indicating ELISA titer of PRRSV antibodies does not correlate with PRRSV protection.

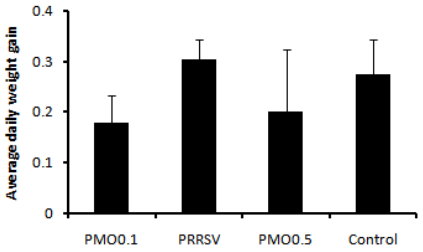

Fig. 10. Average daily weight gain of pigs in PMO animal test 2.

2. *Animal test 2.* The second animal test was conducted as illustrated in Table 2. Based on the results of the first animal test, we designed the second test to further examine the effect of PMO 5UP2 in piglets. Since two doses of PMO 5UP2 showed inhibitory effect, these two doses of 0.1 and 0.5 mg/kg were selected for further test in piglets. Also the PMO 5UP2 was tested in cell culture before used for pig test.

The piglets were weighed before the start of the experimer and again before necropsy. Average daily weight gain was calculate to assess the effect of the PMO treatment and PRRSV infectior There is no significant difference (p = 0.64) between the groups o treatment and controls (Fig. 10), which is consistent with the first tes

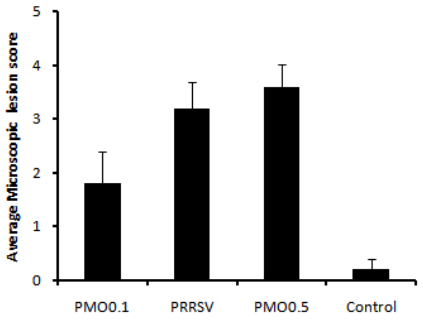

Fig. 11. Average microscopic lesion score in PMO test 2.

Gross lung lesion was scored during necropsy to assess th pneumonia status. But very low scores were noted for many pigs in this test. Thus it was not possible to assess the gross lung lesion score for this test.

The lung tissue was further examined under microscope for interstitial pneumonia as described for test 1. The group of PMO 0.1 had lower average score of interstitial pneumonia than group "PRRSV" and 0.5 mg/kg (Fig. 11). The difference was not statistically significant. However, if one pig with the highest score was removed from each group, the group of PMO 0.1 had statistical lower average score of

interstitial pneumonia than group "PRRSV" (P<0.01). Unexpectedly, the PMO0.5 group had similar score to the PRRSV control group.

ELISA was done and PRRSV antibodies were detected in pigs from all PRRSV-infected groups. Interestingly, the ELISA ratio for PMO0.5 group was the lowest among the three groups tested (Fig. 12). Only two pigs in PMO0.5 group had the ratio above 0.4, which is considered as positive for PRRSV antibody.

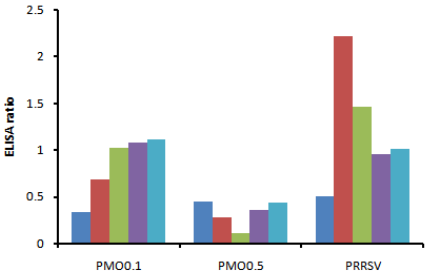

Fig. 12. ELISA ratio of serum samples of day 14. All pigs in PMO0.1 and PRRSV groups are positive on day 14, but only two pigs in PMO0.5 group were positive.

PRRSV viremia was tested for serum samples of 6 and 10 dpi in this experiment. RNA was isolated from serum samples by Trizol LS and reverse-transcribed to cDNA. Real-time PCR was performed to detect PRRSV RNA with primers from PRRSV ORF7. Successful RNA isolation and real time PCR were performed for 3 samples of 6 dpi and 4 samples of 10 dpi pigs in each of the three groups, PRRSV/no PMO, PMO0.1 and PMO0.5. Compared to PRRSV/no PMO group, the PMO0.1 and PMO0.5 groups reduced PRRSV RNA copies significantly (P=0.01) (Fig. 13A). To show the difference between the different groups, relative percentages of the PRRSV RNA copies were calculated and shown in Fig 13B. The PRRSV RNA copies in PMO0.1 and PMO0.5 groups were 27% and 8%, respectively, compared with no PMO group. The viremia test of 10 dpi samples showed the similar trend that PMO groups had lower PRRSV RNA level (Fig. 14). But the reduction of PRRSV RNA copies was much less. The PRRSV RNA copies in PMO0.1 and PMO0.5 groups were 76% and 48%, respectively, compared with no PMO group.

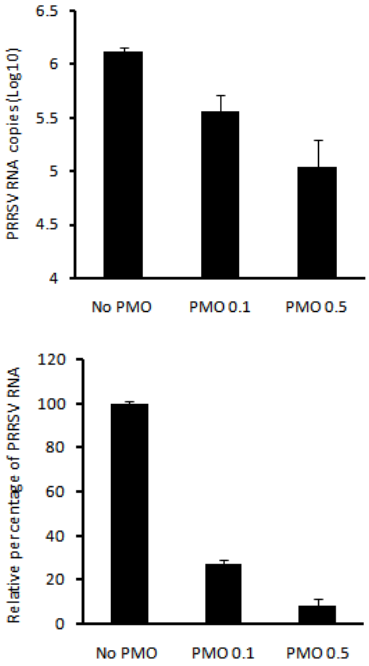

Fig. 13. PRRSV viremia on 6 dpi detected by real-time PCR. A. Average PRRSV RNA copies shown as $\log_{10}$. Error bars show the variation among three pigs in each group. B. Relative percentage of average PRRSV RNA copies. Error bars show the variation among three pigs in each group.

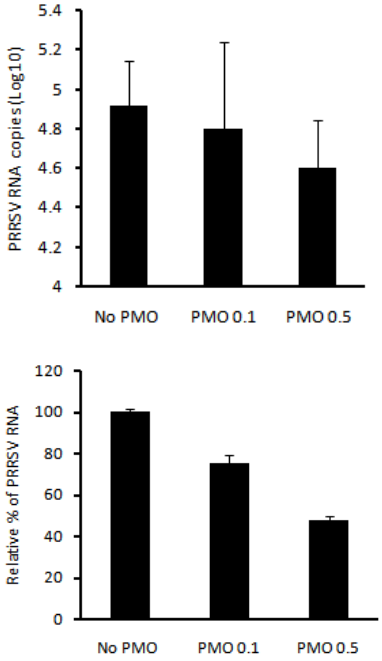

Fig. 14. PRRSV viremia on 10 dpi detected by real-time PCR. A. Average PRRSV RNA copies shown as $\log_{10}$. Error bars show the variation among three pigs in each group. B. Relative percentage of average PRRSV RNA copies. Error bars show the variation among three pigs in each group.

Objective 3. To test safety, efficacy and pharmacokinetics of anti-PRRSV PMOs in pigs with experimental infection.

This objective was designed for year 2 study. It was not conducted since no further funding was given.

Discussion:

In this project, we continued our study on PMO compounds that specifically inhibit PRRSV replication. The 5' terminal UTR of PRRSV genome was found to contain the most productive target sites for inhibition of virus replication with PMO (6, 10). PMO 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 PMO combinations, one of the PMO in the pairs was 5UP1, and the other one was from PMO against translation initiation regions of ORFs 2-7. PMO 5UP1 was tested at 2 and 0.5 µM, respectively. PMO 5UP1 at the concentration of 0.5 µM was paired with 8 or 16 µM of other PMO. Of the total 6 pairs in combination, two pairs, 5UP1+4P1 and 5UP1+7P1 showed effective inhibition of PRRSV replication. But individual PMO alone at the concentration 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 PMO pairs had the combinatory effect of blocking both PMO complementary 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 other PRRSV PMO 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 PMO/target-RNA hybrid did not affect PRRSV replication.

These two PMO pairs were also shown to have an inhibitory effect against 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 ORF2-7 with North American strains. This result further proved that the PMO works in a sequence-specific manner, which is quite a good advantage of PMO as an antiviral compound compared to small chemical drugs. These results indicate that the PMO combination has beneficial implications to protect pigs against infection by heterologous PRRSV isolates.

Application of the PMO compounds to swine primary alveolar macrophages also led to inhibition of PRRSV replication. Since the macrophages are main target cells for PRRSV in pigs, the PMO-mediated inhibition in these cells are meaningful for further testing in host animals. Treatment of the cells with 5UP2 PMO inhibited PRRSV replication and resulted in lower levels of apoptotic-associated gene expression than those observed in infected control samples. Although a low virus yield was detectable in 5UP2-treated PAM, the cells were protected from PRRSV-induced cell death for at least 7 days. This study also provided further insight into the cellular events associated with PRRSV induction of apoptotic-responses.

The presence of a small amount of virus in PRRSV-infected and 5UP2-treated PAM suggests that some infecting virus managed to proceed through replication without being duplexed by PMO. One possible reason is that the mechanism of PRRSV replication is likely somewhat different in primary PAM culture than in a continuous cell line. Previous reports have suggested that PMO targeting the 5' terminal region of the arterivirus genome are likely effective through interference with translation of viral RNA (8, 10). The PMO component of PMO has been shown to remain functionally stable inside cells for several days (9).

Caspases play essential roles in apoptosis. Numerous studies have demonstrated that viruses induce apoptosis via extrinsic and/or intrinsic pathways. Crosstalk between the extrinsic and intrinsic pathways has been demonstrated in PRRSV infection of MARC-145 cells (4). Our study indicates that the activation of caspase 3/7 was the direct result of PRRSV replication in PAM. The 5UP2 PMO treatment of infected PAM reduced viral replication and, as a result, prevented caspase activation. It was recently reported that PRRSV infection of PAM lead to apoptosis after 12 hpi (1). Our data are consistent

with that report, in that the activation of caspase 3/7 in PRRSV-infected PAM was significantly increased at 24 hpi.

Administration of PMO 5UP2 to piglets reduced interstitial pneumonia that was induced by PRRSV infection. In the two animal tests conducted, the PMO 5UP2 did not cause any adverse effect on average daily weight gain, indicating the low toxicity of the compound. Three doses of 5UP2 were given in the first animal test. The 0.1 and 0.5 mg/kg groups had significant lower microscopic lung lesion scores than control group. The 1 mg/kg group did not reduce the lung lesion score, which indicates that the dosage might be too high. Thus, only two doses of 5UP2 were tested in the second animal test. A new batch of 5UP2 was used and verified to be capable to inhibit PRRSV replication in cell culture. The PMO 0.1 mg/kg group reduced interstitial pneumonia in comparison with PRRSV control group. But PMO 0.5 mg/kg group did not lead to any difference. Only two pigs in PMO 0.5 mg/kg group had low antibody production on day 14, indicating the delay of antibody induction by PRRSV infection. So we conducted viremia test by real time PCR to detect PRRSV RNA in serum samples at 6 and 10 dpi, respectively. Both PMO groups reduced PRRSV RNA copies significantly in comparison with PRRSV/no PMO group at 6 dpi. PMO0.5 group had the lowest level of viral RNA in serum samples, which might be the reason for the low ELISA antibody titers in this group at 14 dpi. The difference in viral RNA among the three groups was minimized in serum samples at 10 dpi. This result indicates that the virus replication in PMO0.5 group was suppressed initially but gain speed later.

In summary, we have demonstrated that PMO effectively inhibited PRRSV replication in cell culture, including primary alveolar macrophages. PMO 5UP2 protected PAM from PRRSV-induced cell death for at least seven days. No cytotoxicity was detected in the testing concentrations of the compound. In piglets with experimental PRRSV infection, administration of PMO 5UP2 provided certain level protection against PRRS, shown by the lower levels of lung lesion score, interstitial pneumonia, and viremia. These results indicate that the specific PMO compound can be potential antivirals against PRRSV. Further efficacy test in pigs is warranted to apply the new generation of antiviral compound to assist PRRS control.

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