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Identification and characterization of small molecule inhibitors of porcine reproductive and respiratory syndrome virus

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Abstract
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Keywords
PRRSV, Antivirals, Atractylodinol analogs

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Identification and Characterization of Small Molecule Inhibitors of Porcine Reproductive and Respiratory Syndrome Virus

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is the etiological agent of PRRS, an economically significant disease of swine worldwide. PRRSV is poorly controlled by the currently available vaccines, and alternative control strategies are needed to help prevent the continual circulation of the virus. Previously, we developed a synthetic route for the natural compound atractylodinol and demonstrated anti-PRRSV activity in vitro. However, the synthetic route was inefficient and the yield was poor. To identify PRRSV inhibitors that could be synthesized easily and cost-effectively, we synthesized a series of atractylodinol analogs and characterized their anti-PRRSV activity in vitro. A furan-substituted bis-enyne subunit was found to be critical for PRRSV inhibition. Six analogs had potent inhibitory activity against PRRSV with 50% inhibition concentration (IC50) of 0.4-1.4 μM and 50% cytotoxic concentration (CC50) of 209-1537 □M in MARC-145 cells. Three of the most promising compounds also demonstrated significant antiviral activity and low cytotoxicity in porcine macrophages. Inhibition of PRRSV in MARC-145 cells occurred primarily at a post-entry step during PRRSV replication, between 4 and 12 hr post-entry. These results suggest that atractylodinol analogs are promising antiviral candidates that could augment current PRRSV control strategies.

Keywords: PRRSV; antivirals; atractylodinol analogs

Highlights

- Developed efficient routes for synthesis of atractylodinol analogs that inhibit PRRSV replication in MARC-145 cells and in porcine macrophages
- Determined the presence of a bis-enyne subunit was critical for antiviral activity
- Identified six analogs of atractylodinol with IC_{50} of 0.4-1.4 μM in vitro
- Determined inhibition of PRRSV in MARC-145 cells occurs at a post-entry step during virus replication

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the etiological agent of PRRS, an economically significant disease of swine worldwide. PRRSV causes reproductive losses from abortions and stillbirths in pregnant sows, and respiratory disease in growing pigs, resulting in an estimated $664 million in annual costs to the US pork industry (Collins et al., 1992; Holtkamp et al., 2012; Loula, 1991). Although a number of killed and attenuated vaccines are available, their effectiveness is limited due to the high rate of variation and genetic diversity of the virus (Kimman et al., 2009; Murtaugh and Genzow, 2011; Rose et al., 2015). In addition, PRRSV is capable of persisting in vivo for months after the initial infection, potentially leading to the occurrence of additional outbreaks within herds (Allende et al., 2000; Wills et al., 2003; Wills et al., 1997). Additional strategies for PRRSV prevention and control include biosecurity and containment measures. However, due to the high transmissibility of PRRSV, the virus can quickly spread from one barn and/or farm to others and outbreaks continually occur.

Antivirals could be a useful complement to current PRRSV control and containment strategies. In the past few years, antivirals against PRRSV have been described in the literature that include microRNAs, antisense RNA, immune stimulators, and herbal extracts (reviewed in Du et al., 2017). Many of the antivirals derived from herbs, plants, or mushrooms are used in traditional Chinese medicine, and many have been shown to have antiviral activity against a wide variety of viruses (Mukhtar et al., 2008). Synthetic antivirals such as epigallocatechin gallate (EGCG), EGCG plamitate, and ribavarin were found to inhibit PRRSV-associated cytopathic effects in MARC-145 cells in a dose-dependent manner when added either pre- or post-infection with 100 $TCID_{50}$ PRRSV (Zhao

et al., 2014). To date, few studies have examined anti-PRRSV activity *in vivo*. In one study (Gao et al., 2013), treatment of pigs with extracts of *Cryptoporus volvatus*, a polypore fungus, resulted in reduction in virus replication and clinical symptoms as compared to the control group. However, the treatment protocol included twice daily intramuscular injections of the extract for eight days, which is not practical for field applications. Nonetheless, the results demonstrate the potential of antivirals for reducing the virological and clinical burden of PRRSV infection *in vivo*.

One impediment to the use of antivirals for treatment of PRRSV is the cost of treatment. Many of the natural compounds with anti-PRRSV activity are difficult and/or costly to extract in large quantities. In order to feasibly produce large amounts of anti-PRRSV compounds for *in vivo* application, it is therefore necessary to identify potent anti-PRRSV compounds that can be generated easily and cost-effectively. The goal of the present study was to synthesize and evaluate small molecule inhibitors of PRRSV with the potential for large-scale production. Toward that end, we were especially interested in identifying compounds that could be synthesized efficiently, in high yields, and with potential for high bioavailability. We selected the compound atractylodinol, which has been identified as having potent anti-PRRSV properties (Li et al., 2013; Kraus et al., 2016), as the basis for our synthesis strategy. We previously synthesized atractylodinol and demonstrated its anti-PRRSV activity; however the synthesis route was inefficient (Kraus et al., 2016). In the current study, our strategy included designing an efficient synthesis route to produce large amounts of atractylodinol and/or atractylodinol analogs and assess their anti-PRRSV activity in MARC-145 cells and in porcine macrophages. In total, thirteen

compounds were synthesized and screened for anti-PRRSV activities and we identified six atractylodinol analogs with potent anti-PRRSV activity *in vitro*.

2. Materials and Methods

2.1 Synthesis of atractylodinol and analogs

The synthesis strategy for atractylodinol is summarized in Fig. 1A and detailed in an earlier paper (Kraus et al., 2016). The original compound identified by (Li et al., 2013), atractylodinol (compound **1**), was synthesized in seven steps from butenynylfuran (**2**) and bromopentenynol (**4**) in an overall yield of 11% (Fig 1A). Because of the low overall yield, a second synthetic route was developed via ester **7**, which was synthesized in five steps from commercially available materials, and could further be converted to atractylodinol (**1**). Due to the number of steps and the modest overall yields, neither synthetic route was feasible for scale-up. In the present study we synthesized ten additional analogs predicted to increase bioavailability, stability and/or efficiency of synthesis (Fig. 1B-D). Preliminary experiments suggested that the furan enyne subunit was important for activity (not shown). In order to generate compounds bearing this subunit in high yields, dimers **8**, **9**, **10**, and **12** were prepared (Fig 1b). Analogs **8**– **12** had the conjugated bis-enyne skeleton and were more polar compounds that could be expected to have greater bioavailability. The presence of the amine, or its corresponding salts, in analog **9** may further increase bioavability. The analog **8** was synthesized by dimerization of compound **2** in 36% yield. Diamine analog **9** and aldehyde analog **10** were made in one step from **8** in yields of 60% and 33%, respectively. Additionally, alcohol analog **11** and its dimer, diyne **12**, were

synthesized in yields of 53% and 52%, respectively. Analogs **13** and **14** were benzene ring analogs of **7** and **8**, and would be expected to be more stable (Fig 1C). Ester **13** and dimer **14** were synthesized from 3,4-methylenedioxyphenylacetylene. Truncated analogs **15**, **16**, and **17** contained segments of **1**, and were each prepared in one step from **2** (Fig 1D).

All synthesized compounds were analyzed by ^{1}H NMR, ^{13}C NMR and high resolution mass spectrometry to verify their structure. Because some compounds were not readily soluble in water, all compounds were solubilized in DMSO at a concentration of 1 mg/mL, and further dilutions for the anti-viral and cytotoxicity assays were made in culture media.

2.2 Cells and virus

MARC-145 cells were used for anti-viral assays and were maintained in high glucose (4500mg/L) Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The porcine alveolar macrophage cell line, ZMAC, was originally established from lung lavage of porcine fetuses as previously described (Calzada-Nova et al., 2012). ZMAC is a patented cell line and we obtained the cells from the Aptimmune Biologics Inc., Champagne, IL. The ZMAC cells were cultured in RPMI-1640 medium with supplements as previously described (Calzada-Nova et al., 2012).

The PRRSV strain NVSL97-7895 (GenBank accession **AY545985**) was passaged once in MARC-145 cells and used in all assays.

2.3 Antiviral activity of synthetic analogs in MARC-145 cells

Compounds were assayed for anti-PRRSV activity in MARC-145 cells using a focus-reduction assay as previously described (Wu et al., 2011; Evans et al., 2017). For most assays, 10 μg of each compound, or DMSO diluent control, were incubated with varying amounts of PRRSV NVSL97-7895 for 1 hr at 37°C and inoculated in duplicate onto MARC-145 cells seeded the previous day at $3x10^5$ cells/well in a 12-well plate. At 24 hours post infection, cells were fixed in ice-cold methanol:acetone and immunocytochemistry was performed using the PRRSV N protein-specific monoclonal antibody SDOW17 (Rural Technology) as the primary antibody and sheep anti-mouse IgG conjugated to HRP (Jackson ImmunoResearch) as the secondary antibody. Following addition of the HRP substrate, cells were rinsed with distilled water, air-dried, and foci of infected cells were enumerated by light microscopy. Percent virus inhibition was calculated as a reduction in foci as compared to virus-only control wells. Assays were done in duplicate and repeated at least twice.

To determine the concentration of compound capable of inhibiting 50% of input virus (IC_{50}), three-fold serial dilutions of active compounds were incubated with 200 FFU NVSL97-7895 and PRRSV inhibition was assayed using the focus-reduction assay as described above. The IC_{50} of each compound was calculated from replicate assays using normalized inhibition dose response linear regression in GraphPad Prism 7. As another measure of potency, 10 μg of each active analog were incubated with 10-fold serial dilutions of PRRSV NVSL97-7895, ranging from 2.5×10^2 to 2.5×10^5 FFU, and PRRSV inhibition was assayed as described above. Assays were done in duplicate, repeated twice, and the results were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism 7 software.

2.4 Antiviral activity of synthetic analogs in porcine macrophages

The porcine macrophage cell line ZMAC (Calzada-Nova et al., 2012) was used to evaluate the anti-PRRSV activity of lead compounds. For these assays, 10 μg of select compounds or DMSO diluent control were incubated with 200 FFU NVSL97-7895 (2.1 X 10^5 copies viral RNA) for 1 hr at 37°C and inoculated in triplicate onto ZMAC cells seeded at 2 x 10^5 cells/well in 24-well plates. Cells were incubated 1 hr at 37°C, at which time the supernatant was discarded and fresh media was added. Cells were incubated for an additional 16 hrs at 37°C, lysed by freeze-thawing and centrifuged at 3000 xG for 10 min. Virion RNA was isolated from the supernatant fraction using the QIAamp Viral RNA Mini kit (Qiagen) and quantified by RT-qPCR using primers specific for PRRSV ORF7 RNA. Assays were done in triplicate and repeated twice.

2.5 Cytotoxicity assays

Cytotoxicity of the anti-PRRSV compounds was determined using an ATP-based cell viability assay Cell Titer Glo™ (Promega). To assess cytotoxicity for MARC-145 cells, selected analogs were serially diluted in media and inoculated in triplicate onto MARC-145 cells seeded the previous day at 2.5x 10^4 cells per well in a 96-well black and white plate. Controls included media only and DMSO. Cells were incubated at 37°C overnight, equilibrated at room temperature for 30 min, and 100 μl of CellTiter-Glo reagent was added to each well. The plates were placed on an orbital shaker for 2 min to induce cell lysis and incubated at room temperature for an additional 10 min. Luminescence was

quantified as relative light units (RLU) using Centro XS3 LB960 Luminometer (Berthold Technologies).

To assess the cytotoxicity in ZMAC, select analogs were serially diluted in media, incubated at 37°C for 1 hour and added to duplicate wells of ZMAC cells seeded in 24-well plates at 2 X 10^5 cells/well for 1 hour at 37°C. The supernatant was replace with fresh media and cells wells were incubated for 16 hrs at 37°C and equilibrated at room temperature for 30 min. One hundred μl of CellTiter-Glo reagent was added to each well and cells were lysed by shaking for 2 min. The plate was incubated an additional 10 min at room temperature at which time 200 μl from each well was transferred to triplicate wells of 96-black and white plate and luminescence was quantified as above.

Cytotoxicity was calculated as the RLU in treated wells as compared to DMSO-treated control wells, and the mean of replicates was reported. The 50% cytotoxic concentration (CC_{50}) was calculated as the compound concentration that reduced the luminescence by 50%, using normalized inhibition dose response linear regression in GraphPad Prism 7.

2.6 PRRSV binding, entry and replication assays

For attachment assays, 10^5 FFU of PRRSV NVSL97-7895 was incubated with 10 μg of analog or DMSO control for 15 min at 37°C. The sample was chilled on ice and inoculated onto MARC-145 cells at 4°C for one hour to facilitate virion attachment, but not uptake into the cells. After incubation, cells were washed six times with media and were incubated in 300 μl PBS containing 0.5% trypsin for 10 min. Following the addition of 50 μl FBS, cells and supernatant were collected and separated by centrifugation. Virion RNA was isolated

from the supernatant fraction using the QIAamp Viral RNA Mini kit (Qiagen) and RNA was quantified by RT-qPCR using primers specific for PRRSV ORF7 RNA.

For entry and replication assays, PRRSV was incubated with analog or DMSO and inoculated onto MARC-145 cells at 4°C as described above. Following washing to remove unattached virions, fresh media was added and the cells were shifted to 37°C, designated as time 0 hr. At 1, 4, 8, 12, or 24 hr, cells were treated with trypsin and pelleted by centrifugation as described above, and total RNA was isolated from the cell fraction with the RNeasy mini kit (Qiagen). Viral RNA was quantified by RT-qPCR as above. All assays were done in duplicate and repeated 2-3 times and results are reported as mean copy number of viral RNA/well. A student's t-test was used to analyze viral copy number at each time point across virus-only and analog-treated cells.

3. Results

3.1 Identification of small molecule compounds with anti-PRRSV activity

Atractylodinol isolated from rhizomes of *Atractylodes lancea* has been shown to inhibit PRRSV replication in vitro (Li et al., 2013), but isolation procedures resulted in low yields and impurities. To overcome the limitations of isolation, we recently described a route for synthesis of atractylodinal (Kraus et al., 2016). Synthetic atractylodinol and two intermediate compounds (Fig 1A, compounds **1**, **2**, and **7**) were found to have potent anti-PRRSV activity; however, the synthesis routes to **1** and **7** were inefficient and not feasible for scale up to the quantities of material needed for in vivo testing. Analogs such as **2** were too nonpolar to be bioavailable. In the present study, we synthesized ten new analogs of atractylodinol (Fig 1B-D). The synthesis routes to the new analogs were operationally convenient and would permit large-scale synthesis..

For initial screening assays in MARC-145 cells, 10 μg of each compound was incubated with 200 FFU PRRSV and antiviral activity was calculated as the percent reduction in infectious virus as compared to virus-only controls. Consistent with our previous report (Kraus et al., 2016), atractylodinol (**1**) inhibited 100% of input virus (Fig. 2A). Additional compounds with high activity were structures **2**, **7**-**10** and **12**. Analogs **7**-**10** and **12** contain the furan-substituted bis-enyne subunit (Fig 1). Other compounds (**13**-**17**) lacked the furan substituent or the complete bis-enyne subunit and had significantly reduced activity, indicating the presence of the bis-enyne subunit was critical for optimal anti-PRRSV activity. Based on these promising results, atractylodinol (**1**) and the six analogs with >90% PRRSV inhibitory activity (**2**, **7**-**10**, **12**) were selected for further characterization in MARC-145 cells.

3.2 Potency of small molecule inhibitors of PRRSV

To determine the IC_{50} for MARC-145 cells, serial dilutions of each active compound were incubated with 200 FFU of virus and the IC_{50} was calculated using normalized inhibition dose response linear regression in GraphPad Prism 7. The IC_{50} of the active compounds ranged from 392 nM to 2.24 μM (Table 1). Atractylodinol (**1**) and compound **2** had IC_{50} greater than 1 μM, while the other five compounds had IC_{50} in the nanomolar range (Table 1). All seven of the anti-PRRSV compounds inhibited PRRSV in a dose-dependent manner (not shown), and were effective at concentrations lower than that previously reported for other anti-PRRSV compounds (Cheng et al., 2013; Gao et al., 2013; Karuppannan et al., 2012; Zhao et al., 2014). The IC_{50} of synthetic atractylodinol (**1**) was lower than that reported (Li et al., 2013) for atractylodinol isolated from plant rhizomes (39.4 μM), which likely is due to differences in the purity of the material tested and/or to differences in assay conditions used to evaluate antiviral activity. Compound **2** was the least effective of the seven active compounds, with a maximum PRRSV inhibition of 93.4% (Fig 2). The five compounds with IC_{50} in the nanomolar range (**7**, **8**, **9**, **10**, **12**) reached maximum PRRSV inhibition of 97.2 to 100% (Fig 2), The preparation of dimers **8**, **9**, and **12** requires only three or four steps, and diamine **9** could have the best bioavailability. Thus, compounds **8**, **9**, and **12** were considered the most promising leads as small molecule inhibitors of PRRSV replication.

3.3 Cytotoxicity of small molecule inhibitors of PRRSV

To evaluate the cytotoxicity of active compounds, MARC-145 cells were treated with serial dilutions of each active compound and cytotoxicity was measured using the Cell Titer Glo™ assay. The concentration of compound that reduced absorbance by 50% (CC_{50}) as compared to DMSO control was calculated for each compound (Table 1). Compound **1** was the most cytotoxic among the seven active compounds, with only a 9-fold difference in the CC_{50}:IC_{50} ratio. Compound **9** had the lowest CC_{50} of the analogs at 209 μM; however, this was still well above it's IC_{50} of 0.39 μM, resulting in a CC_{50}:IC_{50} ratio of 535. The remaining active compounds had CC_{50} ranging from 558 to 1529 μM, with CC_{50}:IC_{50} ratios greater than 1000.

3.4 Atractylodinol analogs inhibit high-titers of PRRSV

PRRSV replicates to high titers in vivo, and it was therefore of interest to evaluate the effectiveness of small molecule inhibitors in the presence of high-titers of PRRSV. To assess this, 10 μg of each compound was incubated with 10-fold serial dilutions of PRRSV ranging from 10^5 to 10^2 FFU and percent virus inhibition in MARC-145 cells was compared to the virus-only control wells (Fig 2B). Each of the compounds maintained a similarly high level of PRRSV inhibition at all virus concentrations, with no statistically significant differences in inhibition against the range of titers tested. Compounds **1** and **9** were the most active, inhibiting 100 and 99.93% of input PRRSV up to 10^5 FFU, respectively.

3.5 Compound 9 primarily targets a post-entry step of PRRSV replication in MARC-145 cells

To investigate the mechanism by which selected compounds inhibit PRRSV, we examined the early steps in virus replication in the presence of one of the small molecule inhibitors. Compound **9** was chosen for these assays based on its effectiveness against high concentrations of virus (Fig 2B), ease of synthesis, and potential bioavailability. MARC-145 cells were inoculated with 10^5 FFU PRRSV in the presence or absence of 10 μg compound **9**, and PRRSV genomic RNA was quantified at successive steps during virus replication. We observed a small, but statistically significant, reduction in attached virions in compound **9**-treated cells compared to virus-only cells (Fig 3, time 0, P=0.03). Following the shift to 37°C, viral RNA levels decreased through 4 hr in both compound **9**-treated and control cells, indicative of the eclipse phase in the virus replication cycle. Thereafter, an exponential increase in viral RNA occurred in the virus-only cells, starting between 4-8 hr post-entry. In contrast, the increase in newly synthesized viral RNA was delayed in compound **9**-treated cells, and there was significantly less PRRSV RNA detected at 8, 12, and 24 hr post-entry as compared to the virus-only cells (P=0.018, 0.005, and <0.001, respectively). Although there was a modest inhibition of virion attachment, the results indicate that the primary inhibitory activity of compound **9** occurred at a post-entry step in the PRRSV replication cycle.

3.6 Antiviral activity and cytotoxicity of lead compounds in porcine macrophages

In vivo, PRRSV replicates in porcine alveolar macrophages (PAM); therefore, it was important to determine if atractylodinol analogs would inhibit PRRSV replication in the natural host cell. Although primary PAM cultures can be established in vitro, the cultures are short lived and heterogeneity within and between different cultures confounds

reproducibility of experimental results. To overcome these limitations, we assessed the antiviral activity of compounds using the ZMAC porcine alveolar macrophage cell line, which was originally established from fetal pig lung lavage (Calzada-Nova et al., 2011; 2012). To evaluate anti-viral activity in ZMAC, 10 μg of each of our lead compounds (**8**, **9**, **12**) was incubated with 2.1 X 10^5 NVSL97-7895 virions (200 FFU) in 100 μl for 1 hr at 37°C and inoculated onto ZMAC cells as described above. After 16 hr, cells were lysed and PRRSV RNA was isolated and quantified by qRT-PCR. Parallel cultures were assayed for viability using the Cell Titer Glo™ assay. Each of the lead compounds was shown to significantly reduce PRRSV replication 300-2000 fold in ZMAC cells (Fig 4A). Compounds **8** and **12** were most effective at inhibiting PRRSV replication ZMAC; however, we observed ~10% reduction in cell viability of ZMAC in the presence of 10 μg compound **12** as compared to DMSO control (Fig 4A). The CC_{50} of compounds **8**, **9**, and **12** in ZMAC was 277, 266, and 110 μM, respectively (Fig 4B), which was two- to three-fold lower than we observed in MARC-145 cells. While the three compounds differed in their relative activity and cytotoxicity in ZMAC and MARC-145, each of the compounds significantly reduced PRRSV replication in both cell types.

4. Discussion

PRRSV is one of the most economically significant swine pathogens worldwide. Over the last three decades, efforts to control PRRSV have met with some success, but there is an urgent need for new strategies to augment current vaccine and biocontainment control programs. Natural products derived from plants used in traditional Chinese medicine have been shown to have anti-PRRSV activity *in vitro* (Cheng et al., 2013; Gao et al., 2013; Karuppannan et al., 2012; Li et al., 2013; Sun et al., 2014; Yang et al., 2013; Zhao et al., 2014), and we previously reported the synthesis and antiviral activity of one such compound, atractylodinol (Kraus et al., 2016). However, the synthetic routes were largely inefficient and unlikely to be cost-effective in commercial swine operations. In the present study, we synthesized and characterized the anti-PRRSV activity of atractylodinol and twelve additional analogs. Six of the atractylodinol analogs were found to be potent inhibitors of PRRSV replication *in vitro*, with IC_{50} in the 0.4-1.4 μM range. Active, but not inactive, analogs contained the furan-substituted enyne or bis-enyne subunit, demonstrating the importance of this subunit for anti-PRRSV activity. Although synthetic atractylodinol, compound **1,** had high cytotoxicity, the other six compounds were much less cytotoxic, with CC_{50} between 200 to 1500 μM, resulting in CC_{50}:IC_{50} of 500-2000. Importantly, many of the synthetic analogs were effective against high titers of infectious virus, inhibiting >90% of PRRSV up to 10^5 infectious units in MARC-145 cells Based on efficiency of synthesis, high yield, and potential bioavailability, three lead compounds (**8**, **9**, and **12**) were identified and shown to reduce PRRSV replication 100-1000-fold in porcine macrophages at concentrations showing little to no cytotoxicity. Investigation into the mechanism of action revealed that the major anti-viral effect occurred between 4 and 8

hours post-entry, and resulted in a significant reduction and/or delay in production of viral RNA in newly infected cells, suggesting that a post-entry step is targeted. The availability of small molecule inhibitors of PRRSV may aid efforts to curtail PRRSV replication in vivo and reduce economic losses in the swine industry.

In vivo, PRRSV replicates in alveolar macrophages, and it was important to ascertain the effectiveness of the analogs in a relevant cell type. ZMAC cells, which were originally established from fetal pig lung lavage, express the typical macrophage cell-surface markers such as CD14, CD45, CD163, and CD172, and are highly susceptible to PRRSV infection (Calzada-Nova et al., 2011; 2012). Each of the three lead compounds, **8**, **9**, and **12**, significantly reduced PRRSV replication in ZMAC at concentrations with little to no cytotoxicity. Interestingly, the relative effectiveness and cytotoxicity of the lead compounds depended on cell type. Compound **9** was most effective in reducing PRRSV replication in MARC-145 cells, yet also had the lowest CC_{50} of the analogs tested in these cells. In ZMAC cells, however, **9** was less effective than **8** and **12** in inhibiting PRRSV replication**,** but was less cytotoxic than compound **12**, the most cytotoxic compound for ZMAC. The reasons for these cell-type specific differences are not clear. Few studies of small molecule inhibitors have directly compared anti-PRRSV activity in MARC-145 and PAM. In one study (Yang et al., 2013), flavispidic acid AB (FA-AB) had a slightly lower EC_{50} in MARC-145 (3.5 μg/ml) than in PAM (4.2 μg/ml); however, different PRRSV strains were used in each cell type and it is not clear if the small differences in activity were due to host cell and/or virus strain. We observed that ZMAC were more sensitive than MARC-145 to higher concentrations of atractylodinol analogs. Similarly, Yang et al (2103) reported that the antiviral compound FA-AB was more cytotoxic to PAM than MARC-145 cells. It is not clear what in vitro

parameters are most predictive of effectiveness in limiting PRRSV replication in pigs. However the overall effectiveness of compound **8** and **9** in reducing PRRSV replication in ZMAC at concentrations well below their CC_{50} is encouraging and warrants further studies evaluating antiviral activity in vivo.

The mechanism of action of atractylodinol analogs was investigated using compound **9**, which was selected based on its inhibition of high titers of PRRSV in MARC-145 cells (Fig 2B), ease of synthesis, and potential bioavailability. Incubation of PRRSV with compound **9** prior to infection resulted in a small, but significant reduction in virion attachment (Fig. 3). Time of addition studies also revealed a small, but significant reduction in antiviral activity when compound **9** was added 1-3 hr post-infection (not shown). Together, these results indicate compound **9** may inhibit the earliest steps in PRRSV replication, including attachment and entry. However, the major inhibition of PRRSV replication occurred at a post-entry step in the virus replication cycle. Significant inhibition in levels of newly synthesized viral RNA was observed between 4 and 8 hours post-entry, when there was exponential increase in RNA production in non-treated control cells. Viral RNA levels increased in compound **9** treated cells by 12 hpi; however, the overall levels remained significantly lower than in virus-only cells, representing >99.8% inhibition. Additional studies are needed to determine the specific mechanism(s) of PRRSV inhibition, but possible targets include inhibition of virus uncoating and/or synthesis of viral RNA. It is also possible that antiviral activity is due in part to induction of innate antiviral response (Yang et al., 2013).

A number of studies have identified compounds with anti-PRRSV activity *in vitro*. These are primarily comprised of natural products derived from plants used in traditional

Chinese medicine (Cheng et al., 2013; Gao et al., 2013; Karuppannan et al., 2012; Li et al., 2013; Sun et al., 2014; Yang et al., 2013; Zhao et al., 2014). It is difficult to directly compare antiviral activities of compounds across *in vitro* studies, due in part to the variation in protocols to assay virus inhibition, the use of whole extracts versus purified compounds, and differences in the method of reporting IC_{50} (μg/ml vs. μM). Most of the anti-PRRSV compounds previously described had IC_{50} in the range of <1 to 98 μM. When reported, CC_{50} were in the range of 94 to 431 μM, with varying levels of inhibition and potency (Cheng et al., 2013; Gao et al., 2013; Karuppannan et al., 2012; Zhao et al., 2014). Our synthetic compounds had equivalent or somewhat lower IC_{50} (392 nM to 2.24 μM) and were generally less toxic in MARC-145 cells (CC_{50} ~200-1500 μM, Table 1). The optimal levels of antiviral activity needed to reduce PRRSV replication in vivo are not known, and to date only one study has examined the effectiveness of PRRSV antivirals in vivo (Gao et al., 2013). It is interesting to note, however, that all six of the analogs had higher potency and lower IC_{50} *in vitro* than that reported for extracts of *Cryptoporus volvatus*, which was found to reduce PRRSV replication and associated clinical signs *in vivo* (Gao et al., 2013). It is important to note that higher concentrations of the *Cryptoporus volvatus* were likely required because it was a naturally derived mushroom extract rather than a homogenous synthetic compound. Nevertheless, these results provide promising evidence that anti-PRRSV activity *in vitro* can translate to PRRSV inhibition *in vivo*.

In developing a drug for animal health, there is a necessary balance between the level of a compound's antiviral activity, its bioavailability and the cost of preparing the compound. Our synthesis strategy considered both the ease of synthesis and potential for bioavailability. Dimers **8**, **9**, and **12** can be prepared in only three or four steps, while **9** or

its salts may have the best bioavailability. Based on CC_{50} in ZMAC and overall effectiveness in both cell types, compounds **8** and **9** may be the most promising lead candidates for future *in vivo* trials. There are several concerns and hurdles regarding the use of antivirals in food animals, including the cost of treatment and potential for persistence of antivirals in the food chain. However, targeted and/or temporary administration of potent antivirals could be one component incorporated into a multi-pronged PRRSV-control strategy to lessen the economic burden of PRRSV infection. The relatively high and sustained level of PRRSV inhibition suggests that compound **8, 9** or similar small molecule inhibitors may be especially useful in limiting PRRSV spread during outbreaks and/or during periods of high PRRSV susceptibility.

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7. Figure Legends

Figure 1. Synthesis routes for atractylodinol and analogs. A) Previously described (Kraus et al., 2017) routes for synthesis routes for synthetic atractylodinol (**1**). B) Analogs containing the conjugated bis-enyne skeleton; C) Benzene ring containing analogs; D) Analogs prepared in one step from compound **2**.

Figure 2. Anti-PRRSV activity of synthetic atractylodinol (**1**) and related analogs. A. Ten µg of each compound were incubated with $2x10^2$ FFU PRRSV 1 hr at 37°C and inoculated onto MARC-145 cells. Percent virus inhibition represents the reduction in PRRSV FFU compared to virus-only control cells. Assays were done in duplicate and error bars represent mean ±standard deviation of replicate assays. B. Serial ten-fold dilution of PRRSV from 10^5 to 10^2 FFU were incubated with 10 µg compounds and inoculated onto MARC-145 cells. Percent virus inhibition represents the reduction in PRRSV FFU compared to virus-only control wells. Means from two independent experiments are reported. Error bars represent ±standard deviation of the means. No statistically significant differences between virus treatments within compound were detected via one-way ANOVA.

Figure 3. Compound **9** inhibits a post-entry step in PRRSV replication. Results are reported as the average RNA copy number per well as determined by RT-qPCR. Bound indicates attached virions after 1 hr incubation at 4°. Arrow indicates shift of cells to 37°C. Times 1, 4, 8, 12, and 24 hpi represents cell-associated RNA copy number at sequential times following shift to 37°C. Asterisks represent statistically significant differences between virus-only and compound-treated cells as determined by a t-test ($P<0.05$).

Figure 4. Antiviral activity and cytotoxicity of lead compounds in porcine macrophages. A. Viral RNA copy number (black bars) in ZMAC cells at 16 h post-infection with PRRSV treated with media only, DMSO or 10 μg of compound **8**, **9**, or **12**. Grey bars indicate percent cytotoxicity in parallel cultures. Results represent the mean of replicate experiments done in triplicate ± SEM. *P<0.001. B. Cytotoxicity of lead compounds **8**, **9**, and **12** in ZMAC cells determined using ATP-based Cell Titer Glo™ assay. Dotted line indicates 50% reduction in RLU as compared to cells treated with DMSO control. Results represent the mean ± SEM of replicate treatments assayed in triplicate.

Table 1. Potency and cytotoxicity of small molecule inhibitors of PRRSV

Compound	IC_{50} (μM)		CC_{50} (μM)		CC_{50}/IC_{50}
	Mean	95% CI	Mean	95% CI	
1	2.24	1.65 - 3.03	21	18 - 25	9
2	1.42	1.11 - 1.81	1547	1325 - 2894	1087
7	0.50	0.36 - 0.68	769	733 - 814	1532
8	0.55	0.40 - 0.75	566	533 - 605	1025
9	0.39	0.33 - 0.47	209	192 - 229	533
10	0.45	0.37 - 0.55	1113	909 - 2522	2473
12	0.59	0.42 - 0.83	697	627 - 790	1183