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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 (IC_{50}) of 0.4–1.4 μM and 50% cytotoxic concentration (CC_{50}) of 209–1537 μM 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 h post-entry. These results suggest that atractylodinol analogs are promising antiviral candidates that could augment current PRRSV control strategies.

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., 1997, 2003). 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

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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 was to develop 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 (4500 mg/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 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 h at 37 °C and inoculated in duplicate onto MARC-145 cells seeded the previous day at 3×10^5 cells/well in a 12-well plate. At 24 h 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 focus-forming units (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 10^2 to 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 (2.1×10^5 copies viral RNA) for 1 h at 37 °C and inoculated in triplicate onto ZMAC cells seeded at 2×10^5 cells/well in 24-well plates. Cells were incubated 1 h at 37 °C, at which time the supernatant was discarded and fresh media was added. Cells were incubated for an