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Identification of two antiviral inhibitors targeting 3C-like serine/3C-like protease of porcine reproductive and respiratory syndrome virus and porcine epidemic diarrhea virus

Yuejun Shi[a,b,c,d], Yingying Lei[a,b], Gang Ye[a,b,c], Limeng Sun[a,b,c], Liurong Fang[a,b,c], Shaobo Xiao[a,b,c], Zhen F. Fu[a,b,c,f], Ping Yin[d,e], Yunfeng Song[a,b,c,*], Guiqing Peng[a,b,c,*]

[a] State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China
[b] College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China
[c] The Cooperative Innovation Center for Sustainable Pig Production, Huazhong Agricultural University, Wuhan 430070, China
[d] College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China
[e] National Key Laboratory of Crop Genetic Improvement and National Centre of Plant Gene Research, Huazhong Agricultural University, Wuhan 430070, China
[f] Departments of Pathology, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine epidemic diarrhea virus (PEDV) are highly virulent and contagious porcine pathogens that cause tremendous economic losses to the swine industry worldwide. Currently, there is no effective treatment for PRRSV and PEDV, and commercial vaccines do not induce sterilizing immunity. In this study, we screened a library of 1000 compounds and identified two specific inhibitors, designated compounds 2 and 3, which target the PRRSV 3C-like serine protease (3CLSP). First, we evaluated the inhibitory effects of compounds 2 and 3 on PRRSV 3CLSP activity. Next, we determined the anti-PRRSV capacity of compounds 2 and 3 in MARC-145 cells and obtained EC_{50} and CC_{50} values of 57 μM (CC_{50} = 479.9 μM) and 56.8 μM (CC_{50} = 482.8 μM), respectively. Importantly, compounds 2 and 3 also targeted the PEDV 3C-like protease (3CL protease) and inhibited PEDV replication, showing EC_{50} and CC_{50} values of 100 μM (CC_{50} = 533.8 μM) and 57.9 μM (CC_{50} = 522.3 μM), respectively. Finally, our results indicated that the active sites (His39 in 3CLSP and His41 in 3CL protease) were conservative, and contacted compounds 2 and 3 via hydrogen bonds and hydrophobic forces in the putative substrate-binding models. In summary, compounds 2 and 3 exhibit broad-spectrum antiviral activity and may facilitate the development of antiviral drugs against PRRSV and PEDV.

1. Introduction

Nidoviruses are important pathogens of both humans and livestock; they are enveloped, plus-strand RNA viruses comprising the families *Arteriviridae* and *Coronaviridae* (Gorbalenya et al., 2006). Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the family *Arteriviridae* (Snijder et al., 2013), has been a major threat to the worldwide swine industry since its discovery in the early 1990s (Wensvoort et al., 1991; Collins et al., 1992). In 2006, a highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) emerged and prevailed in mainland China, resulting in devastating damage to swine production (Tian et al., 2007). Currently, HP-PRRSV causes high morbidity and high mortality in infected pigs of all ages and has remained a major threat to the swine producers in China and surrounding countries (Han et al., 2017). Porcine epidemic diarrhea virus (PEDV), a member of the genus *Alpha-coronavirus* (Song and Park, 2012), was first identified in the 1980s in China (Xuan et al., 1984). PEDV causes a contagious intestinal disease called porcine epidemic diarrhea (PED), which is characterized by vomiting, diarrhea, and dehydration (Wang et al., 2016). In October 2010, a large-scale outbreak of PED caused by a PEDV variant occurred in China, resulting in tremendous economic losses (Li et al., 2012; Wang et al., 2013, 2016; Tian et al., 2014). PRRSV and PEDV are highly virulent and contagious porcine pathogens that cause respiratory and enteric disease in pigs and result in significant economic losses for the intensive pig production worldwide (Li et al., 2012; Snijder et al., 2013; Wang et al., 2016).

* Corresponding authors at: State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, 1 Shi-zi-shan Street, Wuhan 430070, China.
E-mail addresses: syf@mail.hzau.edu.cn (Y. Song), penggq@mail.hzau.edu.cn (G. Peng).

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In the PRRSV and PEDV genome, ORF1a and ORF1b comprise approximately 80% of the genome and encode polyproteins (pp1a and pp1b) that are cleaved by viral proteases into intermediate and mature nonstructural proteins (Ziebuhr et al., 2000; Gorbalenya et al., 2006). The viral proteases involved in polyprotein cleavage are papain-like proteases (PL1pro and PL2pro) and a 3CL serine protease (PRRSV nsp4; 3CLSP)/3C-like (PEDV nsp5; 3CL) protease (Tian et al., 2009; Ye et al., 2016). During replication, the PL1pro and PL2pro proteases cleave the N-proximal region of pp1a/pp1ab, and the 3CLSP/3CL protease cleaves the viral polyprotein at other conserved inter-domain junctions (Tian et al., 2009; Ye et al., 2016). The viral 3CLSP/3CL proteases play an essential role in *artertivirus* and *coronavirus* replication; therefore, they have received much attention as potential key antiviral targets (including TGEV, FIPV, IBV and SARS-CoV) (Anand et al., 2002; Kim et al., 2013; Lee et al., 2007; Xue et al., 2008).

As RNA viruses, PRRSV and PEDV are prone to mutation under selective pressures in the field, and this may cause the frequent appearances of new variant strains (Wang et al., 2016; Han et al., 2017). Moreover, current commercial vaccination approaches against PRRSV and PEDV are only partially effective (Wang et al., 2016; Du et al., 2017a,b). To date, effective broad-spectrum antiviral drugs for PRRSV and PEDV infections are not available, although antiviral compounds have been reported previously (Karuppannan et al., 2012; St John et al., 2016; Evans et al., 2017). This remains an impediment to identifying new inhibitors that target the PRRSV and PEDV 3CLSP/3CL proteases for use as antiviral therapies during disease epidemics. In this study, our findings provide new insights into the molecular mechanisms underlying 3CLSP or 3CL protease-mediated inhibition of viral replication and may also guide the development of new broad-spectrum antiviral drugs.

2. Materials and methods

2.1. Compounds

A low-molecular-weight fragment-based library containing approximately 1000 compounds (Specs, Delf, Netherlands) was used for antiviral fragment screening. These fragments are defined as small molecules obeying the Congreve 'Rule of three' in which molecular weight (MW) ≤ 300, the number of hydrogen donors and acceptors ≤ 3 and cLogP ≤ 3 (Congreve et al., 2003). All these compounds were dissolved in dimethyl sulfoxide (DMSO; Mpbio) at a concentration of 10 mM. Moreover, compounds 1–5 were prepared in DMSO as stock solutions (50 mM).

2.2. Viruses and cells

Marc-145 cells (a PRRSV-permissive cell line derived from MA-104 cells) (Kim et al., 1993) and African green monkey cells (Vero cells; Cat. No. CCL-81) were cultured in Dulbecco's minimum essential medium (DMEM, HyClone), supplemented with 10% fetal bovine serum (Gibco) at 37 °C with 5% CO_2. Moreover, the HP-PRRSV WUH3 strain (GenBank accession no. HM853673) (Li et al., 2009) and the PEDV FJZZ strain (GenBank: KC140102.1) were used in all assays.

2.3. Expression and purification of PRRSV nsp4 (3CLSP) and PEDV nsp5 (3CL protease)

For expression of PRRSV 3CLSP, the coding sequence was PCR-amplified from the PRRSV WUH3 strain cDNA library and inserted into pET-42b (+) with a C-terminal His_6-tag at the *NdeI* and *BamHI* restriction sites. All constructs were validated by DNA sequencing. The plasmid was transformed into *Escherichia coli* strain BL21(DE3), and cells were cultured at 37 °C in LB medium containing 50 mg/l kanamycin. When the optical density at 600-nm (OD_{600}) reached 0.8, the culture was cooled to 18 °C and supplemented with 0.8 mM IPTG (isopropyl–D-thiogalactopyranoside). After overnight induction, the cells were harvested via centrifugation at 8500 rpm (30 min, 4 °C). Protein purification followed our previously reported procedure (Shi et al., 2016). Finally, the harvested protein was concentrated to a volume of approximately 2.0 ml and filtered using a Superdex 200 gel filtration column (GE Healthcare) equilibrated with buffer (20 mM Tris–HCl and 200 mM NaCl, pH 7.4). The concentration of the purified protein was determined by detecting the absorbance at 280-nm (A280) using a NanoDrop 2000c UV–vis spectrophotometer (Thermo Fisher Scientific). PEDV 3CL protease expression and purification were carried out following our previously reported procedure (Ye et al., 2016).

2.4. Measurement of IC_{50}

The polypeptide substrate Dabsyl-KTAYFQLE↓GRHFE-Edans (Tian et al., 2009) and Dabcyl-YNSTLQ↓AGLRKM-E-Edans (Ye et al., 2016) were chemically synthesized by the GenScript Corporation. PRRSV 3CLSP and PEDV 3CL proteases were used at a final concentration of 2 μM and 0.25 μM, respectively. Compounds at various concentrations (6.25–500 μM) were pre-incubated with protease for 20 min at 37 °C, and 1 μM (3CLSP substrate) or 10 μM (3CL protease substrate) was added to the mixture in a black 96-well plate. The mixtures were then further incubated at 37 °C for 60 min, and enhanced fluorescence due to substrate cleavage by 3CLSP/3CL protease was monitored at 340 excitation and 485 emission. The relative fluorescence units (RFU) were calculated by subtracting the background (substrate control well without protease) from the fluorescence readings. The percentage of inhibition was calculated as follows: Percentage of inhibition (%) = 100 × [1 − RFU of the experimental group (60 min– 0 min)/RFU of the control group (60 min–0 min)].

2.5. Cell viability assay

The 50% cell death toxic concentrations (CC_{50}) of compounds 2, 3 and 5 in MARC-145 cells and Vero cells were determined following our previously reported procedure (Shi et al., 2016). Briefly, confluent cells grown in white 96-well plates (Corning, Tewksbury, MA, USA) were treated with various concentrations (50–500 μM) of compounds for 72 h. MARC-145 cells or Vero cells were treated with 0.1% DMSO as control. Cell cytotoxicity was measured using the Celltiter-Glo Luminescent Cell Viability Assay reagent (Promega, Madison, WI, USA).

2.6. $TCID_{50}$ assay for PRRSV and PEDV

MARC-145 cells were seeded in 12-well plates at a density of 2.5×10^5 cells per well. When cells were grown to 70–80% confluence, PRRSV was added with increasing concentrations of compounds 2, 3 and 5 (6.25 μM, 12.5 μM, 25 μM, 50 μM, 100 μM and 200 μM) at a multiplicity of infection (MOI) of 0.1, and then supernatants containing virus and compounds were used to infect MARC-145 cells for 36 h. Then, the harvested supernatant (include PRRSV and compounds) was used for $TCID_{50}$ assays. Briefly, MARC-145 cells were seeded in 96-well plates, and infected with serial 10-fold dilutions of supernatant using eight replicates in $TCID_{50}$ assays. The plates were incubated for 72–96 h before viral titers were calculated. The $TCID_{50}$ assay for PEDV was also performed following the same methods.

2.7. Western blot analysis

MARC-145 cells or Vero cells were seeded in 6-well plates at a density of 5×10^5–1×10^6 cells per well. When cells were grown to 70–80% confluence, the PRRSV WUH3 strain and the PEDV FJZZ strain were inoculated into cells at an MOI of 0.1. Virus-infected cells were incubated in the presence of compounds (6.25 μM, 12.5 μM, 25 μM, 50 μM, 100 μM and 200 μM) for up to 36 h. Then, compound-treated PRRSV/PEDV-infected or control cells (0.4% DMSO) were lysed in a