Three kinds of treatment and their combination against coronavirus PEDV

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Abstract

**Background** Porcine epidemic diarrhea virus (PEDV) of the family *Coronaviridae* has caused substantial economic losses in the swine husbandry industry. The currently available vaccines are only partially effective and there is no specific drug available for treatment of viral infection.

**Method** In the current study, we use animal coronavirus PEDV as a model to study antiviral agents. Briefly, a fusion inhibitor tHR2, recombinant lentivirus-delivered shRNAs targeted to conserved M and N sequences, homoharringtonine (HHT), and hydroxychloroquine (HCQ) were surveyed for their antiviral effects.

**Results** Treatment with HCQ at 50 mM reduced virus titer in TCID$_{50}$ by 30 fold, and the combination with HHT at 150 nM reduced virus titer in TCID$_{50}$ by 200 fold.

**Conclusion** The combination of two drugs with low dose can more effectively inhibit PEDV infection compared to one alone. The present study offers information to contribute to the development of effective antiviral strategies against coronavirus infection, and demonstrates that the combination treatment, even at low concentrations, presents more potent antiviral activity than the separate components acting alone.

Introduction

Porcine epidemic diarrhea virus (PEDV) belonging to the subfamily Coronavirinae of the family *Coronaviridae*, which causes epidemic diarrhea in swine, was first reported in 1971 in the United Kingdom and was soon identified in many European and Asian countries [1]. Incidence of diarrheal disease in China and Southeast Asia has been reported at 80%-100% in suckling piglets [2–3]. At present, there is no commercially antiviral therapy available.

The current strategies for controlling viral infectivity focus on identification of agents capable of intervening in the essential steps for viral infection, especially viral entry and replication. The PEDV genome encodes 4 structural proteins, including spike glycoprotein (S), nucleocapsid protein (N), membrane protein (M), and envelope protein (E), 15 non-structural proteins (NSP), and accessory protein ORF3 [1]. N protein is a phosphorylated nucleocapsid protein, and the epitope of N protein can
induce the body to produce an effective immune response [4]. M protein plays an essential role in viral assembly [5]. In RNA interference (RNAi), short interfering RNA molecules or short hairpin RNA (shRNA) targeting specific mRNA leads to degradation of mRNA and inhibition of gene expression [6–7]. RNAi targeting viral genes is an effective way to protect cells from virus infection and has been shown to mediate genetic suppression of many human and animal pathogenic coronaviruses [8, 9, 10]. Class I enveloped viruses such as coronaviruses utilize a conserved membrane fusion mechanism, in which highly conserved heptad repeat (HR) regions interact to form six-helix bundle (6-HB) structures, contributing to the fusion of virus and cell membranes. The HIV-1 fusion inhibitor T20, for example, and several modified peptides (e.g., T1249, T2635, T-20 s138A), which are derived from the HR region, have been clinically approved to suppress HIV-1 and T-20-resistant variants [11–14]. Coronavirus S glycoprotein shares a mechanism similar to other Class I virus glycoproteins for membrane fusion [15–17]. Our previous work has shown that the modified HR peptide efficiently blocks coronavirus attack [17, 18].

Viruses are able to utilize cellular machineries for viral replication, and a growing understanding of cellular proteins and associated pathways in viral replication has supported the design of new strategies for developing novel antiviral agents. Small molecule chemicals that are able to target cellular machinery act as efficient antiviral agents. The natural compound homoharringtonine (HHT), for instance, is known to inhibit the first cycle of the elongation phase of eukaryotic translation. HHT antagonizes the phosphorylation level of endogenous and exogenous eIF4E (p-eIF4E), which may regulate the selective translation of specific mRNA [19, 20]. In a previous study, we observed that HHT completely inhibited infection by PEDV at a concentration of 500 nM in cell cultures; treatment with HHT at doses of 0.05 mg/kg significantly reduced viral load and relieved severe symptoms in PEDV-infected animals [20]. One crucial cellular mechanism, autophagy, relies on lysosomes for the clearance and recycling of abnormal proteins or organelles; the deregulation of autophagy is associated with the development of various diseases, including viral infection by coronavirus [21]. Hydroxychloroquine (HCQ), an autophagy inhibitor chloroquine hydroxy-derivative, acts as an anti-inflammatory agent by attenuating tissue injury through downregulation of inflammatory activation
It also triggers the host defense machinery by inducing innate immune activation [23]. HCQ shows efficiently antiviral activity against a broad spectrum of viruses, including Zika virus and Dengue virus [23, 24].

In the present study, we constructed a truncated HR2 peptide and recombinant lentivirus-delivered shRNAs targeted to conserved M and N sequences, as well as small molecule chemicals HHT and HCQ, to evaluate their antiviral activity. We identified that a combination treatment more significantly improved the antiviral effects compared to the action of individual components. This paper may provide a foundation to leverage these agents in treating coronavirus infection in humans and animals.

Materials And Methods
Cells, virus, and antibodies
Vero cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM L-glutamine, nonessential amino acids, sodium pyruvate, 5% or 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (all reagents are purchased from Gibco Invitrogen). Cells were cultured at 37°C in a humidified incubator with 5% CO₂. PEDV strain CV777 from the same passage with 1 × 10⁷/ml plaque forming units (PFU) is used in this paper. Anti PEDV-N (1:1000) mouse monoclonal antibody was obtained from Alpha Diagnostic International. Antibodies to actin (1:1000) and goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (HRP, 1:10000) were obtained from Beyotime Biotechnology.

Design of shRNA plasmids
The shRNA expression vector pGPU6/GFP/Neo was selected to be the parental plasmid. The shRNA sequences were as follows (5’-3’; only the sense strand is shown). shRNA-N: GCAAAGACTGACCCCACTA; shRNA-M: CTGGAATTTCACATGGAAT. The shRNA-MN plasmid carrying two shRNAs was prepared based on the two intermediate plasmids shRNA-N and shRNA-M. Briefly, shRNA-MN DNA sense template was Bbs I- (shRNA-N) - hU6 promoter- (shRNA-M) - BamH I. Empty vector pGPU6/GFP/Neo and shRNA-MN sequence were digested with Bbs I and BamH I respectively. Then the shRNA-MN sense was ligased to the vector pGPU6/GFP/Neo by T4 DNA ligase. All of these nucleotides were chemically synthesized by Invitrogen.
Transfection of shRNA plasmids and virus infection

For transfection experiments, $2 \times 10^6$ Vero cells were cultured in six-well plates to 50–70% confluence. Cells were transfected with plasmid using TransIntro EL according to the manufacturer's protocols. Briefly, for each well, 4 µg shRNA plasmid and 10 µl TransIntro EL were incubated with 100 µl opti-MEM, respectively, for 5 min. Subsequently, mixture of 10 µl TransIntro EL and 100 µl opti-MEM was added to the mixture of plasmid and opti-MEM. After incubation for 10–20 min at room temperature (RT), the mixture was added to the well. After incubation at 37 °C for 5 h, the medium containing the transfection mix was replaced with growth medium. At 24 h post-transfection (h.p.i), cells were infected with PEDV at multiplicity of infection (MOI) of 0.1. Cultures were then incubated at 37 °C, 5% CO2 in a humidified incubator for 1.5 h, at which point the medium was replaced with growth medium. After 48 h of incubation, the plates were prepared for virus titration and real-time PCR analysis.

MTT assay

The MTT assay was conducted according to the manufacturer’s (Beyotime Biotechnology) protocol. Briefly, cells in 96-well plates were treated with shRNA at 37°C and 5% CO2 for 24 h. Then, 10 µl of MTT solution was added to each well and the samples were incubated for 4 h. The medium was then removed and 100 µl of Formazan solution was added to each well. Optical density (OD) values were measured at a test wave length of 570 nm using a Mullikan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, USA).

Preparation of cell lysates and western blotting

The cells in 6-well plate were collected by centrifugation, washed three times with PBS and dissolved in 200 µl lysis buffer (pH7.5 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 2.5 mM Sodium pyrophosphate, 1 mM β-Glycerophosphate, 1 mM NaVO4, 1 µg/ml Leupeptin) containing protease inhibitor cocktail (Roche) for 30 min at ice. The cell suspension was then fractionated by centrifugation at 6000 × g for 10 min at 4°C. Solubilized proteins were harvested, electrophoresed in denaturing polyacrylamide gels, electro blotted onto a polyvinylidene fluoride (PVDF) membrane, and reacted with the antibodies indicated overnight. Protein bands were detected with secondary antibody conjugated to horseradish peroxidase (HRP) for 45 min at room temperature, and actin was
used as a loading control.

**Quantitative real-time PCR (qRT-PCR)**

Cells were harvested, and total RNA was extracted with Trizol (Invitrogen). A two-step RT-PCR (SYBR Green I technology, Applied Roche) was performed using SYBR green super mix (Toyobo) according to the manufacturer’s protocol to measure transcription levels for several genes of interest. The primers used were as follows: PEDV-N: 5’- CTG GGT TGC TAA AGA AGG CG -3’ (forward), 5’- CTG GGG AGC TGT TGA GAG AA -3’ (reverse). IL-1β: 5’- GAC CTG GAC CTC TGC CCT CTG -3’ (forward), 5’- AGG TAT TTT GTC ATT ACT TTC -3’ (reverse). IL-6: 5’- AAC TCC TTC TCC ACA AGC – 3’ (forward), 5’- TGG ACT GCA GGA ACT CCT – 3’ (reverse). GAPDH: 5’-GAT CAT CAG CAA TGC CTC CT -3’ (forward), 5’- TGA GTC CTT CCA CGA TAC CA -3’ (reverse).

Relative fold changes were automatically calculated by the Step One Plus real-time PCR system software (Applied Bio systems), following the $2^{-\Delta\Delta CT}$ method. GAPDH as a control.

**Determination of 50% tissue culture infectious dose (TCID$_{50}$)**

Vero cells in 6-well plates were cultured overnight with 80% confluency. After PEDV infection for 90 min, the culture supernate was replaced with 2% FBS of DMEM and incubated for 48 h. Virus titration was detected. Ten-fold serial dilutions were prepared for each sample and 100 μl/well of each dilution were added to the cells in 96-well plates in quadruplicates. Wells with cytopathic effect were scored as positive for virus growth and TCID$_{50}$ was determined by the method of Reed and Muench.

**Prediction and construction of HR1 and tHR2**

The software packages LearnCoil-VMF (http://nightingale.lcs.mit.edu) and ExPASy-Coils (http://www.ch.embnet.org/software/COILS) were used to study the coiled coils. HR1 and HR2 domains of spike glycoprotein (gS) from the PEDV (GeneBank Accession No. ALS35469.1), derived from amino acids 978 to 1118 and 1263 to 1314 (52Aa), respectively, were predicted. In addition, an optimised Lupas algorithm was used with window widths of 14, 21 and 28 and the MTIDK matrix. The predicted probability of forming coiled coils was nearly 1.0. Optimal focused HR1 and HR2 domains were predicted as follows: NSAIGNITSA FESVKEA ISQTSKGL NTVAH ALTKQEVVN SQGSALNQLT
VQLQHNFQAI, and LTGE IADLEQR SESLRNT TEELRSL INNINNT LVDLEWL (39Aa), respectively. The scramble peptide is LTGE IADVEQR SESVRNT TEEVRL INNVNNT LVDVEWL, which is also derived from the PEDV HR2 domain, as negative control (NC). All of these peptides were chemically synthesized by China Peptides.

**Gel-filtration analysis**
The highly purified HR1 and tHR2 were loaded onto the Superdex G75 column in a solution buffer of 20 mM Tris-HCl, pH 8.0. The peak MW was estimated by comparing the substrate with protein standards running on the same column. The analytical column was calibrated with a series of individual runs of standard molecular mass proteins as markers, including bovine serum albumin (68 kDa), egg white albumin (43 kDa), ribose nucleotides (13.7 kDa), and antimicrobial peptides (5 kDa).

**Immunofluorescence assay**
Cells growing on glass coverslips in 24-well plates were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 15 min, and blocked with phosphate-buffered saline (PBS) containing 10% horse serum plus 1% bovine serum albumin (BSA) for 2 h. The cells were washed three times with PBS and incubated with diluted primary antibodies overnight at 4°C. Cells were washed three times with PBS at room temperature (RT), then incubated with fluorochrome-conjugated secondary antibodies in the dark for 1 h at RT. The cells were then rinsed, mounted and examined; images were captured using an Olympus FluoView™ FV1000. The primary antibody to PEDV-N (1:1000) and the goat anti-mouse IgG (H + L) secondary antibody conjugated to Alexa Flour 488 (Invitrogen).

**Statistics**
All results were expressed as means and standard deviations (SD). Statistical analyses were performed using Prism 5.01 (GraphPad Software). Significance was determined by one-way analysis of variance (ANOVA) and two-way ANOVA with Dennett’s multiple-comparison test. Partial correlation analyses were evaluated by unpaired student's test.

**Discussion**
In the current study, we found that the combination of HHT and HCQ at low concentrations reduced
TCID$_{50}$ by 200 fold (Fig. 4). In our previous study, we found that HHT exerted broad-spectrum antiviral action and completely inhibited infection by PEDV at a concentration of 500 nM in Vero cells [20]. Treatment with HHT at doses of 0.05 mg/kg significantly reduced viral load and relieved severe symptoms in PEDV-infected animals, but treatment with 0.2 mg/kg of HHT led to severe side effects, fatal to about 50% of the animals [20]. The inhibitory effect of HCQ is associated with host defense machinery and immune regulation; HCQ at high concentration also exhibits tissue toxicity [21–24]. The study of preparations therefore provides new ideas, but the damage of host cells resulting from increasing doses is a problem that cannot be ignored.

Recent research has shown that combination therapy can be more effective than monotherapy. The combination of ribavirin with interferon-β, for instance, inhibits SARS-CoV replication very strongly, compared to either single treatment [25]. A combination of RNAi and a virus receptor trap exerts additive antiviral activity in coxsackievirus B3-induced myocarditis in mice [26]. Likewise, antibody administered with Sunitinib significantly reduces vascular leakage and synergizes to provide superior protection from lethal DENV infection compared with either agent alone [27]. Combination therapies targeting viruses at different stages of their lifecycle are a common strategy to improve the efficiency of the antiviral treatment, and the combination of multi-target preparations is expected to be less cytotoxic [28].

The RNAi-based strategy has different mechanisms of action than antibodies, small molecules, and other protein drugs. It is worth mentioning that the RNAi drug Onpattro (patisiran) was the first FDA-approved for adult patients with peripheral neuropathy (polyneuropathy) caused by hereditary transthyretin amyloidosis (hATTR). Methods to express multiple shRNAs from a single vector have been explored, including expression from multiple cassettes using the same promoter [29], or from a single vector containing multiple promoters [30]. The inhibition of viruses by means of RNAi is an approach to antivirals that has gained importance in recent years [10, 31–34], but the use of multiple shRNAs in the elimination of viral infection has been less successful. Our study demonstrates that dual shRNA-expressing plasmids effectively inhibit the replication of PEDV, better than individual shRNA (Fig. 1). We have established a concept for developing transfection vectors that may have
wide applications in gene antiviral strategies, including the delivery of siRNA to combat viral infection. This approach has the potential to be utilized for the prevention and control of coronavirus PEDV. However, the inhibitory effect of double shRNA needs to be further improved. In future work, the human U6 promoter in the vector pGPU6/GFP/Neo will be replaced with the porcine U6 promoter to increase the transfection efficiency of shRNA and enhance the inhibitory effect.

The membrane fusion process has been considered as a significant antiviral target and has received wide attention; the recombinant HR2 region of fusion glycoprotein is the ideal fusion inhibitor [11]. Based on a LearnCoil-VMF prediction result, amino acid L1252 to L1286 (35Aa) from the HR2 domain of S glycoprotein was selected to design a fusion inhibitor that efficiently blocks MERS-CoV entry [16–17]. The predicted HR2 domain (65Aa) from S glycoprotein of PEDV, however, is longer than that in MERS-CoV and other coronaviruses [16–18, 35], and synthetic HR2 peptide at the concentration of 40 µM statistically inhibits PEDV infection [36]. Reducing the length of the active peptide may be an important consideration to reduce the cost of peptide agents. Here we truncated the tHR2 domain to produce the peptide tHR2 (39Aa), with the aim of identifying a minimal domain capable of disrupting the formation of the six-helix bundle required for viral cell entry. Our results suggest that tHR2, but not scrambled peptide, has the ability to inhibit PEDV infection (Figs. 3E, 3F). Furthermore, the antiviral effect of tHR2 is not as strong as expected, perhaps due to the short length or disrupted core structure. It is noteworthy that by 2013 the FDA had already approved the listing of more than 60 peptide drugs [37]. Peptides will be widely used in medicine and biotechnology in the near further.

In this study, we explored how to maximize the antiviral effect of HHT while reducing host cell toxicity. We found that tHR2 peptide designed based on PEDV S genes effectively inhibits the membrane fusion of PEDV, and the site of action is clear and does not affect the cell structure. The combination treatment with HHT and tHR2 results in additive protective effects by inhibiting membrane fusion and viral replication. We also found that the combination of HHT and shRNA-MN did not exhibit improved effects (not shown here), perhaps because they have the same target in the viral replication process. More details remain to be studied. The combination of a low dose of HHT and/or HCQ as host targeting antivirals (HTA) with tHR2 as direct-acting antiviral (DAA) has the
potential to improve safety and increase the antiviral effect. These results provide a reference for the
development of clinical drugs against coronavirus attack and infection in the future.

Abbreviations
h.p.i.
Hour post infection
HCQ
Hydroxychloroquine
HHT
Homoharringtonine
HR
Conserved heptad repeat regions
PEDV
Porcine epidemic diarrhea virus
PFU
Plaque forming units
RNAi
RNA interference
RT
Room temperature
SD
standard deviations
ShRNA
Short hairpin RNA

Declarations

Ethics approval and consent to participate
Not applicable.

Consent to publication
Not applicable.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Competing interests
The authors declare that they have no conflict of interest. None of the authors of this article has a
financial or personal relationship with other people or organizations that could inappropriately
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Authors contribution

Cui-Cui Li designed and performed the experiments, and analyzed the data.

Xiao-Jia Wang directed the experiments and writing the manuscript.

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Legends
Figure 1

The design of shRNA-expressing plasmids and antiviral activity

A, Schematic illustration of construction of single shRNA-expressing plasmids, pGPU6/GFP/Neo-shRNA-M and pGPU6/GFP/Neo-shRNA-N, and the dual shRNA expression plasmid, pGPU6/GFP/Neo-shRNA-MN. B, Cells were transfected with shRNA-expressing plasmids for 24 h, transfection efficiency by vector GFP fluorescent label was observed. C, The effect on cell viability was assessed. C, Cytopathic effects caused by PEDV infection. Vero cells were transfected with shRNA-expressing plasmids for 24 h, and then infected with PEDV at 0.1 MOI. Pictures were taken at 48 h post-infection. D, At 48 h post-infection, cells were harvested and immunoblotted with antibody to viral protein PEDV-N, with actin as a control. N/A, the change in abundance of PEDV-N was analyzed by densitometric analysis using Image-Pro.
Plus Software and normalized to actin. Under the same experimental conditions, total RNA was isolated and PEDV mRNA was quantified by qRT-PCR normalized against GAPDH (E), and viral yields in the medium were determined by TCID50 (F). Values represent means and SD for triplicates. * p <0.05 for t-test.
Construction and antiviral activity of soluble peptide tHR2. A, Proposed helical wheel of tHR2.

B, Gel-filtration analysis of mixed HR1 and tHR2 at the equimolar concentration. The molecular weight (MW) of the HR1 domain from PEDV was approximately 6.5 kDa, and HR2 was 4.3 kDa. C and D, Vero cells were exposed to PEDV (0.1 MOI) in the absence or presence of peptides, including scramble (NC) and tHR2 peptides at concentrations of 40 and 80 μM for 48 h. These experiments were performed two times with three replicates in each experiment. Values represent means and SD. * p < 0.05 compared to PBS-treated group.
The effect of HCQ on autophagy and inflammatory response A, Vero cells were infected with PEDV at 0.1 MOI in the presence of HCQ at 25 and 50 μM for 24 h, cell lysates were prepared, and LC3B, PARP, and PEDV-N were measured by immunoblotting with specific antibodies, with actin as a control. B, cells were infected with PEDV at 0.1 MOI in the presence of the HCQ at 50 μM for 24 h, total RNAs were isolated and expression levels of IL-1β (left) and IL-6 (right) mRNAs were quantified by RT-PCR and normalized to that of GAPDH. C, Vero cells were exposed to PEDV of 0.1 MOI in the presence of HCQ at 25 and 50 μM for 24 h. The infected cells were imaged by fluorescence microscopy using an Olympus IX73 microscope (magnification of 100 x ) equipped with a DP73 camera, and inserted picture is assessed under light microscopy. Each image is representative of three experiments.
Antiviral activity of combination treatment A-B, Vero cells seeded at $2.5 \times 10^6$ cells per well in six-well plates were infected with PEDV at 0.1 MOI in the presence of the HHT at different concentrations for 48 h, electrophoretically separated proteins were analyzed by immunoblotting with antibodies to viral protein PEDV-N and actin (A). Viral yields in the medium were determined by TCID50 (B). These experiments were performed two times with three replicates in each experiment. Values represent means and SD. $^* p < 0.05$; $^{**} p < 0.01$; $^{***} p < 0.001$ compared to PBS-treated group. C-D, Vero cells seeded at $2.5 \times 10^6$ cells per well in six-well, cells were then exposed to PEDV at 0.1 MOI in the presence of peptide tHR2 and HHT (C) or HCQ (D). At 48 h post-infection, virus titer in TCID50 was determined. Values represent means and SD for triplicates. $^* p < 0.05$, $^{**} p < 0.01$ for t-test.