JOURNAL OF NATURAL PRODUCTS

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Article

Phenanthridine Derivative Host Heat Shock Cognate 70 Down-Regulators as Porcine Epidemic Diarrhea Virus Inhibitors

Duo-Zhi Chen,[‖] Shi-Rui Fan,[‖] Bi-Juan Yang, Huo-Chun Yao, Yi-Ting Wang, Jie-Yun Cai, Chen-Xu Jing, Zi-Hao Pan, Miao Luo, Yan-Qiu Yuze, Guang-Jin Liu,* and Xiao-Jiang Hao*

Cite This: *J. Nat. Prod.* 2021, 84, 1175−1184

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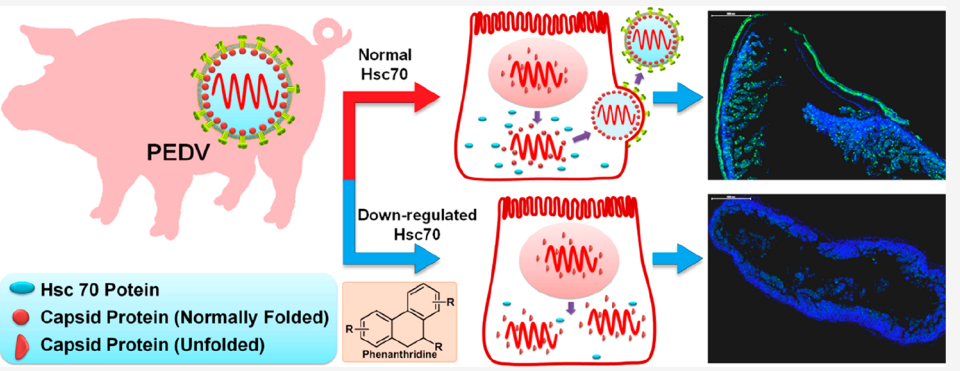


ABSTRACT: Porcine epidemic diarrhea virus (PEDV) has become increasingly problematic around the world, not only for its hazards to livestock but also due to the possibility that it is a zoonotic disease. Although vaccine therapy has made some progress toward PEDV control, additional effective therapeutic strategies against PEDV are needed, such as the development of chemotherapeutic agents. The aim of this work was to identify novel anti-PEDV agents by designing and synthesizing a series of phenanthridine derivatives. Among them, three compounds (compounds **1**, **2**, and **4**) were identified as potent anti-PEDV agents exhibiting suppression of host cell heat shock cognate 70 (Hsc70) expression. Mechanism studies revealed that host Hsc70 is involved in the replication of PEDV, and its expression can be suppressed by destabilization of the mRNA, resulting in inhibition of PEDV replication. Activity against PEDV in vivo in PEDV-infected piglets suggested that phenanthridine derivatives are the first host-acting potential anti-PEDV agents.

Coronaviruses are pathogens that may cause great harm to human and animal health. These viruses can cause severe and life-threatening diseases of the enteric or respiratory systems. For example, zoonotic coronaviruses cause severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) in humans and the deadly porcine epidemic diarrhea virus (PEDV) for newborn piglets.[1−6] Compared to other positive-sense RNA viruses, coronaviruses have an exceptionally large genome and employ a complex genome expression strategy. Next to a role in basic virus replication or virus assembly, many of the coronavirus proteins expressed in the infected cell contribute to the coronavirus−host interplay to create difficulties in combating these infections.[7−10] As a result, despite the harmful impact of such coronavirus infections and the likelihood of future outbreaks of additional pathogenic coronaviruses, the options to prevent or treat these infections remain very limited. This highlights the importance of advancing research on novel antiviral mechanisms and alternative new antiviral agents that not only inhibit viral replication but also prevent the emergence of resistance.[11−14]

It has been shown that regulating some relatively conservative viral replication-related factors in host cells to affect viral replication by affecting the microenvironment of viral replication is also an effective strategy for the prevention of viruses, especially those with high variability. Previous studies have reported that regulation of host heat shock cognate 70 (Hsc70) expression may be a promising antiviral therapeutic strategy.[15,16] Hsc70 is a cytoplasmic adenosine triphosphate binding protein with 646 amino acids. It is a member of the heat shock protein 70 (Hsp70) family.[17] Virology research has shown that Hsc70 might play a role in regulating virion capsid assembly.[18] It has also been demonstrated that host Hsc70 also forms part of the virus

Received: November 18, 2020
Published: March 24, 2021

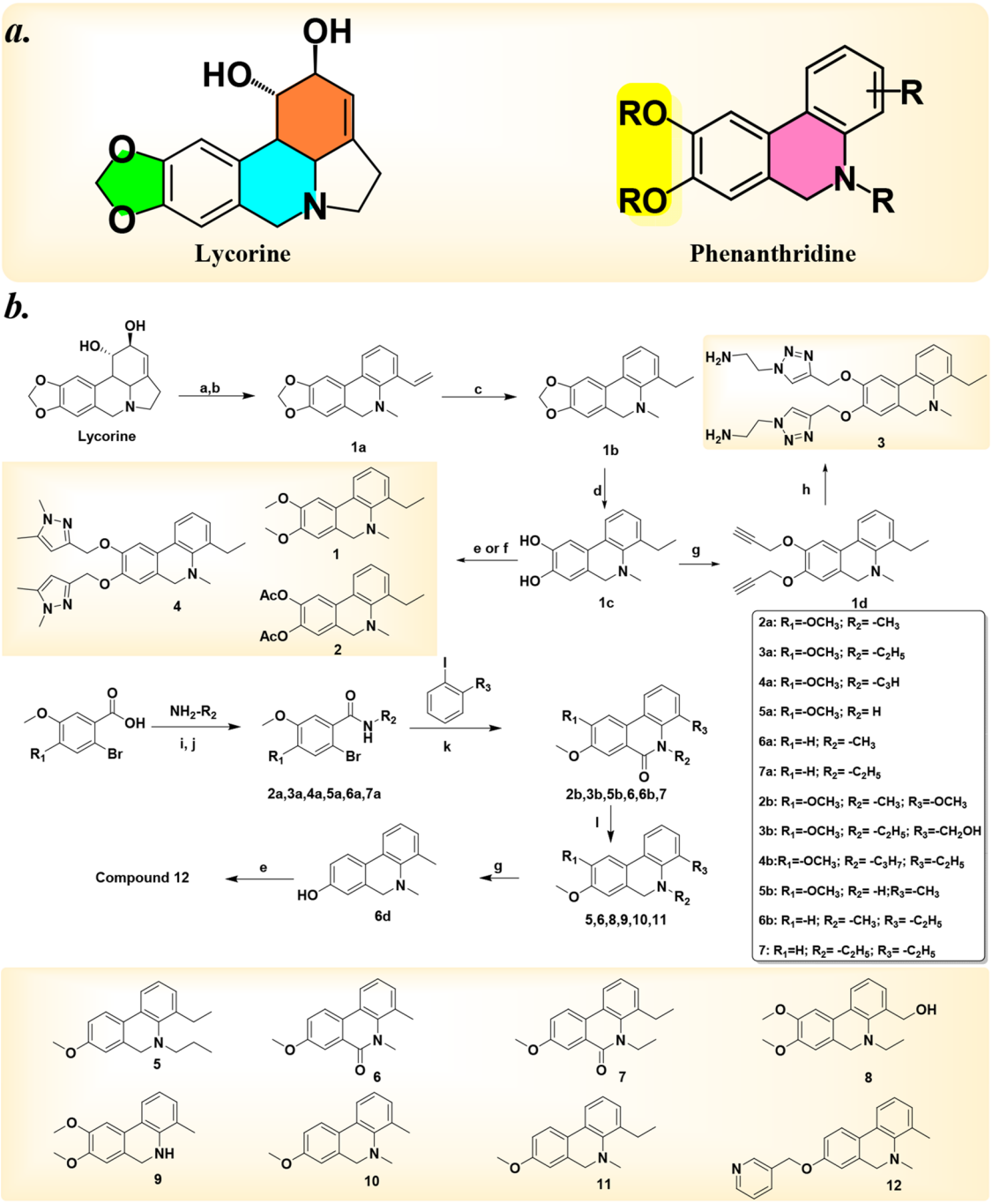
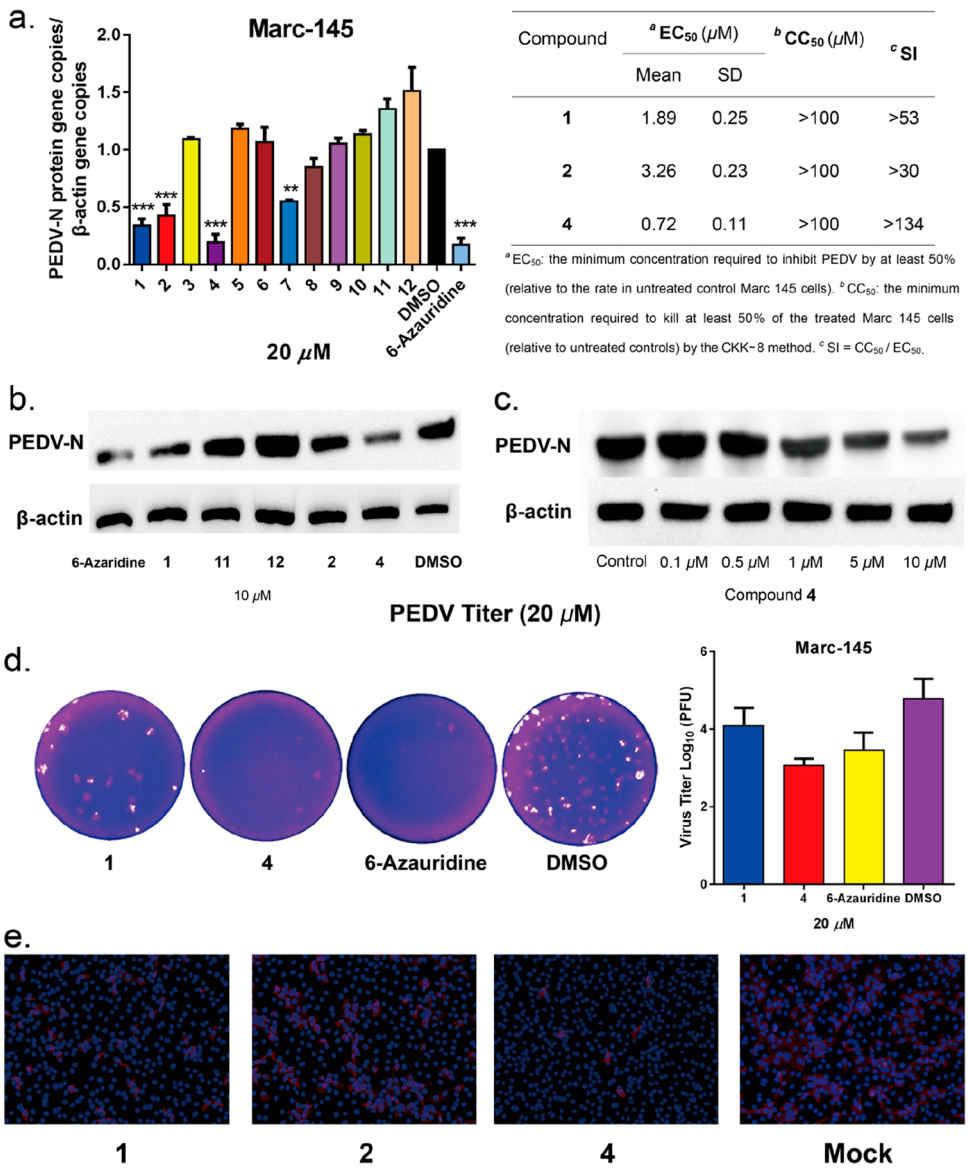


Figure 1. Design and synthesis of phenanthridine derivatives. (a) Regions (colors labeled) of the lycorine and phenanthridine skeleton targeted for modification, i.e., the C-1 and C-2 hydroxy groups, the double bond between C-3 and C-4, the N-5 position, and the benzodioxole group. (b) The 12 phenanthridine derivatives (highlighted in beige) synthesized. In part b, the reagents and conditions are as follows: (a) MeI, DMF, rt, 12 h; (b) *t*-BuOK, *t*-BuOH, N_2, reflux, 4 h; (c) 10% Pd/C, CH_2Cl_2, rt, 24 h; (d) BBr_3, CH_2Cl_2, −78 °C, 4 h; (e) RX, NaH, THF, N_2, rt, 24 h; (f) Ac_2O, pyridine, DMAP, THF, N_2, 60 °C, 6 h; (g) propargyl bromide, K_2CO_3, acetone, N_2, 60 °C, 24 h; (h) 2-azidoethan-1-amine, Cu_2SO_4, sodium ascorbate, H_2O/*t*-BuOH, N_2, 60 °C, 20 h; (i) $SOCl_2$, DMF, THF, 50 °C, 2 h; (j) ammonia reagents, 5 °C; (k) K_2CO_3, norbornene, $Pd(OAc)_2$, TFP, CH_3CN, 85 °C, 6 h; (l) BH_3−THF, THF, −78 °C, 2 h.

particle, and host Hsc70 modulates virus infectivity as well as lipid droplet−dependent virus release after HCV (hepatitis C virus) enters host cells.[19] Down-regulation of Hsc70 in virus-infected host cells reduces the levels of many different types of viruses, such as flaviviruses and hepatoviruses. Hsc70 down-regulators inhibit viral replication while preventing the emergence of resistance.[20,21] Although there have been no reports indicating a correlation between Hsc70 expression and virus replication in animals or studies of the effects of Hsc70 on the coronavirus, the aforementioned findings indicate that Hsc70 may be a useful potential drug target against coronavirus infection. Our previous studies have identified a series of phenanthridine derivatives as potent antiviral agents for HBV (hepatitis B virus), HCV (hepatitis C virus), and TMV (tobacco mosaic virus), which act by down-regulating host Hsc70 expression.[22−24] This implies that Hsc70-

https://doi.org/10.1021/acs.jnatprod.0c01252
J. Nat. Prod. 2021, 84, 1175−1184



Compound	[a] EC_{50} (μM)		[b] CC_{50} (μM)	[c] SI
	Mean	SD		
1	1.89	0.25	>100	>53
2	3.26	0.23	>100	>30
4	0.72	0.11	>100	>134

[a] EC_{50}: the minimum concentration required to inhibit PEDV by at least 50% (relative to the rate in untreated control Marc 145 cells). [b] CC_{50}: the minimum concentration required to kill at least 50% of the treated Marc 145 cells (relative to untreated controls) by the CKK-8 method. [c] SI = CC_{50} / EC_{50}.

Figure 2. Anti-PEDV evaluation of phenanthridine derivatives at a concentration of 10 μM in vitro. (a) Compounds **1**, **2**, **4**, and **11** inhibited PEDV ($p < 0.01$) at the transcription level but did not exhibit cytotoxicity at a high concentration of 100 μM by the CKK-8 method. (b) Compounds **1**, **2**, and **4** inhibited PEDV at the protein level at a single concentration of 10 μM. (c) Compound **4** inhibited PEDV at the protein level at a gradient concentration. (d) The viral titer was determined by a plaque assay and quantified. The data shown are from three independent experiments. (e) Indirect immunofluorescence assay indicated that levels of the PEDV protein (red color) in thenanthridine-treated cells were much less than those in the mock.

regulation is a promising strategy for fighting viral infections and that phenanthridine-type compounds may be suitable as molecular probes to investigate whether a coronavirus may be inhibited by effecting host cell Hsc70 expression, even though it belongs to a different family with a different replication mechanism compared with that of HBV, HCV, or TMV.

Since the coronavirus, porcine epidemic diarrhea virus (PEDV) was first discovered in Belgium in the 1970s; it has spread to many countries, including the People's Republic of China, Japan, the United States, and Canada, and is rapidly becoming problematic across the world.[25−28] PEDV is a deadly virus for newborn piglets with case fatality rates of nearly 100%. Research has shown that PEDV is typical of coronaviruses and is closely related to and shows significant genetic uniformity with other members of the coronaviruses family. In addition, some pilot studies have suggested that PEDV may be a zoonotic disease, even though traditionally it is believed to not infect humans.[29,30] In this investigation, with the purpose to explore new mechanisms for inhibiting the coronavirus in a preliminary manner, PEDV was employed as the model virus system, and a series of phenanthridine derivative inhibitors were rationally designed and synthesized. The antiviral activities in vitro and in vivo and the mechanisms of action of these compounds were evaluated to investigate correlations between the replication of the coronavirus examined and the expression of host Hsc70.

RESULTS AND DISCUSSION

Design and Synthesis of Phenanthridine Derivatives.

Based on previous studies of phenanthridine derivatives and their anti-HBV/HCV activities, down-regulation of host Hsc70, and structure−activity relationships (SARs, Figure

https://doi.org/10.1021/acs.jnatprod.0c01252
J. Nat. Prod. 2021, 84, 1175−1184

1a),[25−28] a series of phenanthridine derivatives was designed and synthesized. As shown in Figure 1b, compounds **1**−**4** were derived from lycorine through a semisynthesis process. Lycorine was initially exhaustively methylated, followed by Hoffman degradation and Pd/C hydrogenation. Subsequent BBr_3 degradation and then alkylation or acylation yielded compounds **1**−**4**. The rest of the compounds were synthesized via one-step palladium-catalyzed sequential aryl−aryl and *n*-aryl coupling between bromobenzoic acid analogues and iodobenzene analogues. In total, 12 compounds were prepared for further evaluation of anti-PEDV activity.

Anti-PEDV Activity of Phenanthridine Derivatives In Vitro. The phenanthridine compounds were initially screened to evaluate their inhibitory effect on PEDV through a PEDV recipient African green monkey embryonic kidney cell line (Marc145 cells). In this assay system, the Marc145 cells were infected with PEDV. The PEDV-positive Marc145 cells then readily produced infectious PEDV particles; incubation of naïve Marc145 cells caused PEDV infection in the cells. The PEDV-infected Marc145 cells were treated with the synthesized phenanthridine derivatives for 24 h; then, PEDV mRNA was detected by real-time quantitative PCR (qPCR), using 6-azauridine[28] as a positive control. The results indicated that some derivatives (**1**, **2**, **4**, and **11**) inhibited the replication of PEDV. Compounds **1**, **2**, and **4** decreased the production of PEDV mRNA by up to 70% (Figure 2a). A dose−effect relationship assay of **1**, **2**, and **4** was examined (EC_{50}), and their cytotoxicity was assessed in vitro by the CKK-8 method (Figure 2a). Overall, the results showed that **1** and **4** exhibited good anti-PEDV activity with EC_{50} values of 1.89 and 0.72 μM, respectively, and no cytotoxicity at a high concentration of 100 μM. Western-blot analysis confirmed that compounds **1**, **2**, and **4** suppressed PEDV at the protein level (Figure 2b), and compound **4** inhibited the replication of PEDV in a dose-dependent manner (Figure 2c). In addition, using a plaque assay, the viral titer of drug-treated cells was determined and, as Figure 2d shows, compounds **1** and **4** clearly decreased the PEDV virion in infected cells at the concentration of 20 μM. To visualize the reduction of PEDV protein in the cytoplasm, indirect immunofluorescence was carried out on phenanthridine inhibitor-treated Marc145 cells. Treatment with 10 μM of the compounds for 24 h caused inhibition of the PEDV protein in Marc145 cells (Figure 2e, red color). All these various test results indicated that compounds **1** and **4**, but especially **4**, can inhibit the replication of PEDV.

Phenanthridine Derivatives Down-Regulate Host Hsc70 Expression. To investigate the mode of action of the phenanthridine derivatives produced, the capacity to down-regulate Hsc70 was evaluated using an Hsc70 mRNA quantitative assay model in Marc145 cells. The infected Marc145 cell line was treated with the top compounds for 24 h, followed by determination of Hsc70 mRNA expression. As shown in Figure 3a, phenanthridine derivatives **1**, **2**, and **4** suppressed host Hsc70 expression at a concentration of 10 μM. Furthermore, according to a qPCR assay, their PEDV inhibition and Hsc70 down-regulation activities were correlated positively (Figure 3b). These results indicate that phenanthridine derivatives inhibit the PEDV while simultaneously suppressing Hsc70 expression. In a subsequent Western blot assay, the same phenomenon could also be observed that compound **4** inhibited the PEDV while down-regulating the expression of Hsc70 at the protein level in a dose-dependent manner (Figure 3c). Together, these results

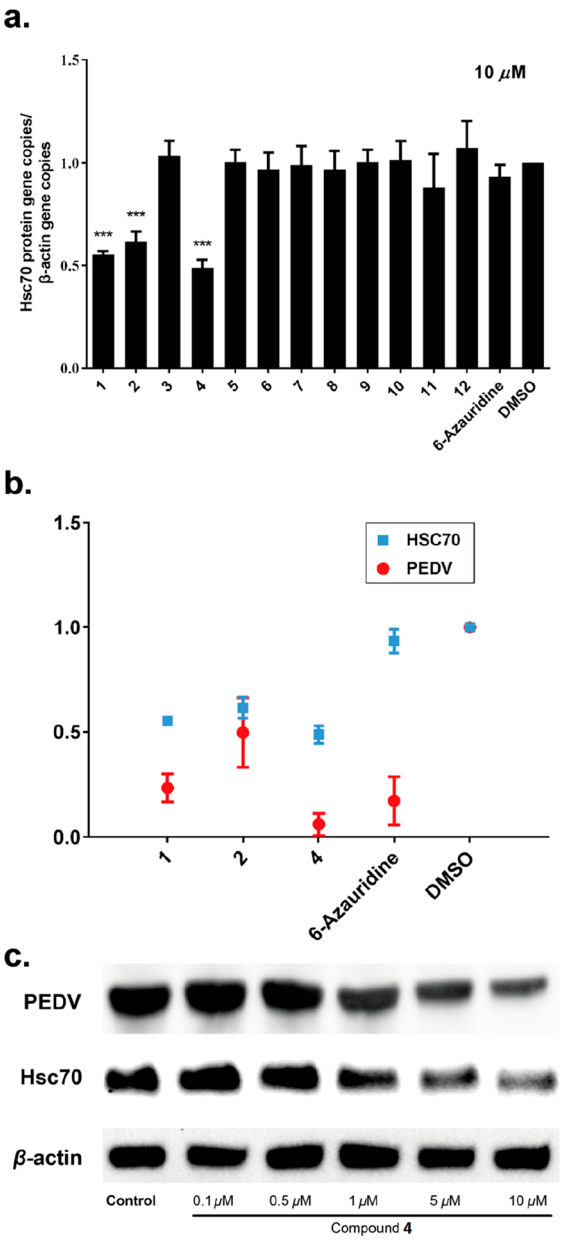


Figure 3. Phenanthridine derivatives regulate the expression of host Hsc70. (a) Intracellular Hsc70 mRNA (detected with qPCR, $p < 0.01$) treated with phenanthridine derivatives. (b) Correlation between inhibition of Hsc70 expression and anti-PEDV activities of the tested phenanthridine-type derivatives (detected with qPCR). (c) Compound **4** suppressed the PEDV-N protein as well as the Hsc70 protein (Western blot) in a dose-dependent manner.

imply that there was a correlation between Hsc70 down-regulation and the anti-PEDV activity of phenanthridine derivatives.

Anti-PEDV Mechanism of Phenanthridine Derivatives. The results suggested that suppression of Hsc70 expression of the host cells may be the main mechanism for the anti-PEDV activities of phenanthridine derivative. To test this, the effects of compound **4** on PEDV assembly were initially evaluated by comparing the proportion between supematant PEDV gene copies and interacellular PEDV gene copies of control and compound-treated groups. As mentioned above, down-regulation in the host would block the assembly of the virus, and the results obtained did indicate that **4**

https://doi.org/10.1021/acs.jnatprod.0c01252
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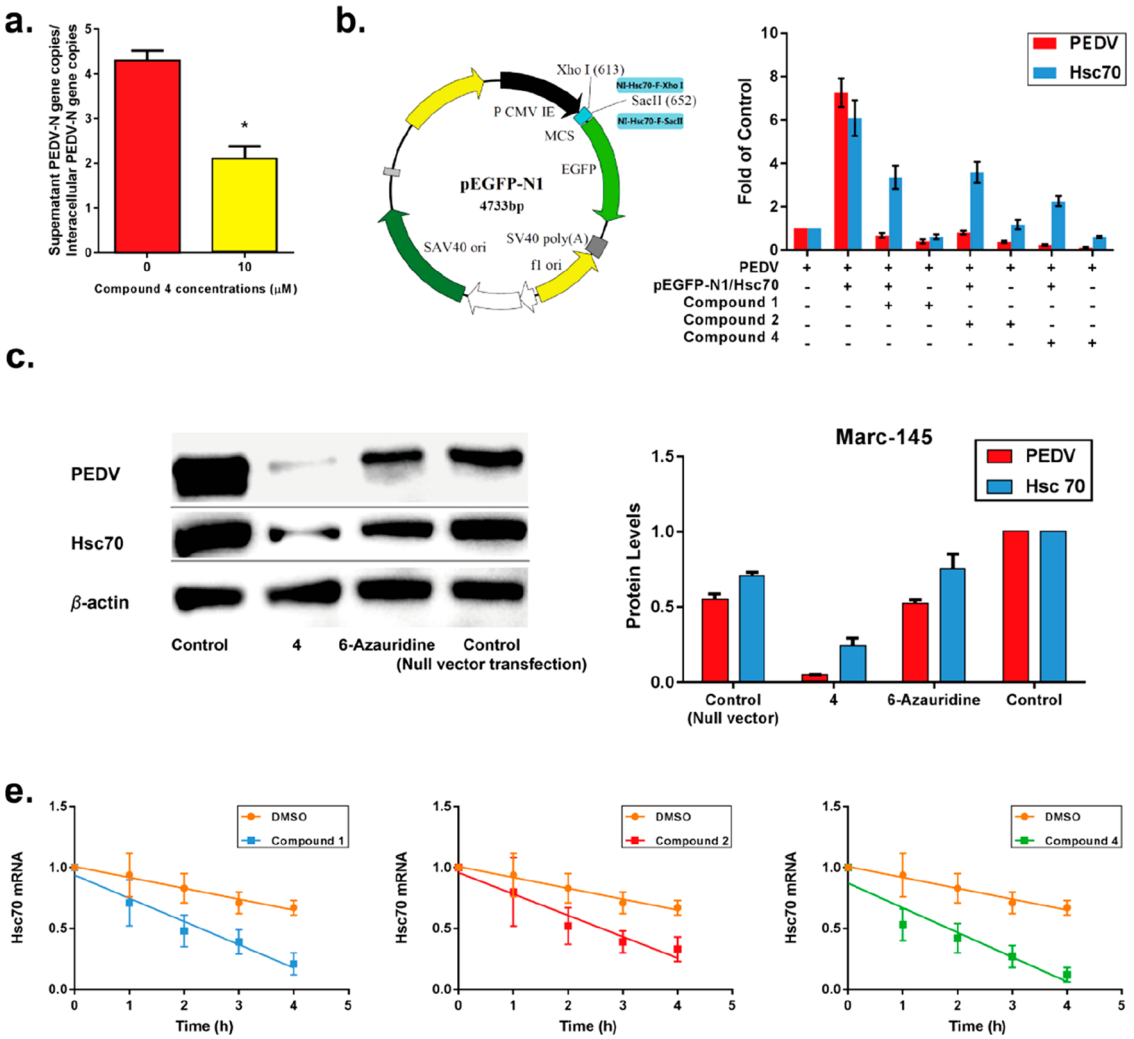

Figure 4. Studies on anti-PEDV mechanism of phenanthridine derivatives. (a) Effects of compound **4** on PEDV assembly ($p < 0.01$). (b) Construction of external Hsc70-containing plasmid. Phenanthridine derivatives strongly inhibited PEDV replication in PEDV-infected Marc145 cells with the addition of external Hsc70. The Marc145 cells were infected with PEDV for 24 h, followed by transfection of the cells with pEGFP-N1/Hsc70 (or pEGFP-N1 as control) for 24 h. These cells were then treated with compounds **1**, **2**, and **4** (20 μM) for 24 h. The level of Hsc70 mRNA as well as PEDV RNA was evaluated with real-time PCR using PEDV-infected Marc145 cells treated with neither top compound nor external Hsc70 as control. For the *y*-axis, the control value of Hsc70 and PEDV mRNA was normalized as 1; the amounts of Hsc70 mRNA or PEDV RNA in the cells with or without drug treatment were plotted relative to that value. The experiment was repeated three times. (c) Compound **4** also showed stronger anti-PEDV activity at protein levels in Hsc70 inserted cells. (d) The half-life ($T_{1/2}$) of Hsc70 mRNA was shortened in Marc145 cells treated with compounds **1**, **2**, and **4** (20 μM) as compared with that in the untreated control.

suppressed the assembly of PEDV. Following this, external Hsc70 was inserted into PEDV-positive Marc 145 cells by transfecting the cells with a Hsc70-containing plasmid (pEGFP-N1/Hsc70, Figure 4b), with the purpose of confirming that the anti-PEDV activities of compounds **1**, **2**, and **4** are Hsc70-dependent. If compounds truly act by down-regulating Hsc70, each compound should be effective against Hsc70-related PEDV replication. Figure 4b shows that addition of external Hsc70 increased PEDV replication in Marc145 cells by 7.2-fold. However, treatment with phenanthridine derivatives such as compound **4** significantly decreased PEDV RNA by 8.1 times (cells without external Hsc70) to 29.3 times (cells with external Hsc70). At the protein level, compound **4** suppressed the PEDV-N protein by 20.5 times in external Hsc70 inserted cells. In contrast, the positive control, 6-azauridine, showed much weaker antiviral activity in Hsc70-inserted cells than in normal cells (Figure 4c). These results indicated that PEDV inhibition by the most active phenanthridine derivatives in this model was more potent than that in the PEDV-positive Marc 145 cells with no addition of Hsc70, suggesting that Hsc70 is a possible mechanism of action for the phenanthridine derivatives evaluated.

A further investigation into the mode of action of these compounds was conducted. Based on the finding that certain phenanthridine derivatives reduced Hsc70 mRNA in Marc145 cells, it was hypothesized that these compounds may suppress Hsc70 expression by destabilizing Hsc70 mRNA. To test this hypothesis, the half-life ($T_{1/2}$) of Hsc70 mRNA was determined in phenanthridine-treated Marc145 cells. In this actinomycin-D-treated quantitative assay model, the phenanthridine derivatives **1**, **2**, and **4** decreased the $T_{1/2}$ value of Hsc70 mRNA by up to 72% (from 6.51 to 2.31, 2.61, and 1.84 h, respectively) in Marc145 cells (Figure 4d), thereby they each exhibited a destabilization effect on Hsc70 mRNA. This indicates that these phenanthridine derivatives may down-

https://doi.org/10.1021/acs.jnatprod.0c01252
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regulate Hsc70 expression of PEDV-infected host cells and then suppress the replication of PEDV mainly by destabilizing the mRNA of Hsc70 as compared to that of untreated cells.

Evaluation of Anti-PEDV Activity of Phenanthridine Derivatives in Vivo. Because the most active phenanthridine derivatives exhibited potent anti-PEDV activity with low cytotoxicity in vitro, it was predicted that these compounds may also be effective in vivo. Thus, pharmacodynamics experiments were conducted on virulent PEDV-infected piglets. Nine newborn piglets were randomly divided into four groups. The dose of administration was converted from the 25% LD_{50} value of compounds **1** and **4** determined in mice. All the piglets remained active, fleshy and with normal feces, prior to infection with the virus. Except for one piglet used as blank control, the other eight were all inoculated with virulent PEDV (NK-2). At 15 h after inoculation, rectal swabs of all piglets except the blank control tested positive for PEDV RNA. Next, three piglets were treated with compound **1** for 72 h at a dose of 50 mg/kg every 24 h, and another three were treated with compound **4** at the same dose, with two of the infected pigs left untreated as a negative control. After the virulent PEDV inoculation for 96 h, the negative control group developed severe watery feces, lethargy, and anorexia and died within the next 12 h. However, the two phenanthridine-treatment groups showed much milder clinical symptoms, and none of the piglets died during the entire experiment. After sacrificing all the piglets, small intestine samples from the negative control group revealed prominent changes characterized by thinner intestine mucosa with increasing transparency, mucosal swelling, and congestion. Microscopic examination of the jejunum, ileum, and colon of all the piglets was then conducted. The results showed that samples from the negative control group exhibited multifocal diffusion of villous atrophy, swelling, sloughing, and attenuation of villi and fusion of numerous villi. However, such pathological changes were reduced significantly in samples from piglets treated with compounds **1** and **4**. Further immunofluorescence assays indicated that, in comparison with that of the negative control, PEDV replication in the intestine of the phenanthridine-treated groups was suppressed considerably. These results show, in a preliminary manner, that compounds **1** and **4** may inhibit replication of PEDV in vivo and reduce PEDV-associated mortality.

EXPERIMENTAL SECTION

General Experimental Procedures. ESI and HRMS data were recorded using a Finnegan MAT 90 instrument and VG Auto Spec-3000 spectrometer, respectively. NMR experiments were conducted on a Bruker AM-400, DRX-500, or Avance III 600 spectrometer using residual $CDCl_3$ and DMSO-d_6 or TMS as internal standards. Column chromatography was performed on silica gel (60−80 mesh, 200−300 mesh, 300−400 mesh, Qingdao Haiyang Chemical Co. Ltd., Qingdao, China). Precoated silica gel 60 GF254 (Merck, Darmstadt, Germany) was used for TLC analyses. Semipreparative HPLC analysis was performed on a Hypersil Gold RP-C_{18} column (i.d. 10 mm × 250 mm, 5 μm, 5 mL/min) developed with CH_3CN−H_2O at room temperature. All regular solvents and reagents were reagent grade and purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA), Acros Organics (Geel, Belgium), or J&K Scientific (Beijing, People's Republic of China). Lycorine was purchased from Laideng Chemical Co. (Kunming, People's Republic of China). The purities of all compounds used in biological assays exceeded 95%, as determined by HPLC. HPLC was performed on an X-Bridge RP-C_{18} column (4.6 mm × 250 mm, 5 μm, and 5 mL/min) with CH_3OH−H_2O at room temperature. All reported yields are for dry compounds that required no further purification for use in other reactions. 6-Azauridine (TCI) was purchased from the discovery platform Inc. PEDV N protein monoclonal antibody was prepared and preserved by Yao's research group.[31] Methanol, isopropanol, agarose, DEPC water, nonfat-dried milk, bovine serum albumin (BSA), Tween-20, PBS (Hyclon) buffer, TAE (10×) buffer, 2× Taq enzyme, GoldView nucleic acid dye, ECL chemiluminescence solution, 0.25% trypsin-EDTA (1×) (Gibco), a NALGENETM Cruo 1 °C freezing container, β-actin monoclonal antibody, Goat Antimouse IgG-HPR (Beyotime), Goat Antirabbit IgG-HPR (Beyotime), Cy3-labeled Goat Anti-Rabbit lgG (H+L) SDS-PAGE sample loading buffer (2×) (Beyotime), Cell Counting Kit-8 (CKK-8) (Beyotime), penicillin−streptomycin solution (100×) (Beyotime), and DAPI staining solution (Beyotime) were purchased from Nanjing Dingsi Biotechnology Co., Ltd. (People's Republic of China). A fluorescence quantification kit (ChamQ Universal SYBR qPCR Master Mix) and RNA reverse transcription kit (HiScript II first Strand cDNA synthesis kit (+gDNA wiper)) were purchased from Nanjing Vazyme Biotechnology Co., Ltd. (People's Republic of China). Tris-MOPS-SDS running buffer powder, transfer buffer powder (GenScript), a Pierce BCA protein quantitative analysis kit (Takara), and Real Band 3-color regular range protein marker were purchased from Nanjing Lanlong Biotechnology Co., Ltd. (People's Republic of China).

Synthesis of Phenanthridine Derivatives. *4-Vinyl-N-methyl-5,6-dihydro[8,9]-dioxolo-phenanthridine (**1a**).*[21] A solution of lycorine (300 mg, 1 mmol) in DMF (10 mL) was poured into a round-bottomed flask, followed by addition of CH_3I (400 μL, 2 mmol). The mixture was stirred at room temperature for 12 h and then evaporated to remove DMF. The vessel was charged with *t*-BuOK (1.1 g, 10 mmol) and *t*-BuOH (10 mL), heated to 90 °C, and stirred for another 4 h. After cooling the mixture to room temperature, the reaction was quenched with 50 mL of saturated NH_4Cl and extracted twice with EtO_2 (20 mL). The organic phase was washed with saturated NH_4Cl and brine, dried over $MgSO_4$, filtered, and concentrated. The resulting residue was purified by column chromatography and eluted with petroleum ether−EtOAc (5:1) to yield compound **1a** (240 mg, 70% yield) as pale yellow crystals (from $CHCl_3$).

*4-Ethyl-N-methyl-5,6-dihydro-[8,9]dioxolo-phenanthridine (**1b**).*[21] A solution of **2** (27 mg, 0.1 mmol) and 10% Pd/C (30 mg) in CH_2Cl_2 (5 mL) was stirred under a H_2 atmosphere for 24 h. The organic layer was filtered and concentrated. The resulting residue was purified by column chromatography and eluted with petroleum ether−EtOAc (15:1) to afford compound **1b** (22 mg, 90% yield) as pale yellow crystals (from $CHCl_3$).

*4-Ethyl-N-methyl-5,6-dihydro-8,9-diphenol-phenanthridine (**1c**).*[21] Compound **1b** (0.2 mmol) was dissolved in 10 mL of CH_2Cl_2. The solution was cooled to −78 °C, and BBr_3 (200 μL, 0.4 mmol) was added. The mixture was stirred for 10 h and diluted in 50 mL of saturated $NaHCO_3$, followed by extraction with CH_2Cl_2 (2 × 20 mL). The organic layer was washed with brine and concentrated. The resulting residue was purified by column chromatography using $CHCl_3$−MeOH (**4**, 25:1; **5**, 20:1) as the eluent to afford **1c** (35.7 mg, 70% yield).

*Compounds **1** and **4**.* Compound **1c** (0.1 mmol) was dissolved in dry THF (10 mL), after which NaH (50 mg, 2 mmol) and the appropriate haloalkane (1 mmol) were added. The mixture was stirred at room temperature for 24 h and then quenched with water (50 mL) in an ice bath. The reaction solution was evaporated to remove THF and extracted twice with CH_2Cl_2 (30 mL). The organic layer was washed with saturated $NaHCO_3$ and then brine, dried over $MgSO_4$, filtered, and concentrated. The resulting residue was purified by column chromatography using petroleum ether−EtOAc as the eluent to afford compounds **1** and **4**.

*4-Ethyl-N-methyl-5,6-dihydro-8,9-diacetoxyphenanthridin (**2**).* Compound **1c** (0.1 mmol) was dissolved in a solution of pyridine (3 mL) containing the appropriate anhydride or acyl chloride (0.5 mmol) and DMAP (30 mg). The solution was stirred at room temperature for 20 h under N_2 and then poured into ice-cold water (50 mL) with vigorous stirring. The mixture was extracted twice with

https://doi.org/10.1021/acs.jnatprod.0c01252
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Table 1. Primer Sequences Involved in This Study

primer	sequence
NI-Hsc70-F-XHOI	CTACCGGACTCAGATCTCGAGATGTCCAAGGGACCTGCAGTT
NI-Hsc70-R-SACII	CGGTGGATCCCGGGCCCGCGGATCAACCTCTTCAATGGTGGGC
Hsc70-F	CATTACCCGTGCCCGATTT
Hsc70-R	ACTCCACCAGCAGTTTCA
β-actin-F	CGGGAAATCGTGCGTGAC
β-actin-R	ATGCCCAGGAAGGAAGGTTG
PEDV-N-F	CCACTAACAAGGGGAATA
PEDV-N-R	CAACTACAATGGGGAGC

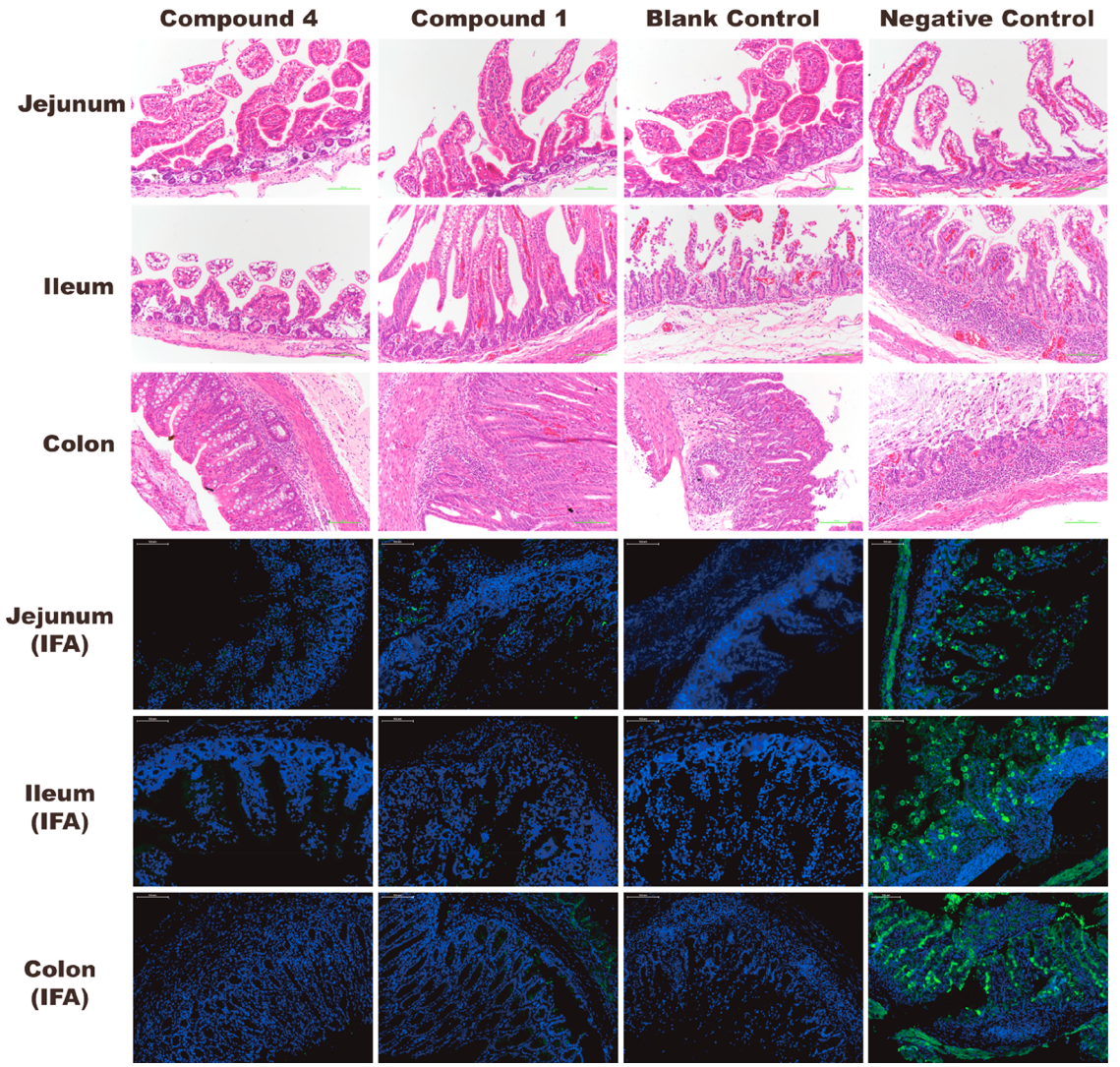

Figure 5. Detection of protective efficiency in piglets during virus exposure. HE (hematoxylin and eosin) staining showed that the impaired structural integrity, slight mononuclear cell infiltration, and vascular congestion of samples from compound-treated groups were much weaker than those of the negative control. In additional immunofluorescence assays of small intestine samples in all four groups, PEDV (green fluorescence) in the samples from compound-treated groups was suppressed significantly. (Amplification: 20× , scale bar represents 100 μM, images with 5× amplification can be found in the Supporting Information, Figure S4).

EtOAc (30 mL) and washed with saturated $NaHCO_3$ and brine, after which it was concentrated, and the crude product was purified by silica gel column chromatography to afford compound **2**.

*Compounds **1d** and **3**.* These two compounds were synthesized according to previous reports.[31]

*Compounds **2a**, **3a**, **4a**, **5a**, **6a**, and **7a**.* Bromobenzoic acid analogues (1 mmol) were dissolved in THF (10 mL), to which DMF (0.1 mL) and $SOCl_2$ (0.5 mL, 4 mmol) were added. In each case, the reaction solution was stirred for 2 h at 50 °C and then concentrated to remove THF. The residue was added to a 30% solution of the corresponding ammonia reagents in water (20 mL) at 5 °C and filtered. The resulting cake was purified by column chromatography to yield compounds **2a**, **3a**, **4a**, **5a**, **6a**, or **7a**.

*Compounds **2b**, **3b**, **5b**, **6b**, **6b′**, and **7**.* A flask was charged under N_2 with $Pd(OAc)_2$ (3.0 mg, 0.013 mmol), tri-2-furylphosphine (6.2 mg, 0.027 mmol), K_2CO_3 (72.3 mg, 0.52 mmol), amides **2a**, **3a**, **4a**, **5a**, **6a**, or **7a** (0.26 mmol), a solution of norbornene (26.9 mg, 0.286 mmol) in anhydrous solvent (5.8 mL), and 1-iodo-2-methylbenzene

https://doi.org/10.1021/acs.jnatprod.0c01252
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(0.26 mmol). The reaction mixture was heated under stirring to 85 °C for 6 h and then cooled to room temperature. After addition of saturated NH_4Cl (30 mL) and extraction with EtOAc (3 × 15 mL), the combined organic extracts were washed with brine (30 mL) and dried over Na_2SO_4. Removal of the solvent under reduced pressure gave the crude product, which was purified by flash chromatography on silica gel to furnish compounds **2b**, **3b**, **5b**, **6b**, **6b′**, or **7**.

*Compounds **5**, **6**, **8**, **9**, **10**, and **12**.* A solution of **2b**, **3b**, **5b**, **6b**, **6b′**, or **7** (0.1 mmol) in THF (5 mL) was added to BH_3−THF (1M, 1 mL) at −78 °C. The reaction was stirred for 2 h and then quenched using H_2O (5 mL). The mixture was extracted with Et_2O (20 mL) twice. The organic phase was washed with brine and concentrated, and the residue was purified by column chromatography to give **5**, **6**, **8**, **9**, **10**, or **12**.

*Compounds **6d** and **11**.* These two compounds were synthesized according to previous reports.[29]

Cells and Viruses. The Marc145 cell line was propagated in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclon) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Gaithersburg, MD). The PEDV strain 85-7 (Passage 5, P5) used herein was isolated in our laboratory and cultured at a multiplicity of infection (MOI) of 0.1 in the presence (10 ng/μL) or in the absence of trypsin. Briefly, a cultured confluent monolayer of MARC-145 cells were inoculated with stock virus 85-7 strain and allowed to adsorb for 1 h at 37 °C with 5% CO_2. The cells were washed three times with DMEM; then, maintenance medium (DMEM with 2% FBS) was added, and the culturing continued at 37 °C with 5% CO_2. The cells were harvested when a cytopathic effect (CPE) of 80% developed. The cell mixtures were subjected to one freeze−thaw cycle and centrifuged at 3000 × *g* for 10 min at 4 °C, and the supernatants were harvested for further propagation or saved at −80 °C.

Quantitative Real-Time PCR (qPCR). Total RNA of the PEDV-infected cells or recombinant plasmid transfected-cells was extracted using the RNAiso Plus reagent (Takara, Japan), according to the manufacturer's protocols. The concentrations of the extracted RNA were measured using a Thermo Scientific Nano Drop 2000c (Thermo Scientific, USA). Gene-specific primers for qPCR are listed in Table 1.[17,19,20] qPCR was performed with ChamQ Universal SYBR qPCR Master Mix using an ABI 7300 real-time PCR system. The β-actin gene served as the endogenous control.[23] Relative quantities of mRNA accumulation were evaluated based on the $2^{-\Delta\Delta Ct}$ method compared with mock-treated results.[22] Each sample was run in triplicate.

Immunofluorescence Assay (IFA). An IFA was performed as follows (Figure 5). The mock or PEDV-infected Marc145 cells were fixed with 4% methanol at 37 °C for 10 min and then permeabilized with 2% Triton X-100 at 37 °C for 5 min. The cells were blocked with 1% BSA at 37 °C for 1 h; then, incubation was carried out with mouse anti-PEDV monoclonal antibody in 1% BSA (1:200, overnight, 4 °C). Afterward, the cells were incubated with Cy3-labeled goat antimouse IgG (H+L) (1:500, 37 °C, 1 h, in the dark) and then stained with DAPI (37 °C, 10 min, in the dark). Finally, the stained cells were examined by fluorescence microscopy (Zeiss, Germany). All solutions were prepared in 1% BSA, and the cells were washed with PBS containing 0.5% Tween-20 (PBST) five times between each step.

Cytotoxicity Assay of Compounds. The Marc145 cell monolayer was digested with trypsin-EDTA, and cell numbers were obtained with a cytometer. Then, 100 μL of a culture with 2 × 10^5 cells/mL was plated into 96-well plates. Fresh culture medium containing each test compound at various concentrations was added 6 h later. Cytotoxicity was evaluated using a CKK-8 assay, according to the manufacturer's instructions.

Overexpression of Hsc70 Assays. The external Hsc70 was amplified from the Hsc70 plasmid by PCR and then cloned into the pEGFP-N1 expression vector to generate pEGFP-N1/Hsc70 plasmids. The PCR primers are listed in Table 1. The recombinant plasmids were transfected into Marc145 cells using Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's instructions. Then, 24 h later, the transfected cells were divided into dishes for either drug treatment or an untreated control. Next, 2 h later, cells were infected by PEDV. Total RNA was extracted after 24 h for measurement using a qPCR assay.

Hsc70 Messenger RNA Half-Life Assay. Marc145 cells were either left untreated or treated with 50 μg/mL compounds for 24 h. Then, 10 μg/mL actinomycin D (A.G. Scientific, San Diego, CA) was added to block transcription. Total cellular RNAs were harvested at different times after actinomycin D treatment to analyze Hsc70 mRNA (mRNA) levels with qPCR Hsc70 mRNA levels which were normalized to β-actin and plotted against time, and the decay rate or half-life of Hsc70 mRNA was calculated.

Western Blot Assay. The extracted total protein or viral lysates were denatured by adding SDS-PAGE sample loading buffer (2×) and then boiled for 5 min at 100 °C. Proteins were analyzed by SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) using a GE TE77 ECL Transphor semidry transfer unit. The membranes were blocked in 5% nonfat dry skimmed milk in PBST solution for 1 h and then washed three times for 10 min with PBST solution. Membrane samples were probed with a monoclonal antibody specific for the PEDV protein (diluted to 1 μg/mL) or Hsc70 (diluted to 0.2 μg/mL; Abcam, Ltd.). As a control, probing experiments were also performed using a polyclonal antibody against actin (diluted to 0.2 μg/mL). After they were washed with PBST, the membranes were incubated for 1 h with an appropriate secondary antibody. The protein signal was visualized and captured using the Immbilon Western chemiluminescent HRP substrate ECL working solution (Millipore Inc.) and a ChemiDoc, Touch imaging system (Bio-Rad, CA).

Anti-PEDV Activities of Phenanthridine Compounds In Vivo. A total of nine colostrum-deprived 1-day-old piglets (Taihu and Changbai binary hybridization) were chosen and serologically confirmed to be negative for PEDV and TGEV antibodies via indirect ELISA. All piglets were tended under strict veterinary supervision and then randomly assigned to four groups (control) and were artificially fed with milk and housed in separate rooms. Three groups (negative control, compound **1** treated, and compound **4** treated) were challenged orally with 5 mL of 10^3 MID/mL of PEDV virulent virus (intestinal tissue from virulent PEDV-infected piglets). Compounds **1** or **4** (50 mg/kg) and the same volume of saline were given orally to the compound-**1**-treated group, the compound-**4**-treated group, and the negative control group, respectively. Following the challenge, infected piglets were examined for clinical symptoms once per day. After 115 h of challenge, both piglets in the negative control group and one from the compound-**1**-treated group died within 5 h. All the surviving piglets were then euthanized by pentobarbital sodium injection for pathological examination. At necropsy, the intestine and other major organs were examined. Jejunum, ileum, and colon samples were placed in 4% paraformaldehyde, dehydrated in graded alcohol, embedded in paraffin, cut into 5 μm sections, and mounted on microscope slides. All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, People's Republic of China) following the guidelines of the National Institutes of Health. The approved protocol number for this investigation was NAU201846, and the approval date is 1 December 2018.

Statistical Analysis. Results were expressed as means ± SD and analyzed with GraphPad Prism 7. One-way analysis of variance was employed to determine significant differences among multiple groups, and a *t* test was employed to identify differences between the two groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Data were combined from at least three independent experiments unless otherwise stated.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c01252.

NMR data, NMR spectra, images of clinical signs of in vivo assays, and IFA images of tissue samples (PDF)

https://doi.org/10.1021/acs.jnatprod.0c01252
J. Nat. Prod. 2021, 84, 1175−1184

AUTHOR INFORMATION

Corresponding Authors

Xiao-Jiang Hao − *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, People's Republic of China*; orcid.org/0000-0001-9496-2152; Email: haoxj@mail.kib.ac.cn

Guang-Jin Liu − *College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, People's Republic of China*; Email: liugj100@njau.edu.cn

Authors

Duo-Zhi Chen − *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, People's Republic of China*

Shi-Rui Fan − *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, People's Republic of China; University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China*

Bi-Juan Yang − *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, People's Republic of China; University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China*

Huo-Chun Yao − *College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, People's Republic of China*

Yi-Ting Wang − *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, People's Republic of China; University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China*

Jie-Yun Cai − *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, People's Republic of China*

Chen-Xu Jing − *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, People's Republic of China*

Zi-Hao Pan − *College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, People's Republic of China*

Miao Luo − *College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, People's Republic of China*

Yan-Qiu Yuze − *College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, People's Republic of China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jnatprod.0c01252

Author Contributions

[‖]D.-Z.C. and S.-R.F. contributed equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was funded by the National Natural Science Foundation of China (81973212, 81773610), The Youth Innovation Promotion Association of CAS (2018429), The Central Asian Drug Discovery and Development Center of Chinese Academy of Sciences (CAM201801), the Fundamental Research Funds for the Central Universities (KJQN201618), and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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https://doi.org/10.1021/acs.jnatprod.0c01252
J. Nat. Prod. 2021, 84, 1175−1184