Arch Virol (2015) 160:1163–1170
DOI 10.1007/s00705-015-2370-x

ORIGINAL ARTICLE

Differential effects of viroporin inhibitors against feline infectious peritonitis virus serotypes I and II

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Received: 28 October 2014 / Accepted: 12 February 2015 / Published online: 21 February 2015
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Abstract Feline infectious peritonitis virus (FIP virus: FIPV), a feline coronavirus of the family *Coronaviridae*, causes a fatal disease called FIP in wild and domestic cat species. The genome of coronaviruses encodes a hydrophobic transmembrane protein, the envelope (E) protein. The E protein possesses ion channel activity. Viral proteins with ion channel activity are collectively termed "viroporins". Hexamethylene amiloride (HMA), a viroporin inhibitor, can inhibit the ion channel activity of the E protein and replication of several coronaviruses. However, it is not clear whether HMA and other viroporin inhibitors affect replication of FIPV. We examined the effect of HMA and other viroporin inhibitors (DIDS [4,4′-disothiocyano-2,2′-stilbenedisulphonic acid] and amantadine) on infection by FIPV serotypes I and II. HMA treatment drastically decreased the titers of FIPV serotype I strains Black and KU-2 in a dose-dependent manner, but it only slightly decreased the titer of FIPV serotype II strain 79-1146. In contrast, DIDS treatment decreased the titer of FIPV serotype II strain 79-1146 in dose-dependent manner, but it only slightly decreased the titers of FIPV serotype I strains Black and KU-2. We investigated whether there is a difference in ion channel activity of the E protein between viral serotypes using *E. coli* cells expressing the E protein of FIPV serotypes I and II. No difference was observed, suggesting that a viroporin other than the E protein influences the differences in the actions of HMA and DIDS on FIPV serotypes I and II.

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Introduction

Feline infectious peritonitis virus (FIP virus: FIPV), a feline coronavirus (FCoV) of the genus *Alphacoronavirus* in the subfamily *Coronavirinae*, causes a fatal disease called FIP in wild and domestic cat species. Cats with FIP develop lesions accompanied by necrosis and pyogenic granulomatous inflammation in several organs, including the liver, lungs, spleen, and central nervous system [14]. Pleural and peritoneal effusion also accumulates in some cats.

The FCoV virion is mainly composed of the nucleocapsid (N), envelope (E), membrane (M), and peplomer spike (S) proteins [13]. FCoV has been classified into serotypes I and II based on the amino acid sequence of its S protein [7, 10]. Both serotypes consist of two biotypes: FIPV and feline enteric coronavirus (FECV) [22]. FECV infection is asymptomatic in cats; however, FIPV infection causes FIP.

Some proteins encoded by the coronavirus genome show ion channel activity. For example, the 3a and E proteins of severe acute respiratory syndrome coronavirus (SARS-CoV) and the 4a protein of human coronavirus (HCoV)-229E have been reported to show ion channel activity [15, 25, 26]. Viral proteins with ion channel activity have also been reported in other viruses and have collectively been termed "viroporins" [12]. The involvement of viroporins in replication and budding has been confirmed in influenza virus, human immunodeficiency virus (HIV), and hepatitis C virus (HCV). The replication of these viruses was shown to be markedly inhibited by viroporin inhibitors. Amantadine, 4,4′-disothiocyano-2,2′-stilbenedisulphonic acid (DIDS), and hexamethylene amiloride (HMA) have been identified as viroporin inhibitors. Wilson *et al.* [25] reported that HMA, an inhibitor

of the E protein ion channel, inhibits viral replication of mouse hepatitis virus (MHV) and HCoV-229E, but the antiviral effect of HMA on FIPV has not been clarified.

In the present study, we examined the effect of HMA and other viroporin inhibitors (DIDS and amantadine) on infection by FIPV serotypes I and II. The inhibitory effects of HMA and DIDS on viral replication varied between the serotypes. Thus, we investigated whether there is a difference in ion channel activity between the E proteins of FIPV serotypes I and II, employing a simple assay system using *Escherichia coli* (*E. coli*) expressing GST fusion protein [17].

Materials and methods

Cell cultures and viruses

Fcwf-4 cells (macrophage-like cells) were grown in Eagle's minimum essential medium containing 50 % L-15 medium, 5 % fetal calf serum (FCS), and antibiotics. The serotype I FIPV KU-2 strain was isolated in our laboratory, and the serotype I FIPV Black strain was supplied by Dr J. K. Yamamoto of the University of Florida. Seroype II FIPV 79-1146 was supplied by Dr. M. C. Horzinek of the State University of Utrecht. These viruses were grown in Fcwf-4 cells at 37 °C.

Compounds

HMA, amantadine, and DIDS were obtained from Sigma Aldrich (USA). These compounds were stored in dimethyl sulfoxide/methanol (1:1), sodium bicarbonate, and ethanol, respectively. On the day of the experiments, these compounds were diluted to the desired concentrations in maintenance medium.

Cytotoxic effect of viroporin inhibitors

Cell viability was measured using the WST-8 assay as described previously [24]. Briefly, Fcwf-4 cells were seeded into 96-well plates. Viroporin inhibitors were added to triplicate wells. After 48 hours, the culture supernatants were removed, WST-8 solution (WST-8 cell proliferation assay kit; Kishida Chemical Co., Ltd., Japan) was added, and the cells were returned to the incubator for 1 hour. The absorbance of formazan produced was measured at 450 nm, using a 96-well spectrophotometric plate reader as described by the manufacturer. The percentage viability was calculated using the following formula: Cytotoxicity (%) = [OD of viroporin inhibitor (or solvent)-untreated cells − viroporin inhibitor (or solvent)-treated cells/ OD of viroporin inhibitor (or solvent)-untreated cells)] × 100. The CC_{50} was defined as the cytotoxic concentration of each viroporin inhibitor that reduced the absorbance of treated cells to 50 % when compared with that of the cell control.

Virus replication in Fcwf-4 cells cultured with viroporin inhibitors

Virus at multiplicity of infection (MOI) of 0.01 was added to the culture and adsorbed by Fcwf-4 cells at 37 °C for 1 hour. After washing, cells were cultured in medium containing viroporin inhibitors. The culture supernatant was collected after 48 h, and the virus titer was measured. The EC_{50} was defined as the effective concentration of viroporin inhibitor that reduced the virus titer of culture supernatant of infected cells to 50 % when compared to that of the virus control. The results were also expressed by using the selectivity index ($SI = CC_{50}/EC_{50}$).

Effect of viroporin inhibitors at low and high multiplicity of infection

Virus at MOIs from 0.001 to 0.1 was added to the culture and adsorbed by Fcwf-4 cells at 37 °C for 1 hour. After washing, cells were cultured in maintenance medium containing viroporin inhibitors with 1.5 % carboxymethyl cellulose (CMC-MEM) or without carboxymethyl cellulose (MEM). In the case of the cells cultured in CMC-MEM, the cell monolayers were incubated at 37 °C for 48 hours and fixed and stained with 1 % crystal violet solution containing 10 % buffered formalin, and the resulting plaques were counted. In the case of the cells cultured in MEM, the culture supernatants were collected at 48 hours postinfection, and the virus titer was determined by $TCID_{50}$ assay.

FIPV E protein expression in *E. coli* cells

The regions encoding the E protein and the S1 domain of the S protein of FIPV serotype I strain KU-2 and the E protein of the FIPV serotype II strain 79-1146 were amplified by RT-PCR using the method described by Takano *et al.* [24]. The primers used to amplify each region are shown in Table 1. The PCR products were inserted into pENTR/D-TOPO (Invitrogen, USA), and then into pDEST15, using recombination. This construct was then used to transfect *E. coli* strain BL21-AI (Invitrogen, USA). Bacterial cultures were grown for 2-3 h at 37 °C to an OD_{600} of 0.4, and the expression of proteins was induced by the addition of 0.2 % (w/v) L-arabinose (Sigma Aldrich, USA). We normalized GST+E protein and GST+S1 protein expression.

Table 1 Sequences of primers used in this study

	Orientation	Nucleotide sequence	Length (bp)
FIPV KU-2 E	Forward	5′-CACCATGATGTTTCCTAGGGCA-3′	246
	Reverse	5′-TCAAACCAAGAGTGCTTCGTT-3′	
FIPV 79-1146 E	Forward	5′-CACCATGACGTTCCCTAGGGCATTTAC-3′	246
	Reverse	5′-TCAAACCAAAAATGCTTCGTCGGGA-3′	
FIPV KU-2 S1	Forward	5′- CACCGTCACTGATTTCAGCCTGC-3′	932
	Reverse	5′- TTATTCATCTTTACCAAAACAGAGC-3′	

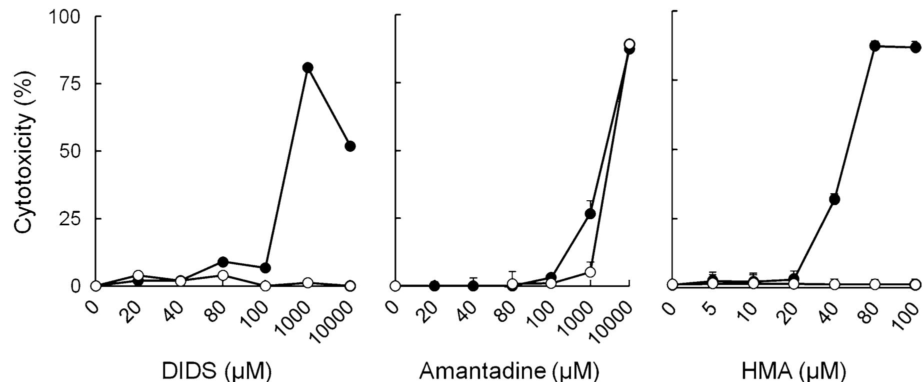

Fig. 1 Cytotoxic effects of viroporin inhibitors in Fcwf-4 cells. Cytotoxicity was evaluated using the WST-8 assay. The black circles indicate treatment with viroporin inhibitors, and the white circles indicate treatment with the solvent as a control. Data represent three independent experiments

β-Galactosidase assay

The *β*-galactosidase assay was performed using a modification of the methods described by Guinea and Carrasco [5] and Liao *et al.* [12]. Briefly, at various times after induction, *E. coli* pellets were resuspended in 1 ml of M9 medium containing 2 mM *o*-nitrophenyl-*β* galactopyranoside (ONPG) (Sigma Aldrich, USA). After incubation at 30 °C for 100 minutes, 1 M sodium carbonate was added to stop the reaction. This reaction solution was centrifuged, the supernatant was collected, and absorbance (OD) at 405 nm was measured. Values for *β*-galactosidase activity were normalized to total *E. coli* cellular protein.

Statistical analysis

Data from two groups were analyzed by Student's *t*-test, and multiple groups were analyzed by a one-way ANOVA.

Results

Cytotoxic and antiviral effects of HMA and other viroporin inhibitors in Fcwf-4 cells

To investigate the cytotoxicity of HMA and other viroporin inhibitors, the viability of Fcwf-4 cells was measured 48 hours after the addition of each reagent (Fig. 1). HMA, DIDS, and amantadine exhibited strong cytotoxicity at 80, 1,000, and 10,000 μM, respectively. The CC_{50} values of the viroporin inhibitors are shown in Table 2. The influence of HMA and other viroporin inhibitors on virus release from FIPV-infected cells was investigated (Fig. 2). HMA treatment drastically decreased the titers of FIPV serotype I strains Black and KU-2 in a dose-dependent manner, but it decreased the titer of FIPV serotype II strain 79-1146 only slightly. In contrast, DIDS treatment decreased the titer of FIPV serotype II strain 79-1146 in a dose-dependent manner but did not decrease the titers of FIPV strains serotype I Black and KU-2. Amantadine treatment did not change the titer of any viral strain in the culture supernatant. The EC_{50} and SI values of the viroporin inhibitors are shown in Table 2.

To further evaluate the mechanism of the inhibitory effects of the viroporin inhibitors on FIPV growth, we analyzed the growth kinetics of FIPV growth in Fcwf-4 cells that were infected at low and high MOI. After washing, we treated the cells with viroporin inhibitors and compared the plaque counts in the cells and the virus titer in the culture supernatants. HMA and DIDS did not inhibit plaque formation by any of the serotypes at low or high MOI (Fig. 3A). However, treatment with10 μM HMA significantly decreased the virus titer in the culture supernatant of cells infected with FIPV serotype I at an MOI 0.01 or 0.001. Treatment with 10 μM HMA or 100 μM DIDS also decreased the virus titer in the culture supernatant of cells infected with FIPV serotype II at an MOI of 0.01. In addition, a reduction in plaque size was also

Table 2 Inhibitory effects of viroporin inhibitors against FIPV

Compound	CC_{50} (μM)	FIPV type I strain KU2		FIPV type I strain Black		FIPV type II strain 79-1146	
		EC_{50} (μM)	SI	EC_{50} (μM)	SI	EC_{50} (μM)	SI
4,4′-disothiocyano-2,2′-stilbenedisulphonic acid (DIDS)	318.7 ± 22.0	185.0 ± 17.1	2.9 ± 0.3	207.8 ± 10.0	2.2 ± 0.1	69.6 ± 6.9	6.1 ± 1.0
Amantadine	2230.9 ± 178.0	>1000	<2	>1000	<2	>1000	<2
Hexamethylene amiloride (HMA)	48.8 ± 1.9	1.9 ± 0.8	25.5 ± 2.2	2.1 ± 0.2	23.3 ± 2.2	10.3 ± 0.1	4.9 ± 0.1

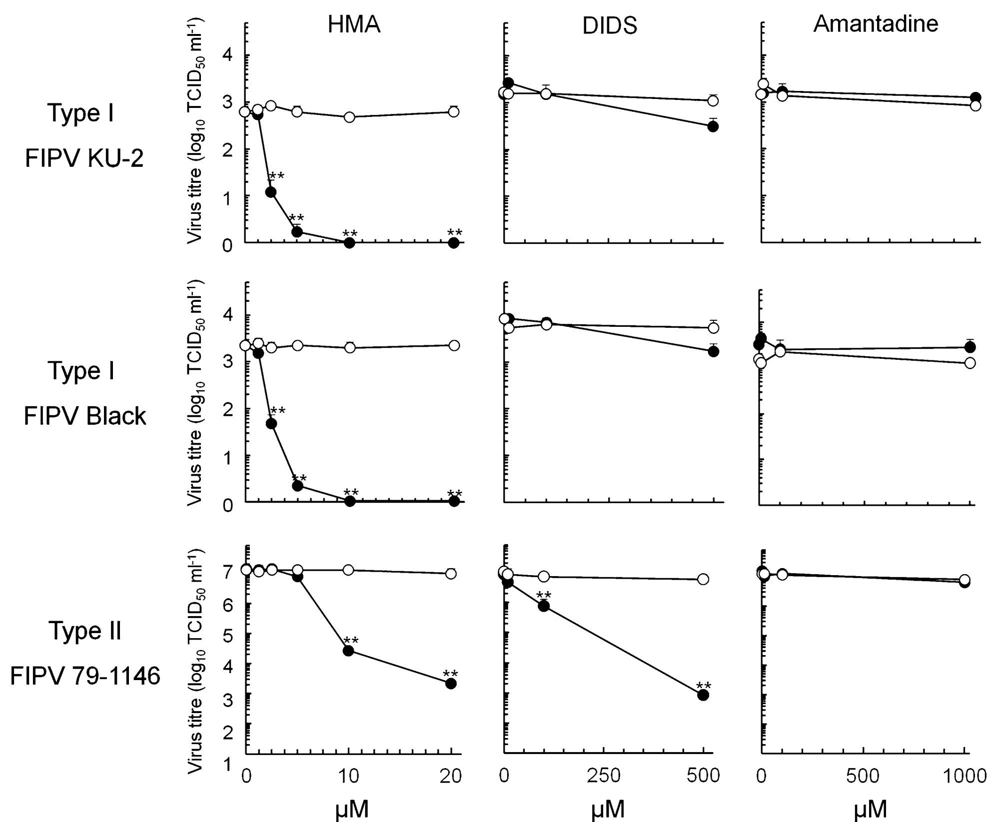

Fig. 2 Virus titers in the culture supernatant of Fcwf-4 cells infected with FCoV and treated with viroporin inhibitors. Fcwf-4 cells were inoculated with FCoV. After adsorption at 37 °C, cells were washed and cultured in medium containing the viroporin inhibitor or solvent as a control. Culture supernatants were collected after 1 day, and the viral titer was measured. The black circles indicate treatment with viroporin inhibitors, and the white circles indicate treatment with solvent as a control. **, $p < 0.01$ vs. solvent control. Data represent three independent experiments

observed in HMA-treated cells infected with FIPV serotype I at an MOI of 0.01 (Fig. 3B) or 0.001, whereas no changes were observed in cells treated with DIDS (data not shown).

Influence of FIPV E protein expression on growth and membrane permeability of *E. coli* cells

The gene regions encoding the E protein of FIPV serotype I strain KU-2 and FIPV serotype II strain 79-1146 were inserted into the pDEST15 vector, and *E. coli* cells were transformed with these vectors (pDEST15 + KU2E and pDEST15 + 79-1146E, respectively). As a negative control, the gene region encoding the S protein (S1 region) of the FIPV serotype I strain KU-2 was inserted into the pDEST15 vector and subsequently introduced into *E. coli* cells (pDEST15 + KU2S1). The expression of a protein with the target estimated molecular weight was confirmed 4 hours after protein induction in all transformed *E. coli* cells (Fig. 4A). The predicted sizes of GST + KU2E, GST-1146E, GST + KU2S1, and GST alone were 36, 36, 62, and 28 kDa, respectively. The influence of E protein expression on the growth of *E. coli* cells was investigated. The growth of cells was significantly lower after 2-4 hours in cells with the induction of the GST + E protein than in cells without induction (Fig. 4B, pDEST15 + KU2E and pDEST15 + 79-1146E). In contrast, no significant differences in growth were observed between cells with the induction of only GST + S1 and GST proteins and cells without induction (Fig. 4B, pDEST15 + KU2S1 and pDEST15). The relationship between inhibition of *E. coli*

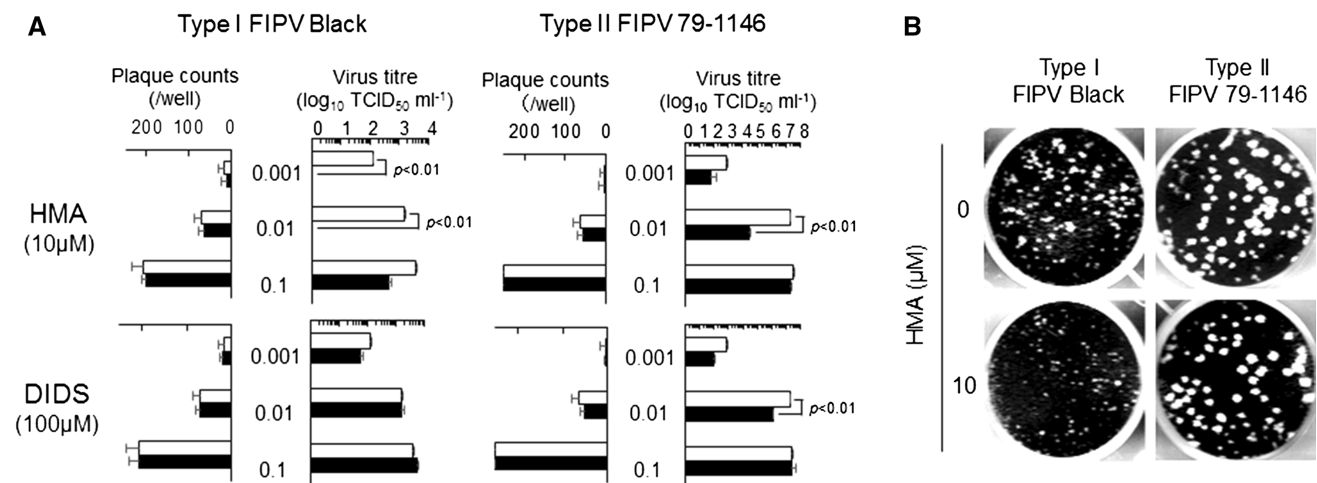

Fig. 3 Effect of viroporin inhibitors on FCoV infection at low and high MOI. (A) Virus at MOI from 0.001 to 0.1 was added to the culture and adsorbed by Fcwf-4 cells. After inoculation, cells were cultured in maintenance medium containing viroporin inhibitors (10 µM HMA and 100 µM DIDS) with 1.5 % carboxymethyl cellulose (CMC-MEM) or without carboxymethyl cellulose (MEM). In the case of cells cultured in CMC-MEM, the cell monolayers were incubated at 37 °C for 48 hours and fixed and stained, and the resulting plaques were counted. In the case of cells cultured in MEM, the culture supernatants were collected at 48 hours postinfection, and the virus titer was determined by $TCID_{50}$ assay. The black bars indicate treatment with viroporin inhibitors, and the white bars indicate treatment with the solvent as a control. Data from two groups were analyzed by Student's *t*-test, and multiple groups were analyzed by one-way ANOVA. (B) Effect of HMA on the plaque morphology of FCoV

cell growth and E-protein-expression-induced changes in membrane permeability was investigated by assessing the incorporation of a substrate of β-galactosidase, ONPG, by *E. coli* cells. The uptake of ONPG in cells was significantly higher after 12-24 hours in cells with the induction of the GST + E protein than in cells without induction (Fig. 4C, pDEST15 + KU2S1 and pDEST15). In contrast, no significant changes were observed in the uptake of ONPG between cells with the induction of the GST + S1 protein and cells without the induction (Fig. 4C, pDEST15+− KU2S1). The uptake of ONPG was significantly lower after 24 hours in cells in which only the GST protein was induced than in cells without induction (Fig. 4C, pDEST15).

Discussion

Viroporins are hydrophobic, low-molecular-weight viral transmembrane proteins [20]. The presence of viroporins has recently been reported in many viruses [1, 3, 8, 9], and their functions have been clarified. A viroporin forms an oligomer and makes a hydrophilic pore in host-infected cells and endosome membranes. These pores have been shown to transport substances inside and outside the cell membrane and are involved in the homeostasis of intracellular ions, changes in cell membrane polarity, localization of intracellular substances, and budding and release of virus particles. Liao *et al.* [13] and Madan *et al.* [16] reported that the E proteins of SARS-CoV and MHV, belonging to the genus *Betacoronavirus*, function as viroporins. The function of the E protein as a viroporin has been also clarified in a member of the genus *Alphacoronavirus* (HCoV-229E) [25]. It was recently reported that the 3a protein of SARS-CoV and the 4a protein of HCoV-229E function as viroporins [15, 25, 26].

We investigated the inhibitory effects of viroporin inhibitors on replication of FIPV serotypes I and II using Fcwf-4 cells. The inhibitory effect of viral replication was investigated by measuring the virus titer in the culture supernatant. Amantadine showed no inhibitory effect on viral replication, whereas HMA drastically inhibited FIPV serotype I replication in a dose-dependent manner but only slightly inhibited FIPV serotype II. HMA drastically decreased the titers of FIPV serotype I strains Black and KU-2 in a dose-dependent manner but decreased the titer of FIPV serotype II strain 79-1146 only slightly. In contrast, DIDS decreased the titer of FIPV serotype II strain 79-1146 in a dose-dependent manner but did not decrease the titers of FIPV serotype I strains Black and KU-2. This may be due to a difference in ion channel activity of viroporin between serotypes I and II FIPV. HMA is a sodium channel blocker [11], whereas DIDS is a chloride channel blocker [19], suggesting that the function of the sodium channel blocker is dominant in the viroporin of serotype I FIPV, whereas the function of the chloride channel blocker is dominant in serotype II FIPV.

To further evaluate the mechanism of the inhibitory effects of the viroporin inhibitors on FIPV growth, we

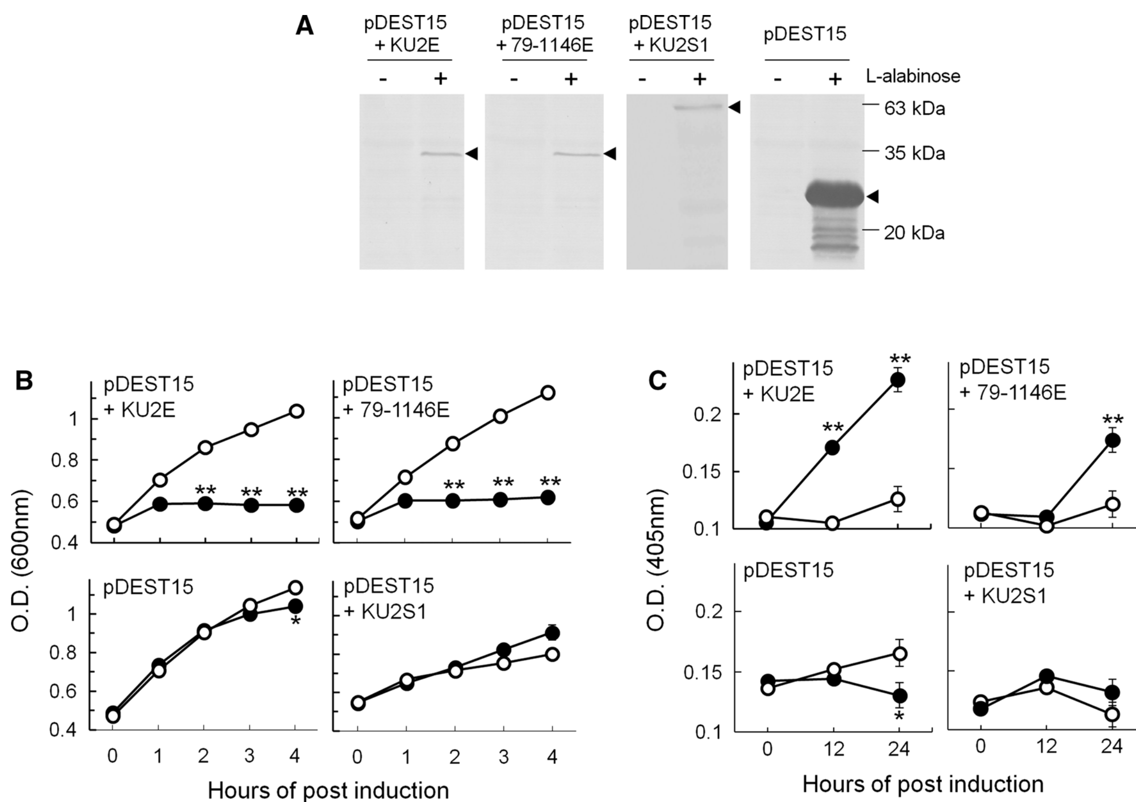

Fig. 4 Influence of FIPV E protein expression on the growth and membrane permeability of *E. coli* cells. (A) Expression of the FIPV E protein and the S1 domain of the S protein. *E. coli* cells transformed with pDEST15 + KU2E, pDEST15 + 79-1146E, pDEST15 + - KU2S1, and pDEST15 were treated with L-arabinose. The expression of protein was determined by Western blot analysis. An anti-GST monoclonal antibody was used to detect protein expression. Arrowheads indicate the protein products. (B) Influence of FIPV E protein expression on *E. coli* cell growth. *E. coli* cells were induced (black circle) or not induced (white circle) with L-arabinose. Cell densities were measured at 600 nm at the indicated times post-induction. (C) Influence of FIPV E protein expression on the membrane permeability of *E. coli* cells. *E. coli* cells were induced (black circle) or not induced (white circle) with L-arabinose. The cells were collected at the indicated times post-induction. Cells were resuspended with ONPG solution and incubated at 30 °C for 2 h. After incubation, β-galactosidase activity was measured using the culture supernatant. *β*-galactosidase activity was measured at 405 nm. ** $p < 0.01$ vs. no treatment with L-arabinose (control). *, $p < 0.05$ vs. no treatment with L-arabinose (control). Data represent three independent experiments

analyzed the growth kinetics of the virus at low and high MOI. HMA and DIDS did not inhibit plaque formation of either serotype at low or high MOI. However, treatment of cells infected with FIPV serotype I at an MOI of 0.01 or 0.001 with 10 μM HMA significantly reduced the size of plaques and decreased the virus titer in the culture supernatant. Treatment of cells infected with FIDV serotype II at an MOI of 0.01 with 10 μM HMA and 100 μM DIDS also decreased the virus titer in culture supernatant but did not reduce the size of plaques. These results suggested that HMA decreases the extracellular level of serotype I FIPV and possibly serotype II FIPV titers.

Various anti-FIPV agents have been identified *in vitro*. As far as we know, these experiments have been performed using serotype II FIPV. However, 70-98 % of FCoV-infected cats in the field have been shown to be infected with serotype I FCoV [6, 10, 14, 23]. Based on this finding, an agent exhibiting an antiviral effect on both serotypes of FIPV is necessary. In this study, HMA was confirmed to exhibit an antiviral effect on FIPV serotype I. In the future, it will be necessary to administer HMA to cats infected with FIPV serotype I and developing FIP to investigate whether HMA alleviates symptoms.

We inserted the E-protein-coding gene regions of FIPV serotype I KU-2 and FIPV serotype II strains 79-1146 into plasmids, which were subsequently introduced into *E. coli* cells by transfection. Using these transfectants, the function of the FIPV E protein as a viroporin was investigated with reference to a study by Liao *et al.* [12]. Since the E protein is hydrophobic, it was assumed that the protein aggregates in the cytoplasm of *E. coli* cells and is not expressed in the cell membrane. To avoid E protein aggregation, a GST fusion protein was prepared. Griffin et al. [13] and Melton et al. [17] reported that the expression of GST-tagged viroporin did not influence the ion channel activity of viroporin in *E. coli* and mammalian cells. Cell growth was

significantly inhibited in *E. coli* cells transformed with pDEST15 + KU2E and pDEST15 + 79-1146E from 2 hours after induction, which suggests that the FIPV E protein expressed on the *E. coli* cell surface functioned as a viroporin. The movement of substances in the bacterial cell membrane may have been altered, and intracellular substances essential for the survival of *E. coli* cells may have exited the cells. A significant elevation in β-galactosidase activity levels was noted 24 and 48 hours after the induction in *E. coli* cells transformed with pDEST15 + KU2E and pDEST15 + 79-1146E, respectively. The FIPV E protein was assumed to be expressed on the *E. coli* surface and to have increased the cell surface permeability, which promoted the incorporation of ONPG into the cells and the reaction of intracellular β-galactosidase with ONPG. Intracellular β-galactosidase may also have exited the cells and reacted with extracellular ONPG. These phenomena have also been reported in *E. coli* cells expressing the SARS-CoV E protein and influenza virus M2 protein [5, 12]. These findings strongly suggest that the FIPV E protein functions as a viroporin.

It was investigated whether there is a difference in the ion channel activity of the E protein between the serotypes using *E. coli* cells expressing the E proteins of FIPV serotypes I and II, but no difference was observed, suggesting that FIPV possesses a viroporin other than the E protein – for example, proteins similar to the 3a protein of SARS-CoV and the 4a protein of HCoV-229E [15, 26]. The ORF3abc gene of FIPV encodes three non-structural proteins, including a transmembrane protein that could potentially be a viroporin. FIPV ORF3abc has been reported to be an important factor for replication in cells [2]. It is necessary to investigate a gene encoding a viroporin other than the E protein in the FIPV genome.

In the present study, the ability of HMA and other viroporin inhibitors (DIDS and amantadine) to inhibit replication of FIPV serotypes I and II was examined. The inhibitory effects of HMA and DIDS on viral replication differed between the serotypes. As far as we know, the influence of differences in the viral serotype on the action of viroporin inhibitors has not previously been reported. It is necessary to express viroporin of FIPV serotypes I and II in mammalian cells or oocytes and elucidate the function of the ion channel activity in detail. Our results strongly suggest that the viroporin inhibitor HMA is applicable as an antiviral agent against FIPV serotype I. The antiviral actions of combinations of HMA and other possible therapeutic agents on FIP need to be examined.

Acknowledgments This work was in part supported by KAKENHI (Grants-in-Aid for Scientific Research (B), No. 25292183) from the Ministry of Education, Culture, Sports, Science and Technology.

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