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Inhibition of betanodavirus infection by inhibitors of endosomal acidification

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Summary

Betanodaviruses, members of the family *Nodaviridae*, have small positive-stranded bipartite RNA genomes and are the causal agent of viral nervous necrosis (VNN) in many species of marine farmed fish. In the aquaculture industry, outbreaks of betanodavirus infection and spread in larval and juvenile fish result in devastating damage and heavy economic loss. Although an urgent need exists to develop drugs that inhibit betanodavirus infection, there have been no reports about anti-betanodavirus drugs. Recently, it was reported that betanodaviruses were detected in the endosomes of infected cells, suggesting that betanodaviruses enter fish cells by endocytosis. This finding prompted us to examine whether blocking this endosomal pathway could provide a target for antiviral drug development. In this study, we examined the inhibitory effect of several lysosomotropic agents against betanodavirus infection in fish E-11 cells. The presence of 1 mM NH_4Cl or 1 μM chloroquine in the medium inhibited the entry of betanodaviruses into cells and inhibited viral infection. The lysosomotropic agents bafilomycin A1 and monensin also inhibited virus-induced cytopathology and virus production. Our data demonstrate that inhibitors of endosomal acidification are candidates as antiviral agents against betanodavirus.

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Introduction

The family *Nodaviridae* is comprised of the genera *Alphanodavirus* and *Betanodavirus*, which predominantly infect insects and fish, respectively. Viruses belonging to the genus *Betanodavirus* are the causative agents of viral encephalopathy and retinopathy, also known as viral nervous necrosis (VNN). Nodaviruses are small (25–30 nm diameter), spherical, non-enveloped viruses with a genome that is composed of two single-stranded, positive-sense RNA molecules [17]. The larger genomic segment, RNA1 (3.1 Kb), encodes the RNA-dependent RNA polymerase (RdRp) [10, 19, 31], and the smaller genomic segment, RNA2 (1.4 Kb), encodes the coat protein [20]. It has recently been determined that a subgenomic RNA transcribed from the 3′ end of

RNA1, termed RNA3, encodes a protein, B2. This protein is highly conserved among the betanodaviruses and is an RNAi antagonist [7, 8, 13].

VNN devastates many species of marine fish culture worldwide [12]. Betanodaviruses have been isolated from more than 30 marine fish species from 14 families and are classified into four genotypes based on a phylogenetic analysis of the coat protein sequences. The genotypes are: striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV) and redspotted grouper nervous necrosis virus (RGNNV) [18, 20, 21]. Effective control of betanodavirus infection is urgently needed to reduce the significant economic loss caused by the virus in the fish industry. It is widely accepted that a vaccine capable of preventing VNN would be a major improvement. Partial immunity has been obtained using recombinant betanodavirus coat protein expressed in *E. coli* [11, 32]. Immunization with virus-like particles (VLPs) of betanodavirus has been reported to induce a protective immune response against VNN [33]. However, in most cases, VNN occurs in larval and juvenile fish that cannot be easily vaccinated due to their small size. Therefore, the development of drugs that inhibit betanodavirus infection is of critical importance. The mechanism of betanodavirus infection is still unclear. An electron-microscopic study of betanodavirus-infected cells suggested that the entry of the betanodavirus into a fish cell line depends on the endocytic pathway [16].

The use of inhibitors of endosomal acidification such as NH_4Cl and chloroquine has been applied against many viruses [14, 26]. In this study, we examined the inhibitory effect of lysosomotropic agents (NH_4Cl, chloroquine, bafilomycin A1, and monensin) on betanodavirus infection. Our results demonstrate that these agents inhibit the entry of betanodavirus into cells.

Materials and methods

Cells, viruses and chemicals

E-11 cells were maintained in Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) at 25 °C. The fish nodavirus used in this study was isolated in 2001 from a seven-band grouper in Nagasaki, Japan. This virus belongs to the RGNNV genotype, determined by our own RNA2 nucleotide sequence analysis. E-11 cells were inoculated with the betanodavirus for propagation, and viruses were harvested when nearly all of the cells in the monolayer showed a cytopathic effect (CPE). NH_4Cl, chloroquine, bafilomycin A1, and chlorpromazine were purchased from Sigma (St. Louis, Mo.), and monensin was purchased from Wako (Osaka, Japan).

Virus infection and titration

E-11 cells were inoculated with virus, either in the presence or absence of chemicals at 28 °C for 1 h. The cells were then maintained in growth medium containing 2% FBS at 28 °C. Unless otherwise described, cells were pretreated with an agent for 1 h at 28 °C and inoculated with virus at a multiplicity of infection (M.O.I.) of 1. The virus titer was expressed as 50% tissue culture infectious dose ($TCID_{50}$), assayed using E-11 cells.

Detection of viral RNA by RT-PCR

Total RNA was prepared from RGNNV-infected cells (5×10^5) using Trizol reagent (Invitrogen). To detect (+) RNA1, (−) RNA1, and 18S rRNA, the RNA samples were reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) using RGRNA1-2490R (5′-GTCAGTGTAGT CTGCATACTG-3′) (for (+) RNA1), RGRNA1-1868F (5′-TGCGTGAGTTCGTCGAGTTT-3′) (for (−) RNA1), and 18S rRNA-R (5′-GCTGGAATTACCGCGGCT-3′) (for 18S rRNA). PCR amplification was performed with a primer pair (RGRNA1-1868F and RGRNA1-2490R for RNA1 and 18S rRNA-F [5′-CGGCTACCACATCCAAGGAA-3′] and 18S rRNA-R for 18S rRNA). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The band intensities were semi-quantitated using the Image J software program (NIH).

Results

Effects of NH_4Cl and chloroquine on the development of betanodavirus-induced CPE

The effect of the lysosomotropic agents on betanodavirus infection was initially examined by the appearance of virus-induced CPE. NH_4Cl and chloroquine diffused into the endosome and served as a proton sink, which inhibited endosome acidification [22]. E-11 cells were inoculated with RGNNV at an M.O.I. of 1 in the presence of different concentrations of the agents (Fig. 1). The uninfected E-11

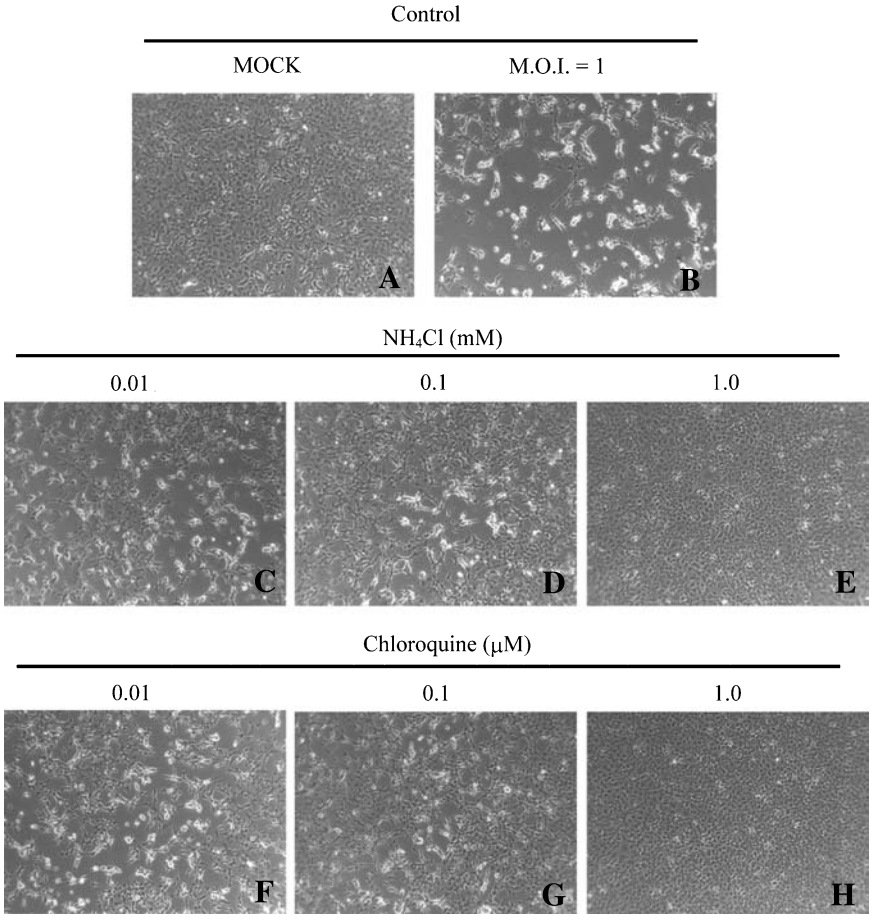

Fig. 1. Inhibition of CPE in betanodavirus-infected cells by NH_4Cl or chloroquine. Cells were inoculated with RGNNV in the presence of the indicated concentrations of NH_4Cl (**C**–**E**) or chloroquine (**F**–**H**). The morphology of the cells was photographed 6 days after infection. Control cells were infected at the indicated M.O.I. and incubated without an agent (**A**, **B**)

cells showed a flat adherent shape (Fig. 1A), and virus-infected cells showed a typical CPE shape and detached from the dish by 6 days after inoculation (Fig. 1B). In contrast, when E-11 cells were infected with RGNNV in the presence of NH_4Cl (Fig. 1C–E) or chloroquine (Fig. 1F–H), the development of CPE was suppressed in a dose-dependent manner. CPE was completely suppressed at a concentration of 1 mM NH_4Cl (Fig. 1E) and 1 μM chloroquine (Fig. 1H), respectively. The effect of NH_4Cl and chloroquine on viral infection may result from the cytotoxicity of these agents. Therefore, we investigated the cytotoxicity of these agents to E-11 cells by WST-1 assay. The degree of cell proliferation and morphologic change with NH_4Cl and chloroquine was not affected at a concentration of up to 12.5 mM and 25 μM, respectively (data not shown).

Lysosomotropic agents do not affect the attachment of betanodavirus to cells

To investigate the mechanism of the inhibitory effect on betanodavirus infection by agents, we next examined the effect of inhibitors on the attachment of betanodavirus to E-11 cells. As shown in Fig. 2A and B, the genomic (+) RNA1 band was detected in cells that were inoculated with RGNNV in a dose-dependent manner. When E-11 cells were inoculated with virus in the presence of 1 mM NH_4Cl or 1 μM chloroquine, the viral (+) RNA1 was still detected in the cells (Fig. 2C). The intensity of (+) RNA1 detected in the presence of drugs was comparable to that of the control cells (Fig. 2D). This result suggested that neither of the agents inhibited the attachment of betanodavirus to the cells.

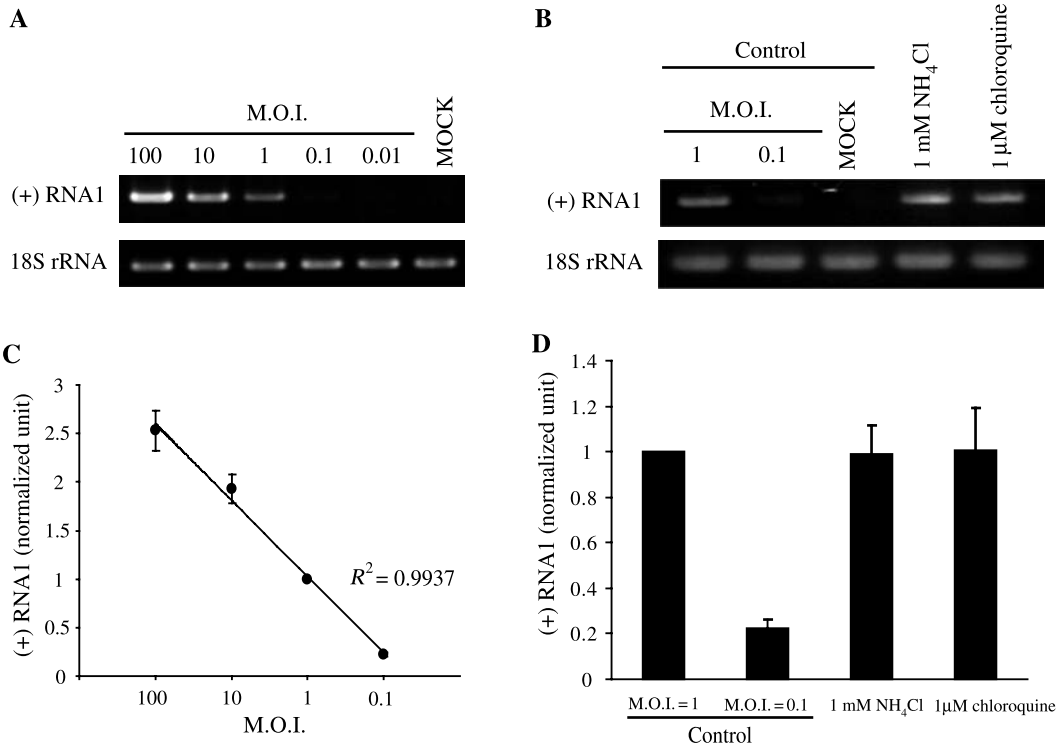

Fig. 2. No influence on the attachment of betanodavirus to E-11 cells by NH_4Cl and chloroquine. (**A**) Cells (3 to 4×10^5) were inoculated with RGNNV at the indicated M.O.I., and the cells were washed four times to remove unbound virus. The total RNA was prepared, and (+) RNA1 and 18S rRNA were detected by RT-PCR and then subjected to agarose gel electrophoresis. (**B**) The band intensity of (+) RNA1 and 18S rRNA were calculated and normalized to cellular 18S rRNA and expressed as the ratio of (+) RNA1 compared to that of the cells at an M.O.I. of 1. The data represent the means and standard deviations from three independent experiments. (**C**) Cells were inoculated with virus in the presence of 1 mM NH_4Cl or 1 μM chloroquine and the cells were washed with a medium containing the same concentration of inhibitor. The total RNA was subsequently prepared and subjected to RT-PCR. Control cells were inoculated at an M.O.I. of 0.1 or 1 without an agent. (**D**) The band intensity of (+) RNA1 was normalized as indicated in panel **B** and calculated as the ratio of (+) RNA1 compared to that of the control at an M.O.I. of 1. The data represent the means and standard deviations from three independent experiments

NH_4Cl inhibits the entry of betanodavirus into cells

To further determine the detailed mechanism of the inhibitory effect of NH_4Cl and chloroquine, we next analyzed the viral genomic (+) RNA1 and template (−) RNA1 accumulation in infected cells. E-11 cells were inoculated with virus in the presence or absence of these agents, and the infected cells were incubated for specific periods of time, and the total RNA was subsequently prepared from the cells. (−) RNA1 was synthesized from (+) RNA1 as the replication template; thus the presence of (−) RNA1 in the cells indicated that the replication of viral genomes had occurred. As shown in Fig. 3A and B, the detection of (+) RNA1 bands in the control cells gradually increased and reached a maximum level at 18 h post-inoculation. No significant (−) RNA1 bands were detected in the cells at 1 h post-inoculation, but their intensity gradually increased until 9 h post-inoculation and significantly increased from 9 to 12 h post-inoculation. These data imply that, after attaching to the cell membrane, the betanodavirus enters the cell, and the synthesis of the (−) RNA1 begins immediately thereafter. No significant (−) RNA1 bands were detected in cells that were inoculated and cultured in the presence of 1 mM NH_4Cl or 1 μM chloroquine. In addition, the band intensity of (+) RNA1 was steady over the sampling period.

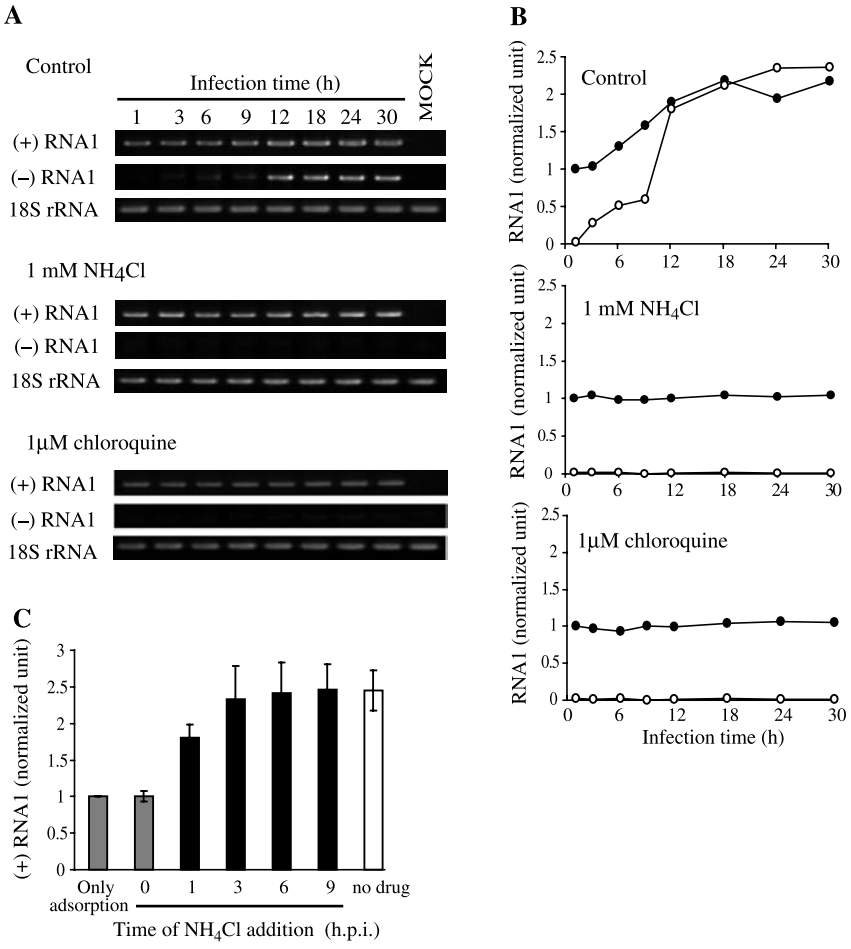

Fig. 3. NH_4Cl and chloroquine inhibit an early step of betanodavirus entry. (**A**) Cells were inoculated with RGNNV in the presence of 1 mM NH_4Cl (*middle panel*) or 1 μM chloroquine (*lower panel*). Control cells were inoculated and incubated without an agent throughout the experiment (upper panel). At the indicated times after inoculation, total RNA was extracted, and the (+) RNA1, (−) RNA1 and 18S rRNA were detected by RT-PCR. (**B**) The band intensity of RNA1s was normalized and calculated as the relative value of (+) RNA1 (closed circle) and (−) RNA1 (open circle) to that of (+) RNA1 which was seen at 1 h post-inoculation. The data represent the means from two independent experiments. (**C**) Cells were inoculated with virus and incubated in the presence (*gray bar*) or absence (*open bar*) of 1 mM NH_4Cl throughout the experiment. NH_4Cl was added to virus-infected cells to a final concentration of 1 mM at the indicated time post-inoculation (*black bar*). At 12 h post-inoculation, total RNA was extracted and subjected to RT-PCR. The band intensity of (+) RNA1 were normalized and expressed as the ratio of (+) RNA1 compared to that of the cells that were only infected with virus (only adsorption). The data represent the means and standard deviations from three independent experiments

NH_4Cl was next added to virus-infected E-11 cells at various times after infection (Fig. 3C). With the continuous presence of 1 mM NH_4Cl during the assay, no (+) RNA1 accumulation was detected in comparison to the control, as shown in panel B. However, a significant accumulation of (+) RNA1 was detected when NH_4Cl was added to the cells 1 h after inoculation. When NH_4Cl was added at 3 h post-inoculation, the amount of (+) RNA1 that accumulated in the cells was almost equal to the level of (+) RNA1 in the untreated cells. Taking these results into consideration, NH_4Cl inhibits an early step of betanodavirus entry into cells and has no effect on viral replication once the virus has entered the cells.

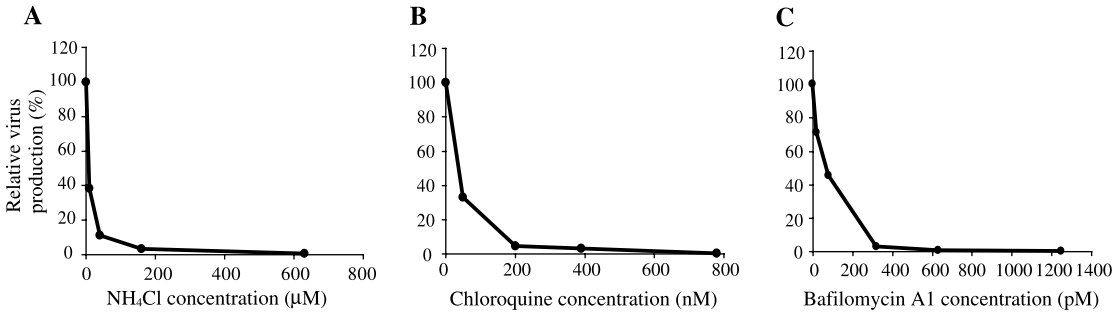

Fig. 4. Lysosomotropic agents inhibit the multiplication of betanodavirus. E-11 cells were inoculated with RGNNV in the presence of increasing concentrations of NH_4Cl (**A**), chloroquine (**B**), and bafilomycin A1 (**C**). The culture supernatant was harvested at 4 days post-infection and the infectivity titer was determined by a $TCID_{50}$ assay. The relative amount of virus production was calculated as the percentage of viral titer to that of the supernatant of the cells infected in the absence of agent. The data represent the means from two independent experiments performed in duplicate

Table 1. Effect of reagents that target the endocytic pathway in betanodavirus infection

Drug	Effect	CPE inhibition[a]
Ammonium chloride	inhibits acidification of endosomes	1 mM
Chloroquine	inhibits acidification of endosomes	1 μM
Bafilomycin A1	inhibits vacuolar H^+-ATPase, inhibits acidification of endosomes	1 nM
Monensin	carboxylic ionophore, inhibits acidification of endosomes	1 μM
Chlorpromazine	prevents assembly and disassembly of clathrin lattices at cell surface and on endosomes and inhibits clathrin-mediated endocytosis	10 μM

[a] The drug concentration that completely inhibited the CPE after 6 days of infection.

Inhibitory effect on viral replication by lysosomotropic agents

We examined the amount of infective virus released into the culture supernatant after betanodavirus infection in the presence of lysosomotropic agents. As shown in Fig. 4A, incubation of the cells with increasing concentrations of NH_4Cl resulted in a reduction in infectious virus. At a concentration of 160 μM NH_4Cl, the number of infectious viruses decreased to about 90% of the control. An almost complete inhibition of virus production was observed when cells were treated with 630 μM NH_4Cl. Chloroquine had an effect identical to NH_4Cl on the reduction of virus production (Fig. 4B). The amount of infectious virus was reduced to less than 5% of the control in the presence of 200 nM chloroquine. Treatment with bafilomycin A1, a lysosomotropic agent that is a specific inhibitor of the vacuolar H^+-ATPase [24], also showed an inhibitory effect against viral production in a dose-dependent manner (Fig. 4C). No virus-induced CPE was observed with cells that were treated with 1 nM bafilomycin A1 for 6 days after inoculation (Table 1). In addition, monensin, another lysosomotropic agent, and chlorpromazine also inhibited the development of virus-induced CPE at the concentrations listed in Table 1. These results strongly suggested that the various lysosomotropic agents could be utilized as inhibitors against betanodavirus infection.

Discussion

We have identified lysosomotropic agents as effective antiviral agents against betanodavirus infection in fish cell cultures. We utilized these agents because the electron microscopic study by Liu et al. [16] suggested that the entry of betanodavirus into cells depends on the endocytic pathway. We initially used NH_4Cl and chloroquine to examine whether they can inhibit the development of CPE after betanodavirus infection. Both agents completely inhibited virus-induced CPE at non-cytotoxic concentrations (Fig. 1). NH_4Cl and chloroquine had no affect on virus attachment to the cells (Fig. 2C and D). We also showed

that NH_4Cl inhibited a very early step of infection rather than blocking polymerase activity, since the level of (+) RNA1 accumulation was not affected when NH_4Cl was added to the culture medium at 1 h post-inoculation (Fig. 3C). The effective doses of NH_4Cl (1 mM) and chloroquine (1 μM) in this experiment were more than 10-fold lower than those used for other viruses [1, 2, 15, 29]. The infection of betanodavirus was also inhibited by bafilomycin A1 and monensin (Fig. 4 and Table 1), thus confirming that lysosomotropic agents can be useful for the prevention of betanodavirus infection.

Chloroquine, first synthesized as an anti-malarial drug, has been shown to have an antiviral effect on a wide range of viruses, including human immunodeficiency virus (HIV) [25, 27], influenza virus [23], and the SARS coronavirus (SARS-CoV) [4]. As for the enveloped viruses, chloroquine and NH_4Cl inhibit the glycosylation of viral envelope proteins [34], the transportation of glycoprotein to the plasma membrane [6, 9], and the envelopment of viral nucleocapsids [14]. It has also been suggested that the terminal glycosylation of angiotensin-converting enzyme-2 (ACE-2), a functional receptor of SARS-CoV, is inhibited by chloroquine and NH_4Cl [34].

Recently, in betanodavirus, sialic acid on the fish SSN-1 cell line has been demonstrated to be involved in binding of virus to the cell [16], although the specific functional cellular receptors for the virus have not yet been identified. Our data suggested that the inhibitory effect against an early step of betanodavirus infection (binding to the cell surface) was not affected by the agents (Fig. 2C and D). Feline calicivirus (FCV), a member of the family *Caliciviridae* and a non-enveloped virus, also requires the acidification in endosomes for its entry pathway [29]. The α2,6-linked sialic acid present on an N-linked glycoprotein acts as a receptor for FCV infection [30], and it has been suggested that the inhibitory mechanism of chloroquine against FCV entry into the cells is not due to a defect of the functional cellular receptor [29].

Treatment of E-11 cells with chlorpromazine inhibited the development of betanodavirus-induced CPE (Table 1), which suggested clathrin-mediated endocytosis as an entry pathway of betanodavirus into cells. This agent is a cationic, amphiphilic molecule that inhibits viral infection by shifting clathrin and the AP-2 complex to the late endosomal compartment [28, 36]. Many non-enveloped viruses have been shown to use the clathrin-mediated endocytic route to infect cells [3, 5, 35], and this pathway requires an acidic environment in the endosomes. Further detailed experiments are needed to clarify the mechanism of the endocytic pathway utilized in betanodavirus infection.

At present, to prevent the spread of betanodavirus, the selection of putative virus-free spawners and disinfection procedures are used, however these procedures are not enough to prevent re-emergence of betanodavirus infection on the same farms.

In conclusion, we have demonstrated for the first time that lysosomotropic agents can inhibit the infection of betanodavirus in vitro. These agents can be considered candidate anti-betanodavirus drugs. However, the agents discussed in this paper have not yet been approved for application in fish culture. Further studies are thus called for to explore this question.

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