

Antiviral effect of lithium chloride on feline calicivirus *in vitro*

Hongxia Wu¹ · Xiaozhan Zhang¹ · Chunguo Liu¹ · Dafei Liu¹ · Jiasen Liu¹ · Jin Tian¹ · Liandong Qu¹

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Abstract Feline calicivirus (FCV) is a highly contagious pathogen that causes oral and upper respiratory tract disease in cats. Despite widespread vaccination, the prevalence of FCV remains high. Furthermore, a high gene mutation rate has led to the emergence of variants, and some infections are lethal. To date, there is no effective antiviral drug available for treating FCV infection. Here, we show that lithium chloride (LiCl) effectively suppresses the replication of FCV strain F9 in Crandell-Reese feline kidney (CRFK) cells. The antiviral activity of LiCl occurred primarily during the early stage of infection and in a dose-dependent manner. LiCl treatment also inhibited the cytopathic effect. LiCl treatment exhibited a strong inhibitory effect against a panel of other two reference strains and two recent FCV isolates from China. These results demonstrate that LiCl might be an effective anti-FCV drug for controlling FCV disease. Further studies are required to explore the antiviral activity of LiCl against FCV replication *in vivo*.

Introduction

Caliciviruses are small, nonenveloped, positive-strand RNA viruses belonging to the family *Caliciviridae*, which is divided into five genera: *Norovirus*, *Sapovirus*,

Lagovirus, *Nebovirus*, and *Vesivirus*. The genus *Vesivirus* includes feline calicivirus, a highly contagious pathogen with a widespread distribution in the feline population. Oral ulcers, upper respiratory symptoms and high fever are the main clinical signs in FCV-infected kittens, followed by subclinical syndrome, including chronic stomatitis, pneumonitis and limping syndrome. Cats with ‘virulent systemic FCV disease’ variably show pyrexia, cutaneous edema, ulcerative lesions on the head and limbs, and jaundice. The disease is more severe in adult cats, with higher mortality [7, 25]. In addition, persistent infections lead to a high prevalence of FCV, ranging from approximately 10 % in the general cat population to as much as 90 % in some colonies [24, 36]. Therefore, treating and preventing FCV infection is challenging.

FCV exhibits one of the highest evolutionary rates among viruses. Indeed, evolutionary rates of the variable regions of the capsid protein have been estimated to be up to 1.3×10^{-2} to 2.6×10^{-2} substitutions per nucleotide per year [8]. Accordingly, older vaccines cannot provide cross-protection against newer strains [22, 23]. In addition, the genetic and antigenic landscape is highly complex in each geographical location; thus, vaccine application must be based on an assessment of the situation at both the local and national level [9]. FCV vaccines are generally effective at reducing the severity and duration of clinical signs but do not prevent infection or shedding [24]. Given the insufficiency of FCV vaccines, it is necessary to develop a safe and effective antiviral drug as a monotherapy or combination treatment. In addition to its significance for feline health, FCV is frequently used as a model for human noroviruses, an important cause of vomiting and diarrhea in people [32, 33].

Several types of therapeutics for the treatment of FCV have been reported. Feline-calicivirus-specific antiviral phosphorodiamidate morpholino oligomers (PMO) were

✉ Jin Tian
tj6049345@126.com

✉ Liandong Qu
qld@hvri.ac.cn

¹ State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, 427 Maduan Street, Nangang District, Harbin 150001, People’s Republic of China

tested in naturally occurring outbreaks of FCV, and the results demonstrated that PMO can work as a high-efficiency drug to treat FCV disease [30]. Mefloquine was employed as monotherapy or combination treatment with rFeIFN- ω [20], but deficiencies in a number of drug metabolism pathways have been recognized in humans [20]. For more than fifty years, lithium chloride (LiCl) has been used as a major treatment for bipolar disorder [18], and the mechanisms of LiCl have been explored, particularly for treating Alzheimer's disease [10], diabetes [16], and serous ovarian cancer [21], and also as an antimicrobial agent. Studies have demonstrated that LiCl can inhibit the replication of some viruses, such as herpes simplex virus 1 [29], coronavirus [27], infectious bronchitis virus [17], and porcine reproductive and respiratory syndrome (PRRS) [11], and it can suppress inflammatory responses [11], modulate cell apoptosis [27], and restore host protein synthesis in infected cells [35]. However, it remains unknown whether LiCl can inhibit FCV replication. In this study, the antiviral activity of LiCl on FCV was investigated *in vitro*, and we found that LiCl inhibited FCV replication in a dose-dependent manner.

Materials and methods

Cells, virus and reagents

Crandell-Reese feline kidney (CRFK) cells (ATCC) were grown in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10 % fetal bovine serum (FBS), 100 U of penicillin and 100 μ g of streptomycin per mL. FCV strains F9, Bolin and 2280 were purchased from ATCC, and the HRB-SS strain of FCV (GenBank accession no. KM016908) has been described previously [19]. The WZ-1 strain was isolated in Haerbin, China, in 2014 and the sequence has not been published. The isolates HRB-SS and WZ-1 were from sick pet cats. CRFK cells were grown in a 5 % CO₂ incubator at 37 °C and used to propagate FCV. The virus stock was centrifuged at 4 °C, 10,000 *g* for 10 min to remove cell debris and then stored at -80 °C. The virus stock was titrated using a TCID₅₀ (tissue culture-infective dose) assay. LiCl purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA) was prepared in DMEM at a concentration of 1 M and sterilized by passage through a 0.22- μ m filter.

Cytotoxicity assay for LiCl

Confluent CRFK cell monolayers in 96-well plates (10⁴ cells) were incubated overnight at 37 °C and 5 % CO₂ in complete medium. The cell supernatant was removed, and the monolayers were overlaid with 100 μ L of LiCl at a series of concentrations (20, 40, 60, 80 and 100 mM) in serum-free DMEM for 24 h and 72 h; the monolayers were then washed

with PBS. Mock-treated CRFK cells served as a control. Cytotoxicity assays were performed according to the instructions of the manufacturer of the Cell Counting Kit-8 (CCK8; Donjindo, Japan). After washing with PBS, the CRFK cells were incubated with medium (80 μ L/well) and CCK8 solution (20 μ L/well) at 37 °C for 1-4 h. The optical density (OD) was measured at a wavelength of 450 nm using an EnSpire® Multimode Plate Reader (PE, USA). The relative cell viability rate was determined as a percentage for each concentration as (OD₄₅₀ drug)/(OD₄₅₀ control) \times 100. LiCl concentrations below the 50 % cytostatic concentration (CC₅₀) were defined as non-toxic concentrations.

Effect of LiCl on viral attachment

To evaluate whether LiCl affects the attachment of FCV to CRFK cells, CRFK cells in 24-well plates were incubated overnight at 37 °C and 5 % CO₂ in complete medium. Nontoxic concentrations (0, 10, 20, 30, 40, 50, and 60 mM) of LiCl mixed with the FCV solution were incubated at 37 °C for 1 h. The mixture was inoculated onto cells at 4 °C for 1 h, and the multiplicity of infection (MOI) was 0.1 TCID₅₀/cell. After removing the upper mixture, total RNA was extracted from the monolayer and subjected to real-time PCR. Relative viral RNA levels were then calculated.

Effect of LiCl on viral entry

To evaluate whether LiCl affects the entry of FCV into CRFK cells, CRFK cells in 24-well plates were incubated overnight at 37 °C and 5 % CO₂ in complete medium. After the cell supernatant was removed, the monolayer was infected with FCV solution at an MOI of 0.1 TCID₅₀/cell containing different concentrations of LiCl (0, 10, 20, 30, 40, 50 and 60 mM) at 4 °C for 1 h and then incubated at 37 °C for 1 h. After removing the upper mixture, total RNA was extracted from the monolayer and subjected to real-time PCR. Relative viral RNA levels were then calculated.

Effect of LiCl on viral replication

To evaluate whether LiCl affects the replication of FCV in CRFK cells, CRFK cells in 24-well plates were incubated overnight at 37 °C in complete medium. The cell supernatant was removed, and the monolayer was infected with FCV at an MOI of 0.1 TCID₅₀/cell at 37 °C for 1 h. After removing the upper mixture and washing with PBS, the monolayer were covered with LiCl 500 μ L/well (0, 10, 20, 30, 40, 50 and 60 mM) and incubated at 37 °C for 12 h. The cytopathic effect (CPE) at 12 h postinfection was assessed, and then the cell supernatants were collected for the measurement of virus titers. After removing the upper mixture, total RNA was extracted from the monolayer and

subjected to real-time PCR. Relative viral RNA levels were then calculated.

Effect of LiCl on different phases of infection

To compare antiviral activity in pre-treatment, simultaneous treatment and post-treatment, CRFK cells in 24-well plates were incubated overnight at 37 °C in complete medium. For pre-treatment, the monolayer was inoculated with 30 mM LiCl at 37 °C for 1 h and then infected with FCV at an MOI of 0.1 TCID₅₀/cell. For simultaneous treatment, 30 mM LiCl and FCV were incubated with cells at 37 °C for 1 h. For post-treatment, the monolayer was infected with FCV at a MOI of 0.1 TCID₅₀/cell and then inoculated with 30 mM LiCl (500 µL/well) at 1, 3, 6 and 9 h postinfection. The duration of exposure to the compound in the pre-treatment assay was from 1 h prior to infection to the assay endpoint, and in the simultaneous-treatment assay, it was from the time of infection to the assay endpoint. The cell supernatants were collected for virus titration at 12 h postinfection. Total RNA was extracted from the monolayer and subjected to real-time PCR. Relative viral RNA levels were then calculated.

Determination of inhibitory activity of LiCl at different time points after infection

CRFK cells in 24-well plates were incubated overnight at 37 °C in complete medium. The mixture of 30 mM LiCl and FCV at an MOI of 0.1 TCID₅₀/cell was incubated with cells at 37 °C for 1 h. The duration of exposure to the compound in the assay was from the time of infection to the assay endpoint. The cells were collected at 3, 6, 9, 11 and 12 h post-inoculation, and total RNA was extracted from the monolayer and subjected real-time PCR. Relative viral RNA levels were then calculated.

Antiviral efficacy against field isolates and other reference strains of FCV

CRFK cells in 24-well plates were incubated overnight at 37 °C in complete medium. The monolayer was inoculated with 30 mM LiCl at 37 °C for 1 h and then infected with FCV F9, Bolin, 2280, WZ-1 and HRB-SS at an MOI of 0.1 TCID₅₀/cell. The duration of exposure to the compound in the assay was from 1 h prior to infection to the assay endpoint. The cell supernatants were collected for virus titration at 12 h postinfection.

Virus titration

Briefly, tenfold dilutions were prepared, and 0.1 mL of each dilution was added to the wells of a microtiter

plate. After 1 h of viral adsorption, the medium was removed, and fresh DMEM containing 1 % FBS and 100 IU of penicillin/mL and 100 µg of streptomycin/mL was added to each well. The viral titers were determined by cytopathology at 72 h post-inoculation and expressed as the median tissue culture infective dose log₁₀ (TCID₅₀/mL) according to the method of Reed and Muench [26].

Real-time PCR

Total RNA from FCV-infected cells was prepared with an Axygen Multisource Total RNA Miniprep Kit according to the manufacturer's instructions. cDNA was obtained using a One Step PrimeScript™ RT-PCR Kit (Takara, Japan). Real-time quantitative PCR targeting the Pro-Pol gene of FCV was carried out using a LightCycler 480 SYBR Green I Master (Roche, Swit) according to the manufacturer's instructions. The relative mRNA expression levels were calculated by the 2^{-ΔΔCT} method using GAPDH as an internal control for normalization. The mean mRNA level of the mock-treated group was set at 1. The following primers were used: FCV-forward (5'-ATGATTTGGGGTTGTGATGT-3'), FCV-reverse (5'-TGGGGCTRTCCATGTGAT-3'); GAPDH-forward (5'-TGACCACAGTCCATGCCATC-3'), GAPDH-reverse: (5'-GCCAGTGAGCTTCCCGTTCA-3').

Indirect immunofluorescence assay (IFA)

After washing with PBS, CRFK cells were fixed with 4 % paraformaldehyde in PBS for 15 min and then permeabilized with 0.2 % Triton X-100 in PBS for 15 min. After washing, the CRFK cells were incubated with a cat anti-FCV antibody (1:50) at 37 °C for 1 h. Subsequently, FITC-conjugated AffiniPure Goat Anti-Cat IgG (1:100) (Jackson, USA) was used as a secondary antibody. After washing three times with PBS, 4,6-diamidino-2-phenylindole (DAPI) (1:100) was added, and the samples were incubated at room temperature for 15 min. After washing, fluorescence was observed under an AMG EVOS F1 inverted microscope (AMG, USA).

Statistical analysis

All experiments were performed in triplicate and repeated in three independent experiments, with the data presented as the mean ± standard deviation (SD). The significance of differences between experimental groups was determined with a paired *t*-test and a one-way ANOVA using the Prism 5.0 software (GraphPad Software). A *p*-value <0.05 was selected to indicate significance.

Results

Assay of cytotoxicity of LiCl in CRFK cells

Cytotoxicity assays were performed according to the instructions of the manufacturer of the CCK8 kit. The relative cell viability was above 90 % after treatment with LiCl at concentrations of 20, 40 and 60 mM for 24 h and 72 h, whereas the viability was under 50 % after treatment with 80 and 100 mM LiCl for 24 h and 72 h (Fig. 1). It was necessary to verify that the concentration of LiCl was nontoxic to the cells to ensure that the results of the experiments were unaffected by the drug. A LiCl concentration under the 50 % cytostatic concentration (CC₅₀) that inhibited the proliferation of exponentially growing cells by 50 % was defined as a non-toxic concentration. At a concentration of 60 mM LiCl, which is below the CC₅₀ value, no difference in cell morphology compared with mock-treated cells was observed (data not shown); this concentration was therefore chosen as the maximum concentration of LiCl for antiviral assays.

No effect on FCV attachment and entry

To evaluate whether LiCl affects FCV attachment to CRFK cells, nontoxic concentrations (0, 10, 20, 30, 40, 50, and 60 mM) of LiCl were mixed with FCV suspension and then inoculated onto cells and incubated at 4 °C for 1 h. After removing the upper mixture, the relative viral RNA levels were calculated by real-time PCR. The result showed that treatment with LiCl at a concentration of 10, 20, 30, 40, 50 or 60 mM did not significantly affect the relative FCV genomic RNA levels compared with mock-treated CRFK cells (Fig. 2A), indicating that LiCl had no effect on the attachment of FCV to CRFK cells. For viral entry assays, the monolayer was infected with FCV at an MOI of 0.1

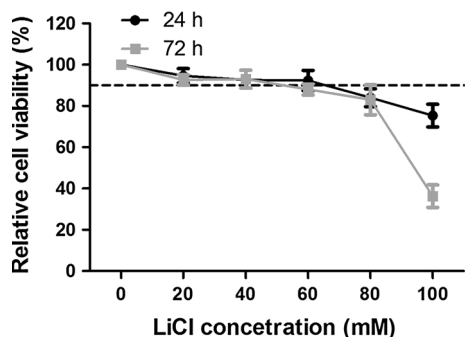


Fig. 1 The cytotoxic effect of LiCl treatment on CRFK cells. Cells were treated with 0, 20, 40, 60, 80 or 100 mM LiCl for 24 h and 72 h. Relative cell viability was determined by the CCK8 assay and normalized to the value of the 0 mM group (set at 100 %). Data are expressed as the mean \pm standard deviation (SD) of three independent experiments

TCID₅₀/cell at 4 °C for 1 h and was then covered with different concentrations (0, 10, 20, 30, 40, 50 and 60 mM) of LiCl (500 μ L/well) and incubated at 37 °C for 1 h. After removing the upper mixture, the relative viral RNA levels were calculated by real-time PCR. As shown in Fig. 2B, the relative FCV genomic RNA levels in all groups were approximately 100 %, and there was also no significant difference between mock-treated CRFK cells and cells treated with 10, 20, 30, 40, 50 and 60 mM LiCl (Fig. 2B), indicating that LiCl had no effect on FCV entry into CRFK cells.

Effect on FCV replication

To analyze the effect of different LiCl concentrations on FCV replication in CRFK cells, monolayers were infected with FCV at an MOI of 0.1 TCID₅₀/cell at 37 °C for 1 h and were then covered with LiCl (500 μ L/well; 0, 10, 20, 30, 40, 50 and 60 mM) and incubated at 37 °C for 12 h. The CPE, virus titers in the cell supernatants and viral RNA levels were analyzed at 12 h postinfection.

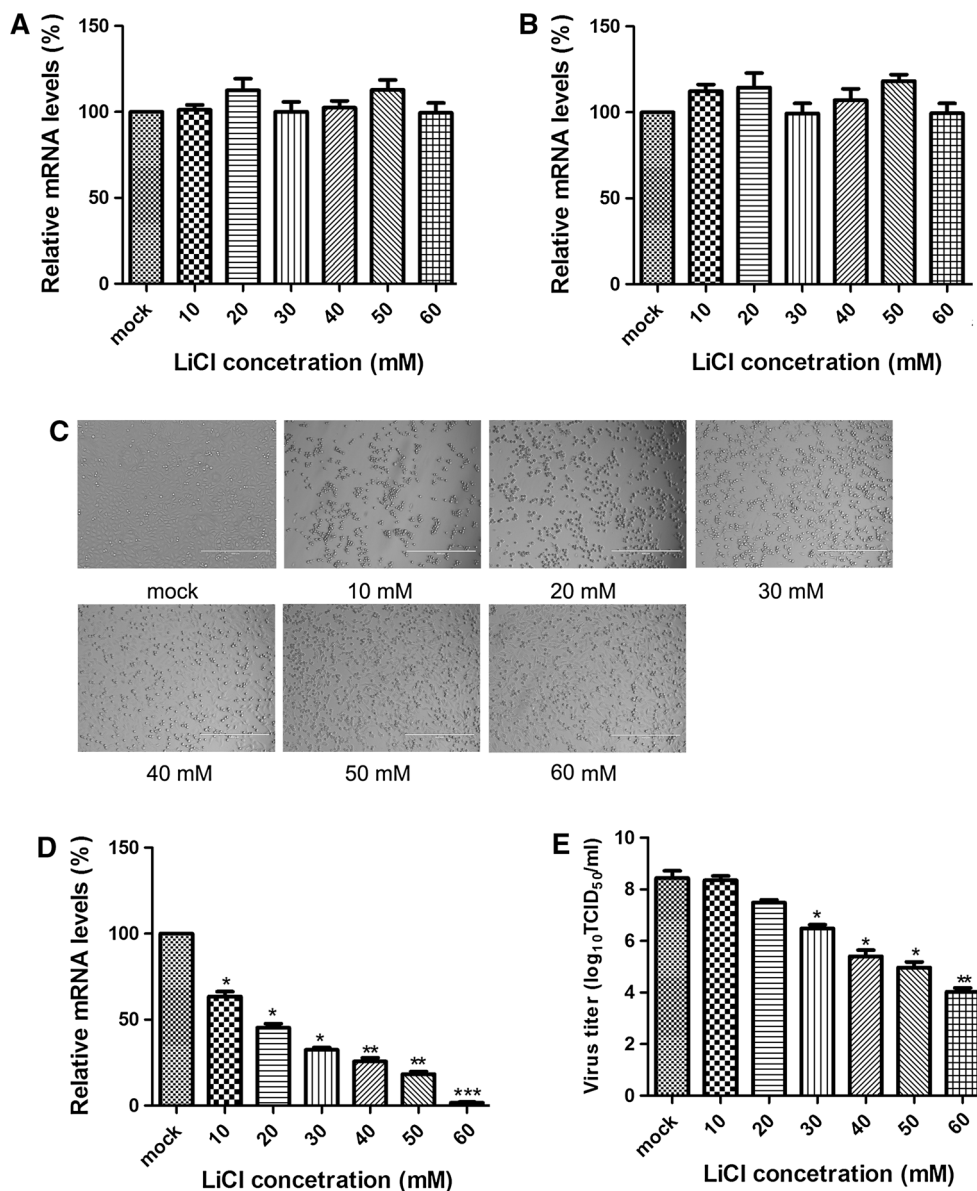
Although treatment with 10 and 20 mM LiCl did not inhibit CPE (Fig. 2C), treatment with 30 to 60 mM LiCl significantly inhibited CPE (Fig. 2C). In addition, the CPE ratio declined as the concentration of LiCl gradually increased from 30 to 60 mM (Fig. 2C). The relative viral RNA levels in the cells treated with 10, 20, 30, 40, 50 and 60 mM LiCl were 63.49 %, 45.33 %, 32.45 %, 25.75 %, 18.29 %, and 1.73 %, respectively, compared with mock-treated cells (Fig. 2D), indicating that LiCl treatment suppressed the synthesis of viral RNA. Furthermore, the virus titers of the cells treated with 10 and 20 mM LiCl were not decreased compared with mock treatment (Fig. 2E), although treatment with 30 to 60 mM LiCl significantly inhibited FCV replication (Fig. 2E). Treatment with 60 mM LiCl led to an approximate 1000-fold decrease.

With regard to IFA, strong fluorescent signals were observed in CRFK cells that were infected with FCV without LiCl treatment at 12 h postinfection, but the signals declined when the cells were treated with 30, 50 and 60 mM LiCl (Fig. 3). No fluorescent signals were detected in the mock-infected cells.

Effect of time of addition of LiCl on FCV replication

Time-of-addition experiments were carried out to explore in further detail the effect of LiCl treatment on viral replication. CRFK cells were treated with 30 mM LiCl at different time points before and after FCV entry. Viral RNA was barely detectable at -1, 0 and 1 h postinfection (Fig. 4A), but the relative viral RNA levels were 32.0 %, 63 % and 103 % at 3 h, 6 h, and 9 h post-infection,

Fig. 2 Viral RNA levels, load and CPE in CRFK cells treated with different concentrations of LiCl at different stages of the viral cycle. **A.** Relative viral RNA levels of cells treated with LiCl at the viral attachment stage. **B.** Relative viral RNA levels of cells treated with LiCl at the viral entry stage. **C.** CPE of CRFK cells treated with different concentrations LiCl at 12 h postinfection. **D.** Relative viral RNA levels of cells treated with LiCl at the viral replication stage. **E.** Viral load of the supernatant of cells treated with LiCl at the viral replication stage. Values represent the mean \pm SD for three independent experiments. The asterisks indicate significant differences between mock-treated and drug-treated groups (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)



respectively, in the drug-treated cells (Fig. 4A). In contrast to the control, the replication of the virus was significantly decreased after the addition of LiCl at -1 , 0 , 1 , 3 and 6 h postinfection, but no significant reduction occurred at 9 h postinfection (Fig. 4B), which agreed with the results for relative viral RNA levels. Thus, the most significant inhibitory effect of LiCl on FCV replication occurred primarily in the early stages.

Increased inhibition of virus replication by LiCl at longer treatment times

To examine whether the inhibition efficiency would increase when increasing the duration of LiCl treatment, relative viral RNA levels were determined at 3 , 6 , 9 , 11 and 12 h post-inoculation. Compared with mock treatment

groups, viral replication was significantly inhibited at all of the time points examined (Fig. 5). The mean relative viral RNA levels were 58.1% , 5.23% , 9.28% , 6.65% and 9.35% at 3 h, 6 h, and 9 h postinfection, respectively, in the drug-treated cells (Fig. 5). The inhibition efficiencies from 6 h to 12 h post-inoculation were higher than that at 3 h post-inoculation. This result indicated that the antiviral efficiency of LiCl against FCV infection increased with increasing treatment time.

Antiviral efficacy against field isolates and other reference strains

LiCl strongly inhibited CPE induced by the reference strain FCV F9 as well as its replication *in vitro*. To assess the efficacy of LiCl against different isolates, an experiment

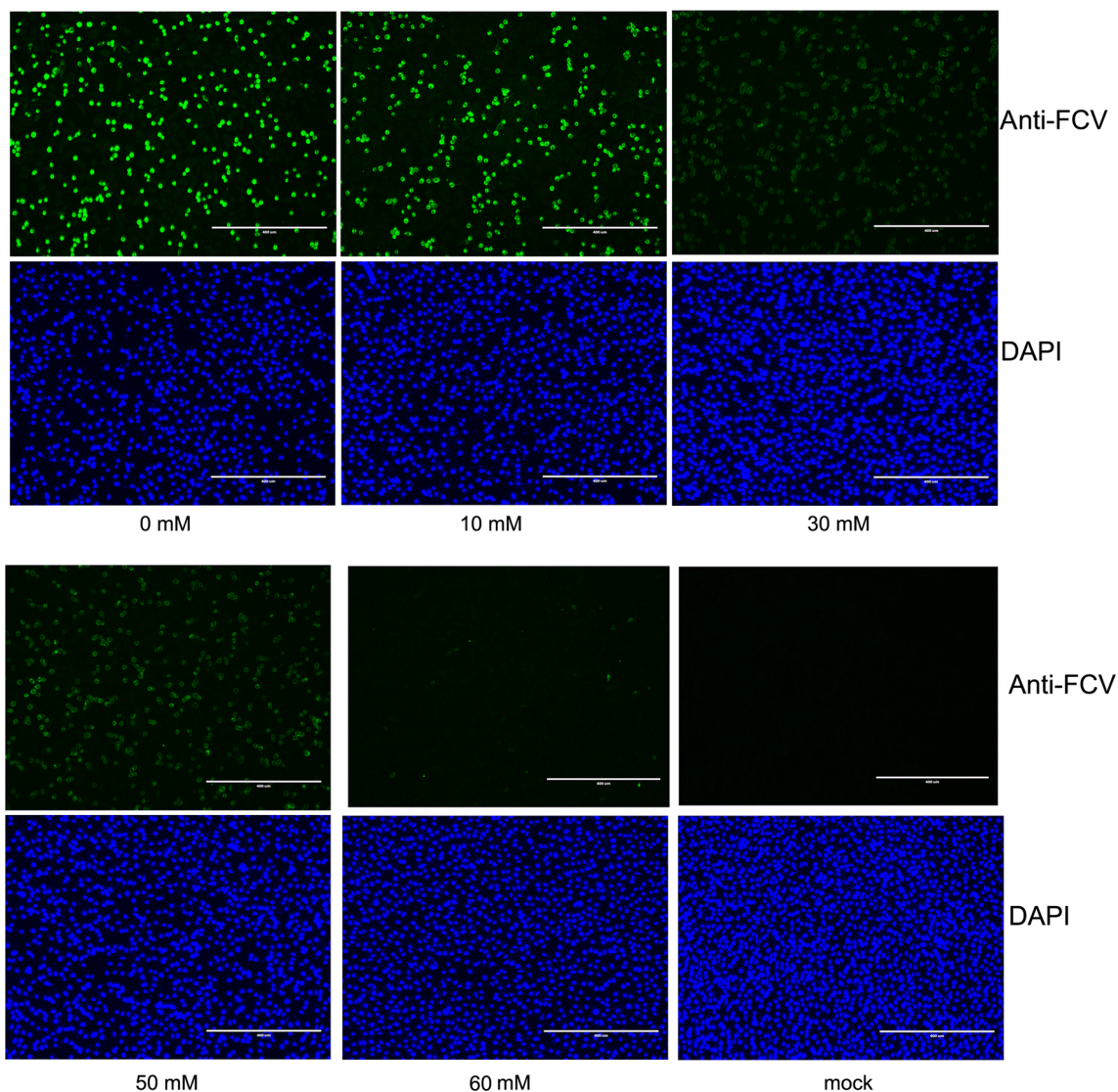


Fig. 3 Inhibitory effects of LiCl on FCV observed using immunofluorescence assays (IFA). CRFK cells were infected with FCV at an MOI of 0.1 TCID₅₀/cell at 37 °C for 1 h and then treated with the

indicated concentrations LiCl. Mock FCV-treated cells were used as a control. The fluorescence analysis ($\times 40$) was performed at 12 h postinfection

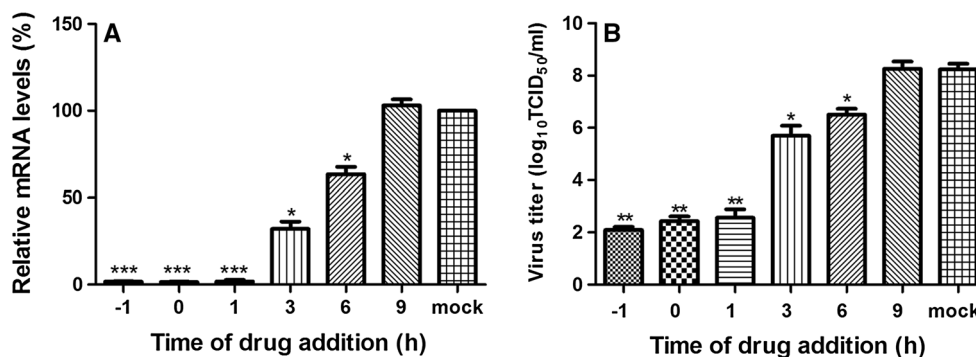


Fig. 4 Time course of F9 replication in CRFK cells with 30 mM LiCl treatment. CRFK cells were infected with F9 (MOI = 0.1 TCID₅₀/cell), followed by treatment with LiCl at the indicated time points. Virus titers were determined at 12 h postinfection. ‘-1 h’ indicates

that the cells were treated with LiCl 1 h before infection. Values represent the mean \pm SD for three independent experiments. The asterisks indicate significant differences between mock-treated and drug-treated groups (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

was conducted using field isolates and other reference strains. Following 1 h of exposure to LiCl or mock treatment, CRFK cells were infected with strain F9, Bolin, 2280, WZ-1 or HRB-SS at an MOI of 0.1 TCID₅₀/cell at 37 °C for 1 h and then covered with 30 mM LiCl (500 µL/well) at 37 °C for 12 h. The virus titers in the cell supernatants were analyzed at 12 h postinfection. The results showed that LiCl strongly inhibited the replication of the reference strains FCV F9, Bolin, and 2280 and field isolates WZ-1 and HRB-SS (Fig. 6). Compared with mock treatment, LiCl pre-treatment led to a nearly tenfold decrease for the infectivity of field isolates WZ-1 and HRB-SS (Fig. 6). These results demonstrated that LiCl could inhibit the growth of both reference strains and field isolates *in vitro*.

Discussion

Previous studies have demonstrated that LiCl has an antiviral effect on herpes simplex virus [2, 31], porcine parvovirus [4], infectious bronchitis virus (IBV) [12], and transmissible gastroenteritis virus (TGEV) [27]. However, inhibition was not observed with influenza viruses and encephalomyocarditis virus [29]. In this study, we showed that LiCl effectively suppressed replication of FCV strain F9 in CRFK cells. The inhibitory effect of LiCl occurred primarily in the early stages and was dose dependent. LiCl treatment also reduced the FCV-induced cytopathic effect. These results demonstrated that LiCl might be an effective anti-FCV drug for controlling FCV disease. Further studies

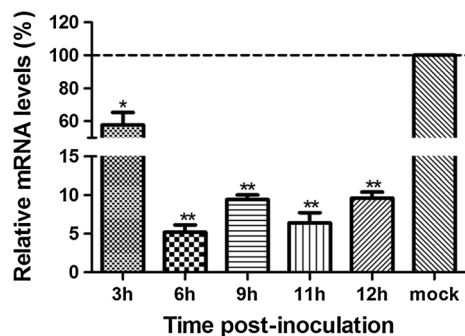


Fig. 5 Comparison of antiviral efficiency at different time points after infection. CRFK cells in 24-well plates were incubated overnight at 37 °C in complete medium. The mixture of 30 mM LiCl and FCV at an MOI of 0.1 TCID₅₀/cell was incubated with cells at 37 °C for 1 h. The duration of exposure to the compound in the assay was from the time of infection to the assay endpoint. The cells were collected at 3, 6, 9, 11 and 12 h post-inoculation, and total RNA was extracted from the monolayer and subjected to real-time PCR. Relative viral RNA levels were then calculated. Values represent the mean ± SD for three independent experiments. The asterisks indicate significant differences between mock-treated and drug-treated groups (*, $P < 0.05$; **, $P < 0.01$)

are required to explore the antiviral activity of LiCl against FCV replication *in vivo*.

In this study, we found that LiCl had no effect on F9 attachment and entry at every concentration tested, although a significant inhibitory effect on F9 replication was observed. Inhibition by LiCl occurred primarily in the early stages and was dose dependent. LiCl treatment also inhibited the cytopathic effect of FCV. These results are consistent with the inhibitory effect of LiCl on other viruses such as HSV-1, transmissible gastroenteritis coronavirus, and infectious bronchitis virus and indicate that the antiviral effect of LiCl may be a general feature of this drug on certain viruses, e.g., coronaviruses [12]. Although the frequency of FCV gene mutation is high, all FCV strains belong to the same serotype. In addition, F9 as well as another seven FCV strains show the same sensitivity to the antiviral drug mefloquine [20]. While the antiviral efficiency varied for different FCV strains infection, LiCl could function in the currently circulating viruses in China.

We found that the viability of CRFK cells was not affected by LiCl at concentrations ranging from 10 to 60 mM. In addition, when the concentration of LiCl was increased from 10 mM to 100 mM, the viability of CRFK cells decreased by less than 20 % at 24 h post-inoculation. However, the viability decreased to under 50 % at 72 h post-inoculation. F9 and other FCV strains replicate and spread rapidly. FCV-induced CPE on CRFK cells is obvious at 12 h postinfection at an MOI of 0.1, with nearly all cells becoming rounded and sloughing at 24 h postinfection. Thus, it is reasonable to use a high dose of LiCl to evaluate its antiviral effects against FCV replication *in vitro*, but high concentrations of LiCl in blood can cause side or toxic effects [34]. Symptoms associated with it are generally mild, including tremor, nausea, diarrhea, vertigo, and confusion [14]. However, higher lithium levels in the

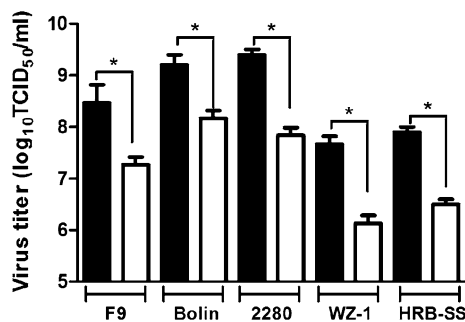


Fig. 6 Antiviral efficacy of LiCl against reference stains and recent field isolates of FCV from China. Cells were pre-treated with 30 mM LiCl for 1 h prior to infection with FCV F9, Bolin, 2280, WZ-1 and HRB-SS at an MOI of 0.1. The virus titre was calculated at 12 h postinfection using a TCID₅₀ endpoint assay. Values represent the mean ± SD for three independent experiments. The asterisks indicate significant differences between mock-treated and drug-treated groups (*, $P < 0.05$)

plasma rarely lead to persistent neurological deficits [3]. Lithium does not appear to be carcinogenic or mutagenic but may lead to renal and liver damage at prolonged exposures to serum levels of 2 mM or more [5].

As a specific inhibitor of glycogen synthase kinase-3 β (GSK3 β), LiCl causes inhibition by competing with native Mg²⁺ for metal-binding sites [28]. LiCl regulates multiple biological processes [34] and is considered to be a nonspecific inhibitor of GSK3 β . LiCl has been shown to effectively suppress EV-A71 replication, apoptosis, and inflammatory cytokine production (interleukin 6, interleukin-1 β) in infected cells [15]. However, LiCl attenuates LPS-, poly(I:C)- and Sendai-virus-induced IFN- β production in a GSK3 β -independent manner in macrophages [34], which appears to be contradictory with regard to the antiviral effects of LiCl. LiCl is widely used as a long-term mood stabilizer in the treatment of psychiatric diseases due to its inhibition of GSK3 β , a multifunctional kinase involved in cellular processes, including glycogen metabolism, cell proliferation, neuronal function, oncogenesis, and development [5, 6], and recent studies have provided evidence for the involvement of GSK3 β in innate immune responses [1]. GSK3 β is also a crucial regulator of the balance between pro- and anti-inflammatory cytokine production in both the peripheral and central nervous system and influences T-cell proliferation, differentiation and survival [1]. LiCl is not only a specific inhibitor of enzymes belonging to the GSK family but also of pyruvate kinase, polyphosphate 1-phosphatase, and inositol monophosphate [6, 13]. The precise mechanism of the antiviral effect of LiCl needs to be investigated further.

Conclusion

This study revealed that LiCl acts as a potent inhibitor of FCV *in vitro* and that the antiviral activity occurs in a dose-dependent manner in the early phase of viral replication. This is the first report of the antiviral activity of LiCl against a calicivirus and expands the antiviral spectrum for this virus. Further investigation and optimization of this compound for clinical use in treating FCV infections should be performed, and consideration should be given to investigating the effectiveness of LiCl against other viruses in cats.

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