Short communication

Antiviral activity of anisomycin against spring viraemia of carp virus in epithelioma papulosum cyprini cells and zebrafish

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A B S T R A C T

Spring viraemia of carp (SVC) caused by spring viraemia of carp virus (SVCV) is an acute and highly lethal viral disease of cyprinid fish. However, effective therapy for SVC is still scarce until now. Here we evaluated the inhibition of anisomycin (Ani), a metabolite produced by *Streptomyces griseolus*, on the replication of SVCV *in vitro* and *in vivo*. Our results demonstrated that Ani could suppress SVCV replication with the maximum inhibitory rate > 95% in epithelioma papulosum cyprini (EPC) cells. And the half maximal inhibitory concentrations (IC_{50}) of Ani on SVCV glycoprotein (*G*), nucleoprotein (*N*) and phosphoprotein mRNA expressions were 21.79, 13.13 and 12.24 nM, respectively. Besides, Ani decreased SVCV-induced cytopathic effects and nucleus damages. As expected, Ani also showed a strong anti-SVCV activity *in vivo*, as indicated by inhibiting viral gene expression and increasing the survival rate of zebrafish. Intraperitoneal injection of Ani increased the survival rate of zebrafish by 30% and markedly inhibited the expressions of *G* and *N* mRNA by > 60% in kidney and spleen at day 1 and day 4 post-infection. Results so far suggest that Ani as a powerful agent against SVCV can be applied to the control of SVC in aquaculture.

1. Introduction

Along with fish demands increase, it is critical to reduce losses from viral diseases that economically devastate the aquaculture industry (Balmer et al., 2017). Considered as one of the most serious viral threats for cyprinids, spring viraemia of carp (SVC) has caused large-scale deaths and serious economic losses in the aquaculture industry in the past decades (Ashraf et al., 2016). The causative agent of SVC is spring viraemia of carp virus (SVCV) which is classified as a member of the *Rhabdoviridae* family (Ashraf et al., 2016; Liu et al., 2019). SVCV is an enveloped, bullet-shaped RNA virus encoding a negative-sense, ssRNA genome. The virus has widely spread over Europe, Asia, North America and South America ever since its first detection in 1971 (Fijan, 1972; Dikkeboom et al., 2004; Garver et al., 2007). Young fish are especially susceptible to SVCV with up to 90% mortality (Baudouy et al., 1980; Ashraf et al., 2016). Given that SVC causes high mortality among cyprinids and large economic losses in aquaculture, it is imperative to develop effective measures to control the outbreaks of SVC.

Traditionally, vaccination of host fish is an effective way to protect them from SVCV infection. New DNA vaccines have been developed against SVCV and are evoking protection even after oral application (Emmenegger and Kurath, 2008; Cui et al., 2015; Embregts et al., 2019). However, there is no commercial vaccine currently available for the disease. In addition, some researches have been conducted to develop anti-SVCV drugs. Previous studies reported that several natural products and their derivatives showed a good anti-SVCV potential (Chen et al., 2018a, b; Liu et al., 2017, 2018; Liu et al., 2019; Shen et al., 2018a, b; Shen et al., 2019). These findings indicate that it is feasible to control SVCV infection by drug treatment.

Microbes have a strong potential to biosynthesize distinct metabolites that are promising drugs (Abt et al., 2012; Martinez et al., 2017; Wang et al., 2018). These metabolites mediate important host–microbe and microbe–microbe interactions and show plentiful biological activities (Wang et al., 2018). For instance, toxin oosporein from pathogenic fungus *Beauveria bassiana* interacted with gut microbiota to accelerate mosquito mortality (Wei et al., 2017). Additionally, poly-g-glutamic acid, a polypeptide secreted by Bacillus sp. played an antiviral role on SARS coronavirus and hepatitis C virus (Lee et al., 2013). Anisomycin (Ani), a small bioactive drug produced by *Streptomyces griseolus*, had been verified to possess antiprotozoal and antiviral activities (Ramabhadran and Thach, 1980; Tang et al., 2012). Reportedly, Ani could inhibit the replication of encephalomyocarditis virus (Ramabhadran and Thach, 1980). However, its application to treat viral diseases in aquaculture has not yet been investigated.

In the present study, the antiviral activity of Ani against SVCV was assessed *in vitro* and *in vivo*. To test the anti-SVCV activity *in vitro*, real-

Table 1
Sequences of primer pairs.

Genes	Accession No.		Primer sequences (from 5'to 3')
G	AY527273.1	Forward	GCTACATCGCATTCCTTTTGC
		Reverse	GCTGAATTACAGGTTGCCATGAT
N	KJ513477.1	Forward	AACAGCGCGTCTTACATGC
		Reverse	CTAAGGCGTAAGCCATCAGC
β-actin	AY222742.1	Forward	GCTATGTGGCTCTTGACTTCGA
		Reverse	CCGTCAGGCAGCTCATAGCT
18s	BX296557	Forward	ACCACCCACAGAATCGAGAAA
		Reverse	GCCTGCGGCTTAATTTGACT

"*G*": SVCV glycoprotein; "*N*": SVCV nucleoprotein.

time quantitative PCR (RT-qPCR), titer test, cytopathic effects (CPE) reduction assay and nucleus damage test were carried out. As a model organism, adult zebrafish (*Danio rerio*) are easily susceptible to SVCV. Thus it is to be considered as an ideal experimental model for studying the antiviral effect of Ani on SVCV *in vivo*. The antiviral activity of Ani against SVCV in zebrafish was evaluated by RT-qPCR and survival rate assay. Our results demonstrated the potential application of Ani as anti-SVCV agent in aquaculture.
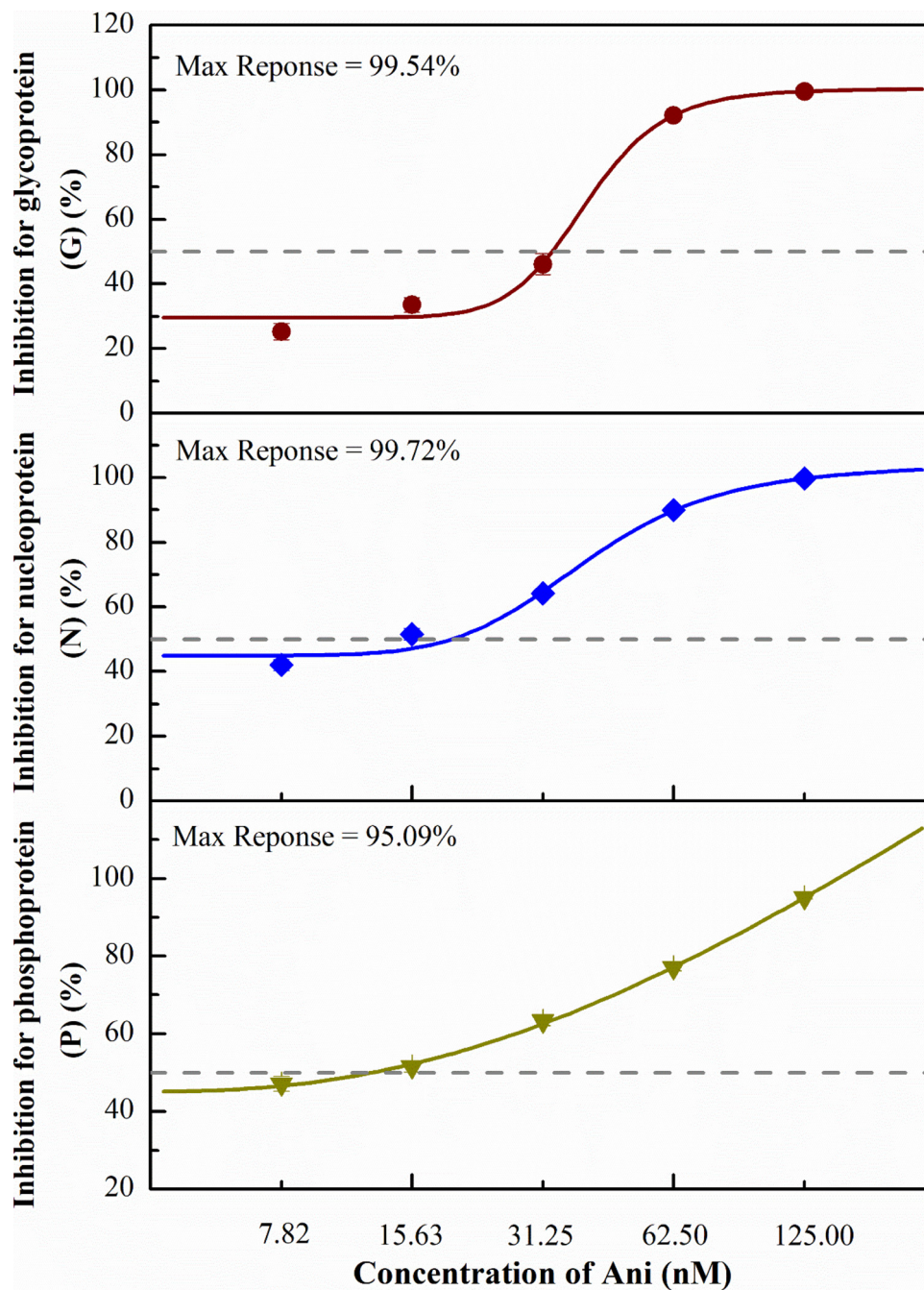
Fig. 1. Five-point dose-response curves for antiviral activities of Ani in EPC cells. The percent inhibition of Ani in the SVCV of glycoprotein (*G*), nucleoprotein (*N*) and phosphoprotein (*P*) expression assay was shown in red, blue and dark yellow. The maximum percent inhibition observed (Max response) of SVCV were indicated. Data were shown as mean ± SEM of three replicate samples of two independent experiments.
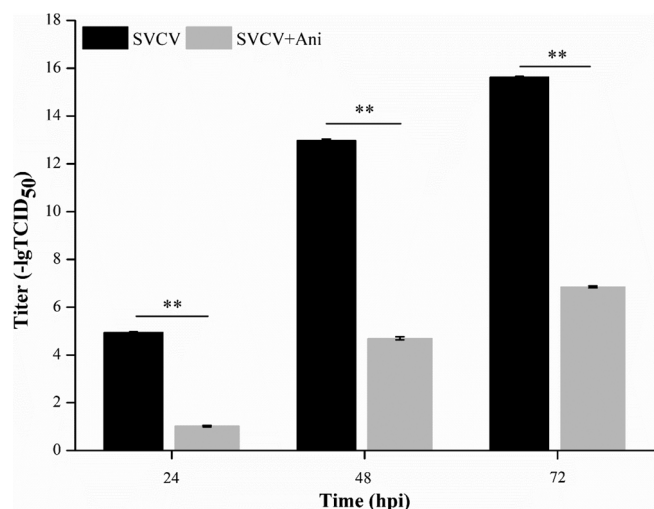
Fig. 2. Ani reduced the titers of SVCV in EPC cells. EPC cells were infected with SVCV with (shown in gray) or without 125 nM Ani (shown in black). Viral titers were determined by $TCID_{50}$ at the indicated time. Error bars indicated the SEM. **$P < 0.01$; *$P < 0.05$.

2. Materials and methods

2.1. Cell, virus, zebrafish and compound

The epithelioma papulosum cyprini (EPC) cells (kindly provided by Prof. Ling-Bing Zeng, Yangtze River Fisheries Research Institute, Wuhan, Hubei, China) were cultured at 25 °C in 5% CO_2 atmosphere in Medium 199 (M199, Hyclone, USA) cell culture with 10% fetal bovine serum (FBS, ZETA LIFE, USA).The strain 0504 SVCV was isolated from common carp in China (Chen et al., 2006), kindly provided from Prof. Qiang Li (Key Laboratory of Mariculture, Agriculture Ministry, PRC, Dalian Ocean University, Dalian, China), and propagated in EPC cells as previously described (Adamek et al., 2012).

Zebrafish (n = 1000, average length was 3.20 ± 0.15 cm) were obtained from the Xi'an Aquarium Market. The fish were acclimatized in five 300 L aquarium with a flow through system of carbon filtered tap water at 28 °C for 4 weeks prior to the beginning of experiments and fed to apparent satiation three times one day (8:00, 14:00 and 20:00) with a diet of commercial fresh blood worms (Aquacube Pet Products CO., LTD, Tianjin, China). Then we moved the aquarium to 17℃ environments so as to lower the water temperature to 17℃. Based on the primers combination strategy of Koutná et al. (2003), fish were checked randomly to verify pathogen free status of SVCV prior to the trial. Ani (CAS No. 22862-76-6) was purchased from Beyotime Biotechnology (Jiangsu, China).

2.2. Anti-SVCV activity of Ani in vitro

To test the anti-SVCV activity of Ani *in vitro*, EPC cells were cultured in 12-well plates to a monolayer and infected with SVCV (1×10^3 $TCID_{50}$) for 2 h at 25 °C with 5% CO_2. Then the media was removed, cells were washed three times and further incubated in 5% FBS M199 containing DMSO as a control or Ani (7.82~125.00 nM) as treatments for 48 h. Afterwards, the media was removed, RNA extracted, cDNA obtained and RT-qPCR carried out as below indicated.

2.3. CPE reduction assay and nucleus damage test

For CPE reduction assay, EPC cells were infected with 1×10^3 $TCID_{50}$ SVCV for 2 h, then the medium was replaced by new maintenance medium containing 125 nM Ani. According to the study of Shen et al. (2018a), the cells were directly observed and photographed under an inverted microscope.

Samples for cell nuclear damage test were prepared in the same way as in CPE reduction assay. The samples were dyed by DAPI (1 mg/L) for 20 min, and thoroughly washed with PBS, and then mounted on glass slide. The cell nuclear morphology was observed with an upright fluorescence microscopy (Leica-DM5000, Germany).

2.4. Titer assay

Titer assay was performed as described previously (Shen et al., 2018a, 2019). EPC cells cultured in 96-well plates were infected with SVCV. After infection for 2 h, the medium was replaced by maintenance medium containing 125 nM Ani and cells were then incubated for 72 h. The viral titer was calculated by the Karber method.

2.5. Antiviral activity of Ani in zebrafish

2.5.1. Survival rate assay

According to our previous studies (Shen et al., 2018b, 2019), healthy zebrafish were divided into three groups: SVCV-free PBS control group, SVCV + PBS group and SVCV + Ani group (each group, one tank, n = 30). The rearing temperatures of each aquaria were kept at 17 °C. Then the following treatments were executed for the three groups: (1) For the control group, each zebrafish was injected intraperitoneally with 10 μL PBS and reared for 14 d; (2) For SVCV + PBS and SVCV + Ani groups, SVCV (200 $TCID_{50}$) was mixed with PBS or Ani in equal volume, then each zebrafish was injected intraperitoneally with 10 μL mixture. The infected fish were fed three times daily with commercial dry feed pellets (Hello Fish Dry Pellets, Beijing, China), and then reared for additional 14 days to monitor mortality.

2.5.2. Gene expression in brain, spleen and kidney of zebrafish

The SVCV infection and Ani treatment processes were in conformity with these processes in the survival rate assay. Four zebrafish were randomly collected after infection for 1, 4 and 7 d, and the brain, kidney and spleen were collected for RT-qPCR.

2.6. RNA isolation, cDNA synthesis and RT-qPCR assays

Total RNA was extracted using Trizol (TaKaRa, Japan) according to the manufacturer's protocols. RNA was reverse transcribed using HiScript Q Select RT SuperMix for qPCR (+gDNA wiper) (Vazyme, China) and 400 ng/μL of RNA was used per reaction in cDNA generation. The RT-qPCR was performed with CFX96 Real-Time PCR Detection System (Bio-Rad, USA) using AceQ® qPCR SYBR® Green Master Mix (Vazyme, China) with the parameters according to the previous study (Shen et al., 2018b). The sequences of primer pairs are listed in Table 1 (Garcíavaltanen et al., 2014; Varela et al., 2014; Yang et al., 2014; Gotesman et al., 2015). Relative mRNA expression was calculated using $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001).

2.7. Statistical analysis

The data were analyzed by probit analysis which was used for calculating the inhibitory concentration at half-maximal activity (IC_{50}) of the compound using SPSS 18.0 for Windows (SPSS Inc. an IBM Company). The data were analyzed by an unpaired, two-tailed Student's *t*-test to determine significance (SPSS 18.0), and were presented as mean ± standard error (SEM). A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Antiviral activity of Ani against SVCV infection in EPC cells

To determine the anti-SVCV activity of Ani in EPC cells, the

A

B
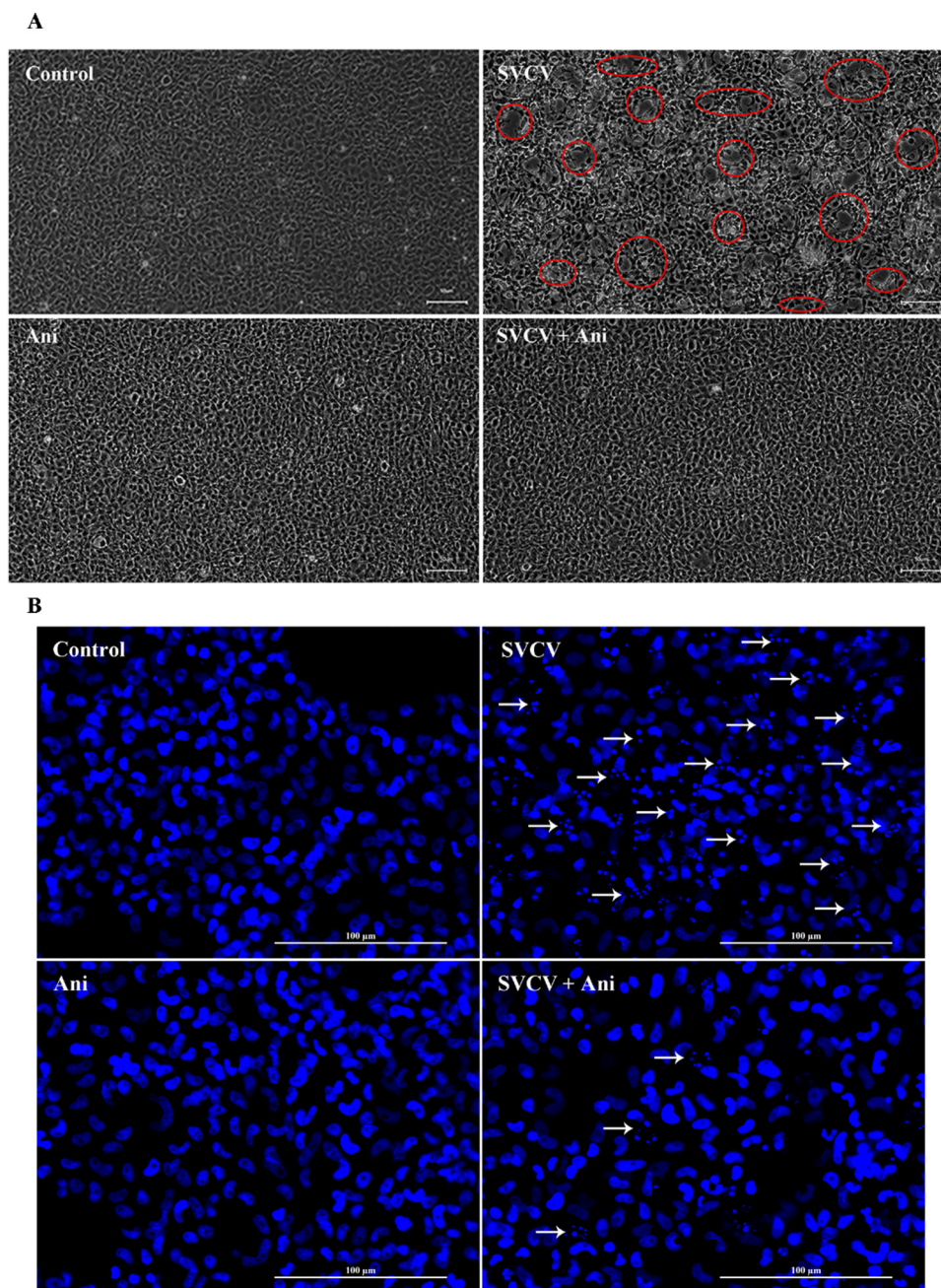
Fig. 3. Morphologically protective effect of Ani on SVCV in EPC cells. EPC cells cultured in 12 well plates were exposed to 10^3 TCID$_{50}$ SVCV for 2 h and then the medium with SVCV was removed and cells were incubated in fresh medium containing Ani for 48 h. After incubation periods, cytopathic effects (A) and nucleus damage (B) in EPC cells were observed. Cytopathic effects were shown in red ovals and nucleus damage was detected as arrows indicating.

expressions of SVCV glycoprotein (*G*), phosphoprotein (*P*) and nucleo-protein (*N*) were analyzed by RT-qPCR. As shown in Fig. 1, the 48 h IC$_{50}$ for Ani on *G*, *N* and *P* were 21.79, 13.13 and 12.24 nM, respectively. Moreover, SVCV was dramatically inhibited after its exposure to 125 nM Ani, with the maximum inhibitory rate > 95%. It should be noted that Ani at 125 nM had no significant toxicity on EPC cells by trypan blue exclusion dye staining test (data not shown).

In addition, we further evaluated the titer of SVCV after Ani treatment (Fig. 2). In consistency with the results of RT-qPCR, significant inhibition of SVCV was also shown in Ani-treated EPC cells in the measurement of the viral titer. SVCV titers were $10^{4.95}$ (24 h post-infection (hpi)), $10^{12.98}$ (48 hpi) and $10^{15.63}$ (72 hpi) TCID$_{50}$/0.1 mL; whereas SVCV titers were $10^{1.03}$ (24 hpi), $10^{4.70}$ (48 hpi) and $10^{6.85}$ (72 hpi) TCID$_{50}$/0.1 mL in Ani-treated group. The results above

indicated that SVCV replication could be significantly inhibited by Ani treatment *in vitro*.

3.2. Morphologically protective effect of Ani on EPC cells

SVCV infection could induce morphological damages in EPC cells (Liu et al., 2018; Shen et al., 2018a, b; Shen et al., 2019). The results in Fig. 3 showed that SVCV infection induced significant CPE and cell death in EPC cells. In accordance with the viral mRNA expressions, CPE of SVCV-infected cells in presence of 125 nM Ani was decreased significantly at 48 h (Fig. 3A).

In addition, SVCV infection induced nuclear fragmentation in EPC cells (Fig. 3B). By contrast, Ani treatment slowed down this critical condition induced by SVCV to some extent. All these results
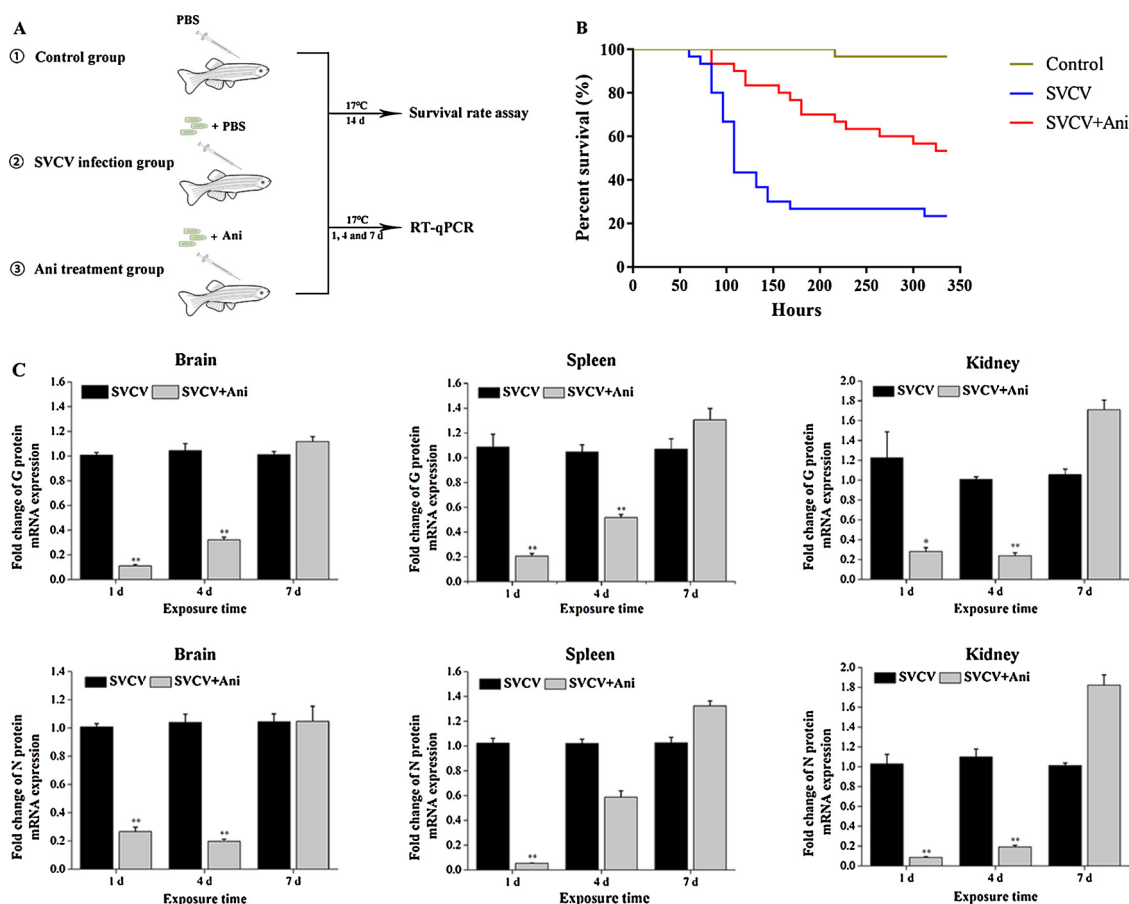
Fig. 4. Antiviral activity of Ani against SVCV in zebrafish. (A) Workflow of the experimental design followed in (B–C). PBS (Control group), SVCV + PBS (SVCV infection group) or SVCV + Ani (Ani treatment group) were injected in zebrafish, and then reared for additional 14 days to monitor mortality. In addition, four zebrafish were randomly collected after being infected for 1, 4 and 7 d, and then the brain, kidney and spleen were collected for RT-qPCR detection. (B) Cumulative survivorship curves of fish intraperitoneally injected with SVCV and Ani. (C) Expressions of SVCV *G* and *N* in kidney and spleen after Ani injection. Each value was represented as mean ± SEM of four fish. **$P < 0.01$, *$P < 0.05$.

demonstrated that Ani showed highly efficient inhibition of SVCV replication in EPC cells.

3.3. Anti-SVCV activity of Ani in zebrafish

Based on pre-test of toxicity, we determined the injection concentration of Ani was 500 nM. As shown in Fig. 4A, the cumulative mortality and the expressions of *G* and *N* in brain, spleen and kidney were evaluated. Fig. 4B showed that zebrafish died within 3th and 13th days post-infection (dpi) and died with a high speed within 3th dpi and 5th dpi. The cumulative morality of SVCV-infected zebrafish reached up to 76.7% at 13th dpi. Due to the anti-SVCV activity of Ani in zebrafish, the cumulative morality of zebrafish was reduced to 46.7% after Ani treatment.

To further confirm the anti-SVCV activity of Ani *in vivo*, the expressions of *G* and *N* in brain, spleen and kidney were also evaluated. Fig. 4C showed that Ani had significant inhibition on SVCV replication at 1 st and 4th dpi. The expression levels of *G* gene were decreased by 88.96 and 67.92, 79.31 and 48.27, and 71.88 and 76.15% in brain, spleen and kidney at 1 st and 4th dpi, respectively. At 7th dpi, Ani did not show any anti-SVCV activity in zebrafish. Altogether, these results implied that Ani was highly effective in the treatment of SVCV infection in zebrafish.

4. Discussion

Severe diseases in aquatic animals caused by rhabdovirus have

inflicted heavy economic losses on aquaculture industry. As one of the most lethal pathogens of the isolated rhabdovirus, SVCV has profoundly threatened the lives of cyprinids (Ashraf et al., 2016). A number of researches have been conducted to probe into its pathogenic mechanisms and develop new vaccines for its prevention (Zhao et al., 2018; Embregts et al., 2019). However, the use of vaccine in aquaculture is still restricted due to the handling stress on fish, as well as high laboring and production costs (Life, 2008). In addition, effective drugs for controlling SVCV in aquaculture is still scarce up to now (Ashraf et al., 2016; Shen et al., 2019). To address the urgent need for therapeutics for the treatment of SVCV, in the present study, we explored the anti-SVCV activity of Ani. Our results indicated that Ani was an effective drug for SVCV infection *in vitro* and *in vivo*.

Up to now, only a few studies have provided valuable insights into discovering antiviral agents against SVCV. Our recent studies reported that four synthesized coumarins inhibited SVCV with IC_{50} 2,2, 3.2, 0.6 and 2.4 mg/L (Liu et al., 2017; Chen et al., 2018b; Shen et al., 2018b). In addition, arctigenin ($IC_{50} = 0.29$ mg/L) and its two derivatives ($IC_{50} = 0.08$ and 0.10 mg/L) were found with anti-SVCV activities (Chen et al., 2018a; Shen et al., 2018a). As natural products, bavachin and saikosaponin D showed efficient inhibition on SVCV infection with IC_{50} 0.46 and 0.21 mg/L (Chen et al., 2018a; Shen et al., 2019). In the present study, the IC50 of Ani on SVCV *G* mRNA expression is only 21.79 nM (0.06 mg/L). Ani is more effective to inhibit SVCV infection in EPC cells, compared with the previously reported compounds. Previous study reported that Ani significantly inhibited the proliferation of T cells in mice (Xing et al., 2008), which indicated that Ani had a

suppressive effect on specific cellular immunity. Several immunosuppressive agents have also been confirmed to possess antiviral activities. For instance, dexamethasone, a classic immunosuppressive agent, has been reported with antiviral activity against Human immunodeficiency virus and Parainfluenza virus (Bourinbaiar and Leehuang, 1995; Liliana et al., 2003). Rapamycin, the third-generation immunosuppressive agent, was found with antiviral activity against infectious hematopoietic necrosis virus, rift valley fever virus and so on (Bell et al., 2017; Ko et al., 2017; Zhao et al., 2017). In addition, these immunosuppressive agents usually possess anti-inflammatory activity and can improve pathological features (Attur et al., 2000; Liliana et al., 2003; Song et al., 2015). Here, we speculated that the suppressive effect of Ani on specific cellular immunity might induce the anti-inflammatory activity and contribute to its protective effect on SVCV-infected zebrafish, which needs further studies.

Due to the highly efficient inhibition of SVCV replication mediated by Ani *in vitro*, we further explored whether Ani was also effective to SVCV infection *in vivo*. Based on the results of previous studies which performed an efficacy comparison between bath infection and intraperitoneal injection of viral infection (Sanders et al., 2003; Encinas et al., 2013), we chose the intraperitoneal injection as the way of SVCV infection. Our previous study indicated that SVCV replicated quickly in zebrafish within 2th and 5th dpi (Shen et al., 2018b). In accordance with this study, zebrafish died with a high speed within 3th and 5th dpi, which suggested SVCV replication sharply expanded in zebrafish within a short period. It should be noted that Ani could inhibit SVCV replication at 4th dpi. This indicated that Ani reduced SVCV replication in fish body to delay virus infection at the peak of virus replication. Thus, Ani as an antiviral agent showed an effective protection on SVCV-infected zebrafish. Take the example of our recently synthesized coumarin derivatives, statistics showed that they increased the survival rates of zebrafish by 17.5 and 22.5% and inhibited the *G* mRNA expression by about 50% in kidney and spleen at the first four days (Shen et al., 2018b; Liu et al., 2019). Compared with these two coumarin derivatives, Ani with an inhibitory percentage of > 60% was more effective in the treatment of SVCV infection in zebrafish. Given the above, Ani has been identified with antiviral activity against SVCV *in vivo* and can be an alternative anti-SVCV agent in aquaculture.

In conclusion, this study demonstrated that Ani inhibited SVCV replication and blocked SVCV-induced morphological damages in EPC cells. More importantly, Ani injection could suppress SVCV infection *in vivo* and prolong the survival time of zebrafish. Altogether, Ani is expected to be used as a therapeutic agent in aquaculture.

Acknowledgment

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