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Short communication

Antiviral efficiency of a coumarin derivative on spring viremia of carp virus *in vivo*

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ABSTRACT

Spring viraemia of carp (SVC) in aquaculture is challenging because there are few preventative measures and/or treatments. The previous study demonstrated that an antiviral coumarin derivative, 7-(4-(4-methyl-imidazole))-coumarin (C2), inhibits spring viremia of carp virus (SVCV) infection by targeting Nrf2-ARE signaling pathway in fish cells. Thus, we hypothesized whether C2 may be used as a potential therapeutic agent for controlling SVCV infection in aquaculture. In this study, SVCV infectivity was significantly inhibited *in vitro* in a dose-dependent manner by preincubation with C2. C2 was verified against SVCV in zebrafish, in which the mortality and viral titer in fish body were decreased. Like other coumarins, C2 was stable with a prolonged inhibitory half-life (3.5 days) at 15 °C in the early stage of SVCV infection. The results show that horizontal transmission of SVCV was reduced by C2 in a static cohabitation challenge model, especially for recipient fish in injection treatment, which suggested that C2 may be suitable as a possible therapeutic agent for SVCV in aquaculture. Overall, this study provides the new insight that a small molecule antiviral drug can be used to control rhabdovirus infection in fish aquacultures.

1. Introduction

As fish and shellfish demands increase, it is critical to reduce losses from viral diseases that economically devastate the aquaculture industry (Balmer et al., 2017). Spring viraemia of carp (SVC) is regarded as an acute hemorrhagic and contagious disease that has resulted in numerous financial losses. Therefore, it is listed as a notifiable animal disease by the Office International des Epizooties (OIE) in 2018 (Su and Su, 2018). SVC is caused by spring viraemia of carp virus (SVCV), the genus *Sprivivirus* of the family *Rhabdoviridae*, which mainly infects a range of cyprinids and some non-cyprinid fish species (like sheatfish and rainbow trout) (Ashraf et al., 2016). Initially, SVCV is enzootic in river systems throughout many European countries; since then, it has been identified in Brazil, the Middle East, China, and North America (Petty et al., 2002; Dikkeboom et al., 2004; Xiao et al., 2014). Young fish are typically most susceptible to SVC, with cumulative mortality rates as high as up to 90% during epizootic outbreaks (Baudouy et al., 1980). The virus enters the fish through the gills, replicates in gill epithelium and spreads to internal organs (Baudouy et al., 1980). Concomitantly, horizontal transmission of SVCV occurs when the virus is excreted *via* feces and urine from infected fish, but vertical transmission is controversial (Ahne et al., 2002). Due to the virus surviving for one month at −20 °C, the virus may be spread to other locations if the frozen infected fish are fed to piscivorous fishes or other animals (Petty et al., 2002). More seriously, SVCV is difficultly eradicated in affected ponds, and is challenging in all aquatic lives. Therefore, the therapy options are needed.

Traditionally, vaccines can elicit both early, nonspecific, and cross-protective antiviral immunity mediated by interferon and are basic prevention strategies for viral pathogens in aquaculture (Kanellos et al., 2007; Min et al., 2012; Cui et al., 2015; Zhu et al., 2015). However, vaccines are high costs of labor and production, lack efficient vector constraints, and have the handling stress, which makes it impractical for large numbers of susceptible fish (Adelmann et al., 2008; Plant and LaPatra, 2011). Thus, their application is still limited for the control of SVCV infection. Lately, a novel small-molecule antiviral coumarin derivative, 7-(4-(4-methyl-imidazole))-coumarin (C2), exhibits antiviral effect on SVCV infection in epithelioma papulosum cyprini (EPC) cells

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at the concentration of 25 μg/mL, and shows to reduce apoptosis in SVCV-infected cells by lower caspase-3, 8, 9 activities (Liu et al., 2017). Like other antiviral coumarins (Liu et al., 2018), C2 triggers Nrf2 translocation into nucleus, and enhances anti-oxidative enzyme gene expression to keep balance of intracellular redox state (Liu et al., 2017). Although C2 decreased EPC cell viability in some extent, it had no significant lethal effect on fish cells at effective antiviral doses (up to 25 μg/mL) (Liu et al., 2017). The use of C2 in cell model is impractical, and it is needed a further study using *in vivo* model.

Due to easy susceptibility to SVCV infection (Encinas et al., 2013), adult zebrafish (*Danio rerio*) are considered as an ideal experimental model for studying the antiviral effect of C2 on SVCV *in vivo*. In this study, we also determined that SVCV infection and horizontal transmission were inhibited by C2, and evaluated the inhibitory half-life of C2 in water environments.

2. Materials and methods

2.1. Coumarin derivative C2, cells and zebrafish

Synthetization and identification of coumarin derivative C2 were performed as described in the previous study (Liu et al., 2017). It was dissolved in 100% DMSO (Beyotime, China), stored at 4 °C, and used within 6 months (the synthetic route of C2 is shown in Fig. 1A).

EPC cells are kindly provided by Prof. Ling-Bing Zeng, Yangtze River Fisheries Research Institute, Wuhan, Hubei, China and maintained in Medium 199 (Hyclone, USA) containing 10% fetal bovine serum (FBS; Every Green, China) (M199-10), penicillin (100 IU/mL) and streptomycin (0.1 mg/mL) at 25 °C in 5% CO_2 atmosphere. The cells were seeded into a 96-well plate until grown to a monolayer. Up to 20 μg/mL C2 or 0.04% DMSO was added to wells in triplicate for 1, 3, 5 and 7 days. Cytotoxicity was measured by using Enhanced Cell Counting Kit-8 (CCK-8; Beyotime, China) following the manufacturer's protocol.

Zebrafish (the total length and body weight of 3.35 ± 0.12 cm and 0.47 ± 0.05 g, data are presented as mean values ± SD) were purchased from the Xi'an Aquarium Market. Fish were feeding in a flow system of active carbon filtered tap water (as aquatic water, pH 7.9 ± 0.4, hardness 6.3 ± 0.2°DH, dissolved oxygen 7.6 ± 0.4 mg L^{-1}) at 28 °C for a month and fed with commercial fresh blood worms at 8:00, 14:00 and 20:00. Fish were randomly checked to verify virus free according to the study of Koutná et al. (2003). Prior to the beginning of *in vivo* experiments, zebrafish were transferred into a new container and acclimatized at 15 °C for 2 weeks.

2.2. SVCV propagation and viral titer assay

SVCV (strain 0504) was isolated from common carp (Chen et al., 2006), kindly provided from Prof. Qiang Li (Key Laboratory of Mariculture, Agriculture Ministry, PRC, Dalian Ocean University, Dalian, China). For *in vivo* studies, SVCV was propagated in EPC cells, and viral titer assay was performed as previously described (Adamek et al., 2012).

2.3. In vitro inhibition

For preincubation, EPC cells were cultured into a 12-well plate and grown to a monolayer in 24 h at 25 °C. SVCV at 1×10^4 $TCID_{50}$/mL and up to 20 μg/mL C2 were incubated together in M199-0 (medium without FBS) for 15, 30 and 60 min, followed by viral titer test. A vehicle control (v/v, 0.04% DMSO) was set.

2.4. In vivo inhibition

Here, a total of 400 zebrafish were randomly separated into 40 zebrafish per aquaria with quiet UV-sterilized water at 15 °C. Based on data (not shown) of the preliminary tests on toxicity of C2 in bath and injection, bath concentration of 5 μg/mL and injection concentration of 10 μg/mL were chosen in this study. After two week for feeding in 15 °C, experimental fish were intraperitoneally injected with 10 μL 1×10^3 $TCID_{50}$/mL of SVCV per fish. After infection 2 h, SVCV-infected fish were treated in bath with 5 μg/mL C2 or intraperitoneal injection with 10 μg/mL C2 for rearing additional 14 days. The DMSO controls were set 0.01% (the final concentration) for bath and 0.02% for injection. The zebrafish were observed at regular intervals (every 6 h). To avoid the deterioration of the water quality, the observed dead fish were removed from the water in time.

Three SVCV-infected fish were collected at 1st, 3rd, 5th, and 7th days respectively after C2 treatment. After being homogenized with M199, the homogenates of fish samples were centrifuged at 3000 *g* for 30 min at 4 °C. The supernatants should be filtered through 0.45 and 0.22 μm membrane (Millipore, USA) for viral titer assay. The entire exposure was conducted in static water with aeration. The fish were humanely euthanized with tricaine methanesulfonate (MS-222) at a final concentration of 40 μg/mL, and frozen at −80 °C until processing.

2.5. Stability in aquatic water

Aquatic water samples were obtained from the flow system of active carbon filtered tap water at 15 °C. C2 at 5 μg/mL was mixed into a water sample and placed at 15 °C with 12/12 h dark/light, remaining for 0–7 days. On each day, SVCV (final viral titer was 1×10^4 $TCID_{50}$/mL) were pre-incubated with C2-exposed water sample for 1 h, followed by viral titer assay (described above), in which 1 part D5 in water was added to 1 part virus with v/v = 1:1 in M199-4 to determine final viral titer.

2.6. Cohabitation analysis

Donor fish were infected with 1×10^5 $TCID_{50}$/mL SVCV or treated with M199 only (as Mock). After 12 h infection, three donor fish were transferred into each challenge container (quiet UV-sterilized water) or injected with 10 μg/mL C2 (0.02% DMSO). Subsequently, recipient fish were treated with C2 at 5 μg/mL for bath (vehicle control was 0.01% DMSO) or at 10 μg/mL for injection (vehicle control was 0.02% DMSO) in each challenge container. Following 72 h of co-habitation (housing infected fish with un-infected fish), the sampled fish were euthanized in the laboratory through washrag soaked with MS-222 and frozen at −80 °C. This process was performed twice. Also, kidney and spleen were taken from recipient fish and immediately frozen in liquid nitrogen for RNA isolation.

2.7. RT-qPCR analysis

Total RNA was extracted by using Trizol (Takara, Japan) according to the manufacturer's protocols. The RNA concentrations were quantified by using the NanoDrop spectrophotometer (ND-1000, Nano-Drop Technologies Inc., Wilmington, DE). Purity of the extracted total RNA was determined by A_{260}/A_{280} ratio. Ratios of the absorbance at 260 and 280 nm ranged from 1.8 to 2.0. DNA contamination was removed by treating with DNase I (Takara, Dalian, China) following manufacturer's instruction. The total of 400 ng/μL of RNA was used per reaction in cDNA generation. The cDNA was constructed by reverse transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). SVCV N gene was quantified by RT-qPCR, and normalized to the expression levels of 18S housekeeping gene (Varela et al., 2014; Gotesman et al., 2015). RT-qPCR was performed using CFX96 Real-Time PCR Detection System (Bio-Rad, USA) and SYBR® Premix Ex Taq™ (Takara, Japan) with the previous parameters (Shen et al., 2018). The primers are listed in Table 1. The RT-qPCR reactions were performed in a final volume of 25 μL, containing 12 μL SYBR Premix Ex Taq™, 0.5 μM of each primer, and 500 ng of cDNA. The mRNA expression levels were

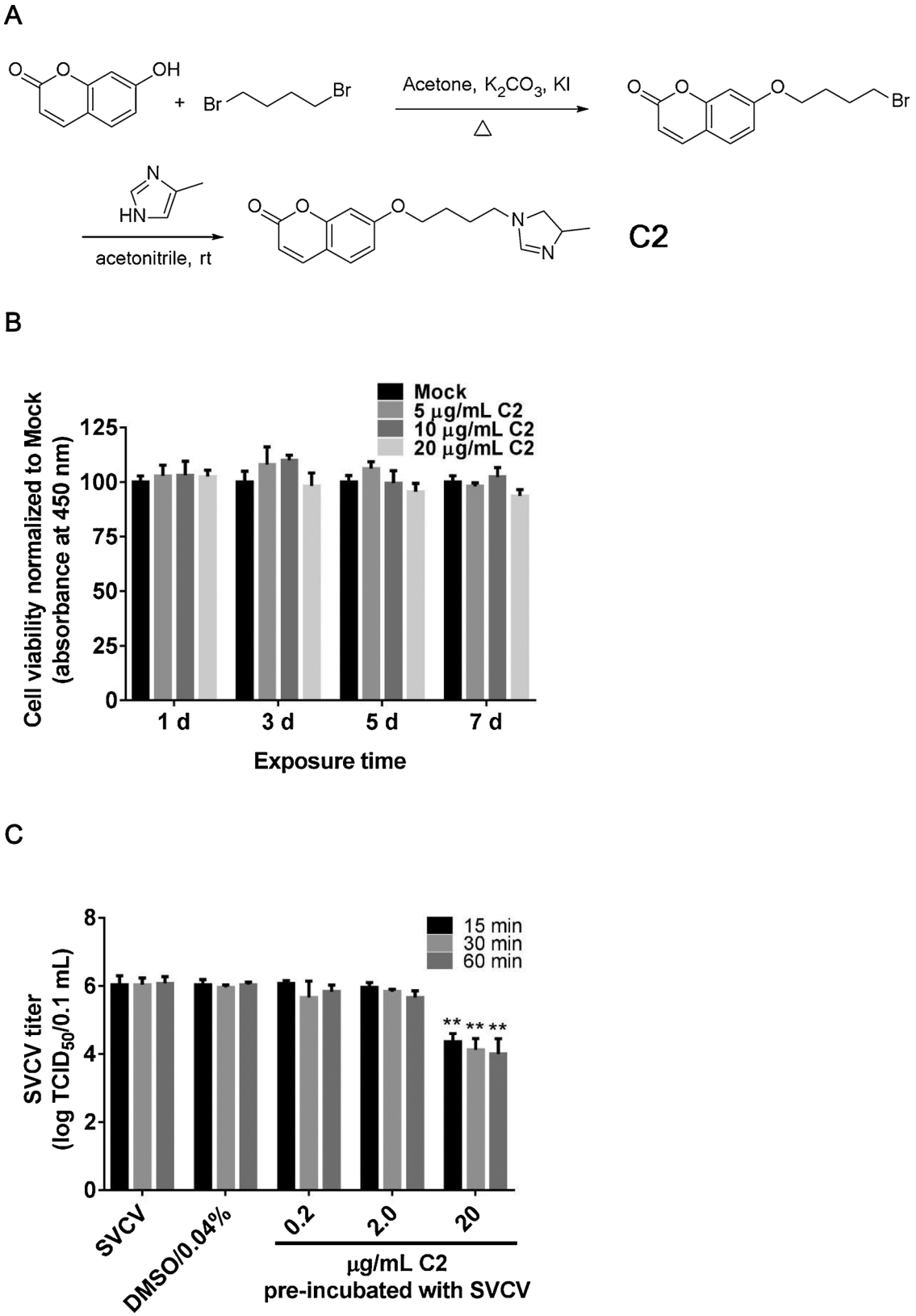

Fig. 1. Cytotoxicity of C2 at antiviral concentrations. (A) Synthetic route of C2. (B) The cells exposed to up to 20 μg/mL C2 (20 μg/mL C2 with 0.04% DMSO, 10 μg/mL C2 with 0.02% DMSO, and 5 μg/mL C2 with 0.01% DMSO). The absorbance was measured at 450 nm by CCK-8 analysis. (C) C2 preincubated with the virus for 15, 30, and 60 min. The SVCV titer was determined. Each value is represented as the mean ± SD by normalized to values for no treatment. The *p* value is determined by Student's *t* tests. $^{**}p < 0.01$; $^{*}p < 0.05$.

Table 1
Sequences of primer pairs used for the analysis of gene expression by qPCR.

Genes		Primer sequences (from 5'to 3')
SVCV nucleoprotein (N)	Forward	AACAGCGCGTCTTACATGC
	Reverse	CTAAGGCGTAAGCCATCAGC
18S	Forward	ACCACCCACAGAATCGAGAAA
	Reverse	GCCTGCGGCTTAATTTGACT
IFNγ	Forward	ATGATTGCGCAACACATGAT
	Reverse	ATCTTTCAGGATTCGCAGGA
IFNφ1	Forward	GAGCACATGAACTCGGTGAA
	Reverse	TGCGTATCTTGCCACACATT
IFNφ2	Forward	CCTCTTTGCCAACGACAGTT
	Reverse	CGGTTCCTTGAGCTCTCATC
MDA5	Forward	GAATCAGAATGTTCGCGTGTGT
	Reverse	CCTCGTCAGGGCTAGATTTGG
ISG15	Forward	ACTCGGTGGTGATGCTCCTC
	Reverse	CCTTCGGCACTCTCTCTTTC

calculated by using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.8. Statistical analysis

Viral titers were $\log_{10}$ transformed prior to statistical analyses for the remaining assays. The data were analyzed using an unpaired, two-tailed Student's *t* test to determine significance (SPSS 18.0), and presented as mean values ± SD. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Cytotoxicity of C2 at the antiviral concentrations

We evaluated the potential cytotoxicity of C2 in EPC cells by using CCK-8 test. EPC cells were exposed to up to 20 μg/mL C2, in which no cytotoxicity of C2 was evident with increasing to 7 days (Fig. 1B). The related results indicated that DMSO control was not cytotoxic at up to v/v = 0.04% when exposure time was reached to 7 days (data not shown). Clinical signs were used to assess the potential toxicity of C2 *in vivo* experiments, none of which was observed at the antiviral concentrations, including prolonged lethargy, decreased respiration rate, loss of equilibrium, circling, swimming slowly or in the bottom of challenge containers, and overt death. Thus, our results also suggested that C2 was not toxic *in vitro* and *in vivo* at the antiviral doses. Besides, C2 preincubated with SVCV in 60 min significantly inhibited SVCV infection *in vitro* at 20 μg/mL final concentration ($p < 0.01$) (Fig. 1C). The DMSO control had no inhibitory effect at a concentration of 0.04%.

3.2. Inhibition of C2 on SVCV infection in zebrafish

As shown in Fig. 2A, C2 could effectively inhibited SVCV infection *in vivo* by reducing the morality of zebrafish. The data showed that there was substantial enhancement of the survival rate in both bath (non-continuous) and injection of C2, in which 17.5% for bath and 32.5% for intraperitoneal injection were detected compared to only SVCV infection. During 7 days, the survival rate of zebrafish was up to 65% under continued C2 bath, which was increased 12.5% more than the discontinued bath (Fig. 2B). Our results demonstrated that the virus-infected fish were efficiently protected by C2 at antiviral concentrations with continued bath and intraperitoneal injection.

In the parallel trial, the further results confirmed that C2 inhibited SVCV infection in a time-dependent manner (Fig. 3A). As expected, viral titers reduced significantly in all time points, suggesting that C2 inhibited SVCV infection in zebrafish. At 3rd day, viral loads under bath treatment of C2 slightly exceeded that in injection. The dead zebrafish were collected for viral titer in 7 days (*in vivo inhibition assay*), and C2 with intraperitoneal injection (n = 11) obviously decreased viral titer

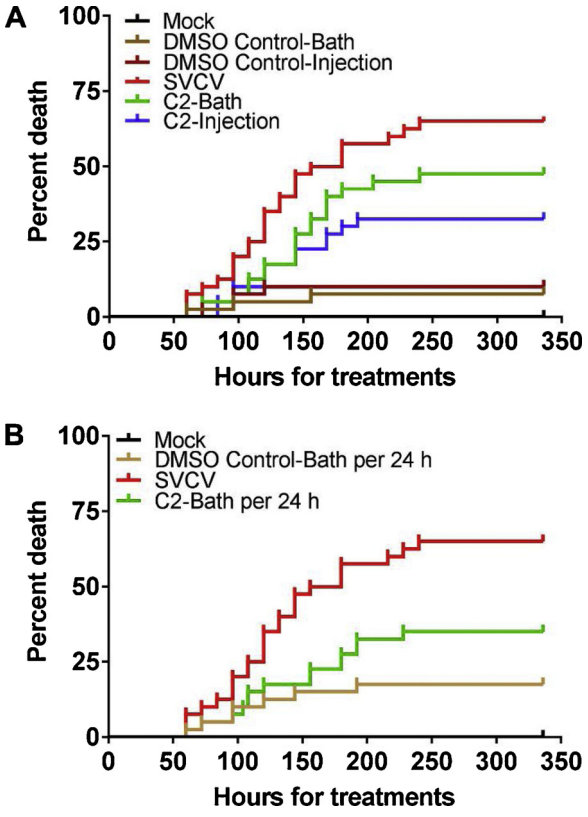

Fig. 2. Antiviral effect of C2 *in vivo*. (A) Survival curves of SVCV-infected fish with DMSO (0.01%) or C2 treatments. SVCV-infected fish were immersed and intraperitoneally injected with C2 at different antiviral doses. (B) Survival curves of SVCV-infected fish with continuous bath of C2. The concentration of 5 μg/mL C2 was updated every 24 h in 7 days. The survival rate of fish was increased compared to immersion treatment without C2 updating.

compared to SVCV group (n = 20), while the bath (n = 16) had no significant influence (Fig. 3B). Overall, our results suggested that SVCV loads can be inhibited *in vivo* by C2.

3.3. The stability of C2 in water

After *in vivo* studies, we tested the stability of C2 water environment. C2 was added to aquatic water to place for up to 7 days at 15 °C, and finally together with SVCV for preincubation, followed by viral titer assays. As shown in Fig. 4, C2 was fairly stable in the earlier stage of SVCV infection (3 days) and had a calculated inhibitory half-life of 3.5 days ($R^2 = 0.95$) when a nonlinear regression, best-of-fit line was applied (data not shown). These data indicated that C2 was relatively stable compound in water environment.

3.4. Inhibition of C2 on horizontal transmission of SVCV

In order to explore antiviral ability of C2 on horizontal transmission of SVCV, donor fish were infected with SVCV for 12 h, followed by C2 treatments and addition of recipient fish in a cohabitation condition (Fig. 5A). SVCV recipient fish in C2 intraperitoneal injection had a lower viral titer ($p = 0.029$ for injection) than that treated with DMSO. SVCV-infected donor fish from C2-treated groups had slightly lower, but not significantly lower ($p = 0.155$ for bath and $p = 0.259$ for injection) (Fig. 5B and C). Under the same experimental conditions, the expression of SVCV N gene was significantly down-regulated in kidney and spleen of recipient fish (Fig. 5D and E). Therefore, the present results suggested that SVCV infection in horizontal transmission process would be blocked by C2 to some extent.

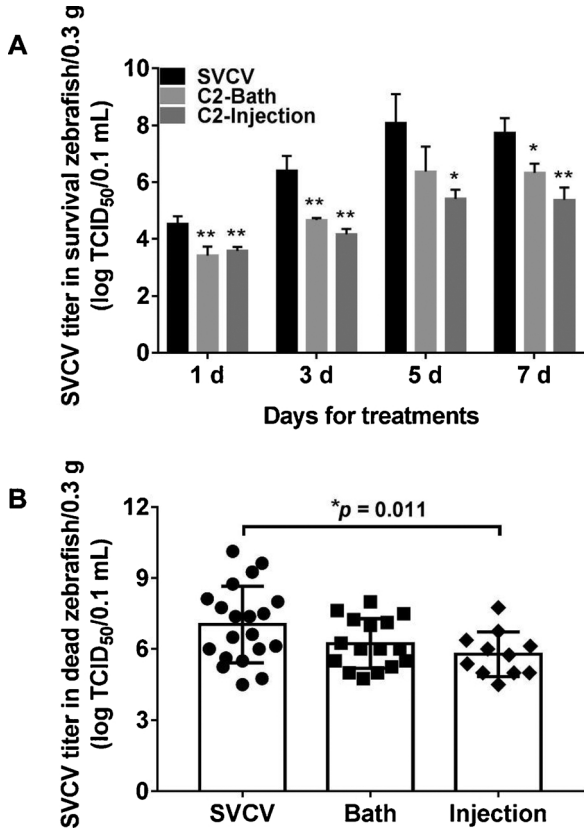

Fig. 3. Inhibition of C2 on SVCV infection in zebrafish. (A) SVCV infectivity titers in fish under C2 treatments (n = 3 fish per treatment). (B) SVCV infectivity titer in dead fish under C2 treatments (n = 20 fish in virus group; n = 16 fish with C2 bath treatment; n = 11 fish with C2 injection treatment). Each value is represented as the mean ± SD by normalized to values for no treatment. The *p* value for each study is determined by Student's *t* tests. $^{**}p < 0.01$; $^{*}p < 0.05$.

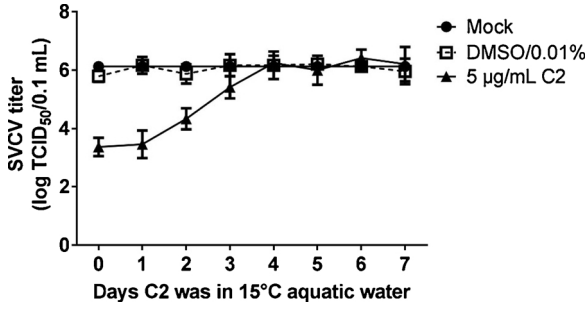

Fig. 4. The related stability of C2 in aquatic water at 15 °C. C2 was added to aquatic water placed at 15 °C for 0–7 days. On each day, SVCV was pre-incubated with treated water sample, followed by viral titer tests. There was inhibition of infection by C2 on day 0–3. Data are represented as the mean ± SD.

4. Discussion

Coumarins are naturally plant-derived and synthetically taken polyphenolic substances, present a wide variety of biological activities and behaviors, and are applied as therapeutic agents for multiple diseases (Katsori and Hadjipavloulitina, 2014), including some novel antiviral agents (Penta, 2015; Torres et al., 2014). Mostly, coumarins are gaining momentous attention on human viral diseases. For example, numerous classes of coumarin compounds are evaluated for inhibitory effects against HIV replication, and some of them show to inhibit different stages of HIV replication cycle excellently (Babé and Craik, 1997; Chen et al., 2011). Furthermore, a new compound library of hybrid coumarin-benzimidazole derivatives is connected through methylenethio linker (-SCH2-) as anti-hepatitis C virus (HCV) agents (Hwu et al., 2008). In our previous study, a total of 44 coumarin derivatives were designed, synthesized and evaluated the anti-SVCV activity in EPC cells (Liu et al., 2017). By comparing the inhibitory concentration at half-maximal activity (IC_{50} = 3.2 μg/mL), C2 was selected, with maximum inhibitory rates on SVCV more than 95% in EPC cells, which is hypothesized a viable way of preventing and controlling SVCV infection (Liu et al., 2017). Therefore, we evaluated the antiviral effect of C2 *in vivo* in this study.

Over the years, many research have focused on antiviral drug discovery against SVCV, but few of these potential antiviral candidates have been characterized in animal models to demonstrate their applicability in aquaculture (Chen et al., 2018). In comparison with the anti-SVCV coumarin derivatives (7-(4-benzimidazole-butoxy)-coumarin (BBC) and 7-(6-(2-methyl-imidazole))-coumarin) (D5) (Shen et al., 2018; Liu et al., 2018, 2019), C2 was lower toxic and more efficacious against SVCV *in vitro* and *in vivo* at a higher antiviral concentrations. High concentrations of C2 (≥ 20 μg/mL for injection and ≥ 10 μg/mL for bath), however, overwhelm the system and establish toxic effects, which is compounded by safety of DMSO at concentrations of 0.04% and 0.02%. Due to toxic differences between bath infection and intraperitoneal injection, the efficacy with injection of C2 was much higher than discontinued bath treatment, and there was a possible correlation with drug doses (≥ 10 μg/mL for injection and ≥ 5 μg/mL for bath). In addition, it was important to emphasize that C2 enhanced the survival rate of fish when C2 was updated every day in 7 days. The viral titer in zebrafish by C2 bath slightly exceeded that by C2 injection at the 3rd day, which represented a degradation of immersion efficiency. In order to verify the hypothesis that C2 doses may reduce in water after 3 days, we explored the stability of C2 in water environment. Generally, compounds degrade more slowly at lower temperatures, and the normal physiological temperature range for SVCV includes 15 °C (Fijan, 1999). In this study, we demonstrated that C2 was more stable at 15 °C, with a higher inhibitory half-life than other antiviral compounds (Balmer et al., 2016; Liu et al., 2019). Since the main limitations of antiviral drug applications in water systems are a relatively short half-life, obviously, C2 in continued bath is more suitable for aquatic application against SVCV infection *in vivo* (although C2 in injection shows an efficient protection on SVCV-infected zebrafish).

Horizontal transmission of SVCV occurs during epizootic outbreaks, in which excretion of SVCV begins shedding virus in the water *via* feces and urine from the infected fish (Ahne et al., 2002). In a water-based environment following acute rhabdovirus infection, fish mortality rate rapidly increase in most cases because viral loads in fish body reach to the highest peak (by an exponential enhancement) in a short period (Workenhe et al., 2010). This phenomenon suggests that horizontal transmission of virus plays an important role in outbreaks of aquaculture. In this study, C2 with intraperitoneal injection significantly inhibited horizontal transmission of SVCV in a cohabitation challenge model, but there was no effect of C2 bath treatment. Unexpectedly, viral loads were also nonsignificantly decreased in SVCV-infected donor fish, which may be relevant to a degree of SVCV multiplication beyond the threshold value of the antiviral activity of C2. Indeed, the higher doses of C2 in fish were expected to reduce SVCV replication under injection treatment. When the amount of virus shed from donor fish were decreased by C2, small amounts of virus in the water can infect fish, followed by low viral replication in the host. Because of lacking of antiviral doses in fish body, horizontal transmission of SVCV was not inhibited by donor fish immersed in continuous bath of C2. As the previous study mentioned that real-world conditions of variable amounts of organic material and high water flow rates/flowthrough systems are also challenges for therapeutic applications in aquaculture (Balmer et al., 2017), C2 may be suitable to inhibit viral transmission in pond aquaculture settings or in a static condition with intraperitoneal

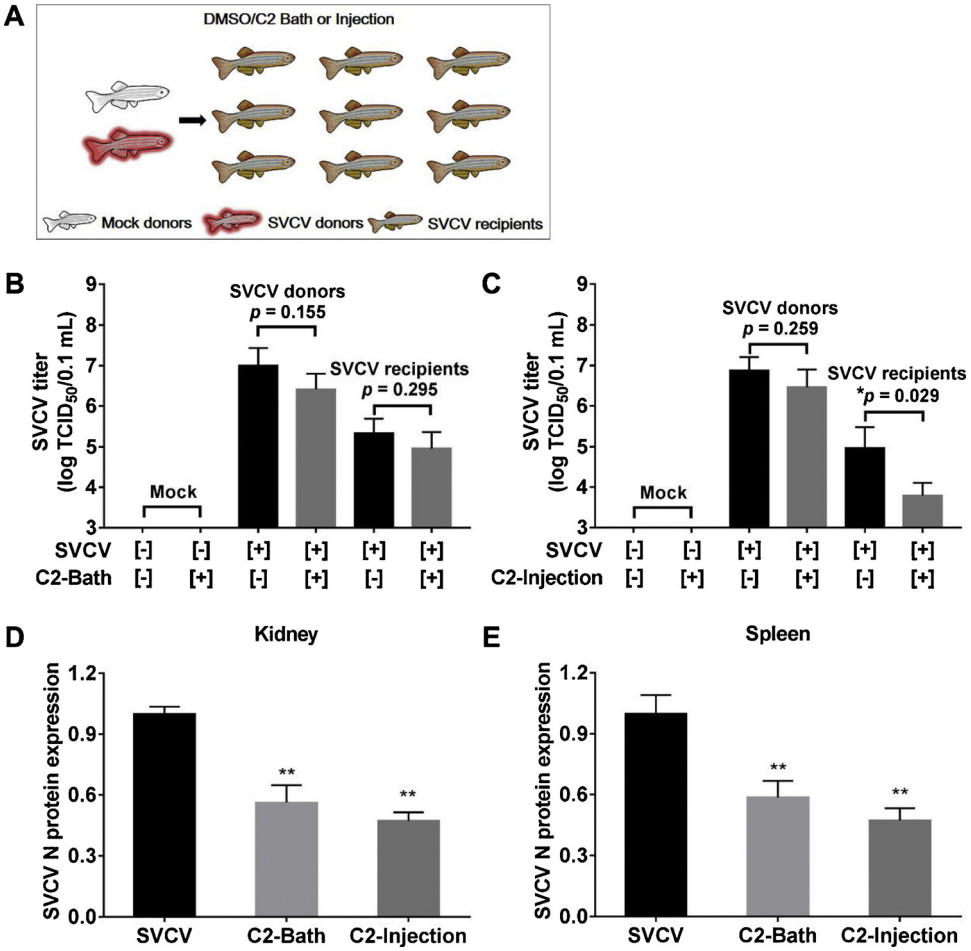

Fig. 5. Inhibition of C2 on horizontal transmission of SVCV. (A) Workflow of the experimental design. (B and C) There was a significant ($p < 0.029$, as determined by Student's *t* test) decrease in viral loads for the C2-treated recipient fish compared to DMSO-treated recipient fish. (D and E) Down-regulation of SVCV N gene expression in recipient fish with C2 treatments. Values represent the mean ± SD of three replicate samples. The *p* value for each study was determined by Student's *t* tests (two-tailed assuming equal variances). Significance between control and exposure groups are indicated by $^{**}p < 0.01$, $^{*}p < 0.05$.

injection way.

In conclusion, a coumarin derivative C2 is verified to be a positive antiviral effect against SVCV infection *in vivo*. Although the use of C2 as a therapeutic for SVCV at antiviral concentrations may be limited, C2 is considered to be useful as a therapeutic against horizontal transmission of SVCV under static-water conditions in some extent. We will be focused on exploring the further antiviral actions of C2 in more complex aquatic environments by performing it in real aquaculture. Therefore, these pleiotropic effects indicate that C2 may be a suitable antiviral agent against SVCV infection in aquaculture.

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