Virus Research 263 (2019) 112–118

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

# An imidazole coumarin derivative enhances the antiviral response to spring viremia of carp virus infection in zebrafish

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ARTICLE INFO

*Keywords:*
Coumarin
SVCV
IFN
Response
Oxidative stress

ABSTRACT

As an efficient pathogen resulting in economic impact in aquaculture, spring viremia of carp virus (SVCV) causes devastating disease in cyprinids. Based on the previous study that 7-(6-(2-methyl-imidazole))-coumarin (D5) exhibited anti-SVCV activity in fish cells, we hypothesized that D5 may be useful as a potential therapeutic agent for controlling SVCV infection *in vivo*. In this study, we verified that D5 inhibited SVCV replication in zebrafish, with reducing 22.5% mortality of SVCV-infected fish. Further data suggested that coumarin D5 was more stable with a prolonged inhibitory half-life in the early stage of virus infection (1–4 days). Consistent with above results, D5 decreased the viral titer in fish body and repressed SVCV glycoprotein gene expression in virus sensitive tissues (kidney and spleen) in the early stage of virus infection. In addition, the results replied that D5 elicited an innate immune response in non-viral infected zebrafish by up-regulating the expression of interferon genes (IFNγ, IFNφ1, IFNφ2 and RIG-1). D5 also enhanced the levels of antioxidant-related gene transcription and enzyme activities in SVCV-infected zebrafish, suggesting that D5 exhibited an antioxidant protection on fish by keeping the balance of redox state. Therefore, D5 is a potential therapeutic agent for the devastating fish rhabdovirus infections.

## 1. Introduction

As a negatively single stranded RNA virus, spring viremia of carp virus (SVCV) belongs to the genus *Sprivivirus* of the family *Rhabdoviridae* (Ashraf et al., 2016). It infects a range of cyprinid fish species (Ahne et al., 2002), induces high contagious mortality rates (Ahne et al., 2002; Baudouy et al., 1980), and is challenging in aquaculture (Fijan, 1999). More seriously, all aquatic life should be destructed in affected ponds to eradicate this virus (Fijan, 1984; Ahne et al., 2002), resulting in more economic losses for the aquaculture industry. Therefore, SVC has been listed as a notifiable animal disease by the Office International des Epizooties (OIE) and recognized as one of the Class I viral disease by animal epidemic prevention law in China (Ashraf et al., 2016; Zheng et al., 2018).

In aquaculture, the applications of the natural products/derivatives and chemical agents on virus infection have been generally explored (Zhi et al., 2011; Zhao et al., 2011). For instance, moroxydine hydrochloride exhibited an anti-GCRV (grass carp reovirus) effect for increasing cell viability (Yu et al., 2016); arctigenin and bavachin were measured the half maximal inhibitory concentration ($IC_{50}$) of 0.29 and 0.46 mg/L, respectively (Shen et al., 2018a). In the previous studies, three coumarin-imidazole hybrid derivatives were regarded as efficient anti-SVCV drugs, suggesting that four or six carbon atoms length of linker of coumarins played a key role in the antiviral activity (Liu et al., 2017a; Liu et al., 2018). The imidazole hybrid coumarin 7-(6-(2-methyl-imidazole))-coumarin (D5) was effective in weakening SVCV infection in a dose-dependent manner, showed low toxicity to fish cells, and regulated SVCV-induced undesirable conditions *in vitro* (Chen et al., 2018). Based on these results, we aimed to evaluating anti-SVCV properties of D5 *in vivo*. Owing to easy susceptibility to SVCV infection (Encinas et al., 2013; Sanders et al., 2003), adult zebrafish (*Danio rerio*) are considered as an ideal experimental model for studying on viral infectious disease and chosen in this study.

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https://doi.org/10.1016/j.virusres.2019.01.009
Received 22 October 2018; Received in revised form 14 January 2019; Accepted 14 January 2019
Available online 15 January 2019
0168-1702/ 

## 2. Materials and methods

### 2.1. Cell, virus and zebrafish

Epithelioma papulosum cyprini cells (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 cell culture medium 199 (M199, Hyclone, USA) cell culture with 10% fetal bovine serum (FBS, ZETA LIFE, USA), 100 μg/mL streptomycin and 100 U/mL penicillin. 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; the total length and body weight of 3.20 ± 0.15 cm and 0.44 ± 0.07 g, mean values ± SD) were purchased from the Xi'an Aquarium Market. Prior to the beginning of experiment, fish were acclimatized in a 300 L aquarium in a flow system of carbon filtered tap water at 28 °C for 4 weeks and fed to apparent satiation three times per day (8:00, 14:00 and 20:00) with a diet of commercial fresh blood worms (Aquacube Pet Products CO., LTD, Tianjin, China). Based on the primers combination strategy of Koutná et al. (2003), zebrafish were randomly checked to verify SVCV free status prior to the trial.

### 2.2. Synthetization and identification of coumarin derivative D5

Synthetization and identification of coumarin derivative D5 were performed as described in the previous study (Chen et al., 2018). The detail data were shown in the Supplemental materials and methods available online. The synthetic route of D5 was shown in Fig. 1.

### 2.3. Anti-SVCV activity of D5 in EPC cells

Based on the previous study of Shen et al. (2018b), quantitative real-time PCR (qRT-PCR) was performed for detecting the effect of D5 on the expressions of SVCV nucleoprotein (N), phosphoprotein (P), glycoprotein (G) and matrix-protein (M) gene in EPC cells.

### 2.4. Anti-SVCV activity of D5 in vivo

In the experiment, a total of 120 zebrafish were randomly divided into six aquaria (20 zebrafish per aquaria) containing 25 L of quiet UV-sterilized water. The rearing temperatures of each aquaria were kept at 17 ± 1 °C, because the optimum temperature of SVCV proliferation was less than 20 °C. After one week for adjusting to low temperature, zebrafish were intraperitoneally injected with viral titer $10^{°-4}$ × $TCID_{50}$ of SVCV per fish. Subsequently, the virus-infected fish were reared for additional 14 days with three times daily of a diet of commercial fresh blood worms. Based on the statistical data of the mortality experiment, we chose $10^2$ × $TCID_{50}$ SVCV for the further tests. Under the same operational procedure, a total of 120 zebrafish were intraperitoneally injected with mixture (SVCV + D5 or M199, 40 zebrafish per treatments), and five samples from each treatment were collected in control (M199), SVCV/M199 and SVCV/D5 groups on the 3rd, 6th and 9th days, respectively.

### 2.5. Stability experiments

Based on the previous study on the anti-SVCV activity of coumarin D5 in EPC cells (Chen et al., 2018), 10 mg/L of D5 was chosen to add to aquatic water samples. Coumarin D5 was added to a new water each day and placed at 17 ± 1 °C, remaining in the water sample for 0–7 days. On the final day (day 7), $10^3$ × $TCID_{50}$ SVCV (final concentration) was preincubated with each D5-treated water sample for 4 h, followed by virus titer assay of 48 h, in which 1 part D5 in water was added to 1 part virus with v/v = 1:1 in medium to determine the final virus titer.

**Table 1**
Sequences of primer pairs used for the analysis of gene expression by quantitative real-time PCR.

| Genes | | Primer sequences (from 5'to 3') |
|---|---|---|
| Actin | Forward | GCTATGTGGCTCTTGACTTCGA |
| | Reverse | CCGTCAGGCAGCTCATAGCT |
| SVCV glycoprotein (G) | Forward | GCTACATCGCATTCCTTTTGC |
| | Reverse | GCTGAATTACAGGTTGCCATGAT |
| SVCV nucleoprotein (N) | Forward | AACAGCGCGTCTTACATGC |
| | Reverse | CTAAGGCGTAAGCCATCAGC |
| SVCV phosphoprotein (P) | Forward | TGAGGAGGAATGGGAATCAG |
| | Reverse | AGCTGACTGTCGGGAGATGT |
| 18S | Forward | ACCACCCACAGAATCGAGAAA |
| | Reverse | GCCTGCGGCTTAATTTGACT |
| IFNγ | Forward | ATGATTGCGCAACACATGAT |
| | Reverse | ATCTTTCAGGATTCGCAGGA |
| RIG-1 | Forward | TTGAGGAGCTGCATGAACAC |
| | Reverse | CCGCTTGAATCTCCTCAGAC |
| IFNφ1 | Forward | GAGCACATGAACTCGGTGAA |
| | Reverse | TGCGTATCTTGCCACACATT |
| IFNφ2 | Forward | CCTCTTTGCCAACGACAGTT |
| | Reverse | CGGTTCCTTGAGCTCTCATC |
| SOD (Mn) | Forward | CCGGACTATGTTAAGGCCATCT |
| | Reverse | ACACTCGGTTGCTCTCTTTTCTCT |
| SOD (Zn/Cu) | Forward | GTCGTCTGGCTTGTGGAGTG |
| | Reverse | TGTCAGCGGGCTAGTGCTT |
| GPx | Forward | AGATGTCATTCCTGCACACG |
| | Reverse | AAGGAGAAGCTTCCTCAGCC |
| CAT | Forward | AGGGCAACTGGGATCTTACA |
| | Reverse | TTTATGGGACCAGACCTTGG |

### 2.6. Viral titration and gene expression in kidney and spleen of zebrafish

A parallel trial following the above procedure in Section 2.4 was set to investigate viral titration and gene expression, and a total of 120 zebrafish were intraperitoneally injected with mixture (SVCV + D5 or M199, 40 zebrafish per treatments). At the timepoints 3rd, 6th and 9th days, five sampled fish were euthanized in the laboratory by soaked with an anesthetic 20.0 g/$m^3$ MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate), and the body was immediately homogenized with ten volumes of M199 medium (v/w = 10/1) in a glass tissue homogenizers under ice-bath cooling. The homogenates were centrifuged at 6000 g, 4 °C for 20 min, and subsequently, the supernatants were successively filtered through 0.45 and 0.22 μm membranes (Millipore, USA) for testing viral titration. In the replicate trial, kidney and spleen in five fish samples from a total of 120 zebrafish at the timepoints 1st, 4th and 7th days were aseptically removed from the abdominal cavity for analyzing the antioxidant activity and virus replication.

### 2.7. Quantitative real-time PCR analysis

The kidney and spleen samples were frozen in liquid nitrogen for RNA isolation. The total RNA was extracted using Trizol (TaKaRa, Japan) according to the manufacturer's protocols and reverse

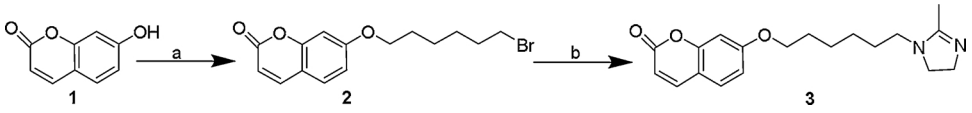


**Fig. 1.** Synthetic route of 7-(6-(2-methyl-imidazole))-coumarin (D5). (a) 1,6-dibromohexane, $K_2CO_3$, triethylamine, dry acetone, 60 °C, 20–24 h; (b) 2-methylimidazole, $K_2CO_3$, $CH_3CN$, r.t..20–24 h.

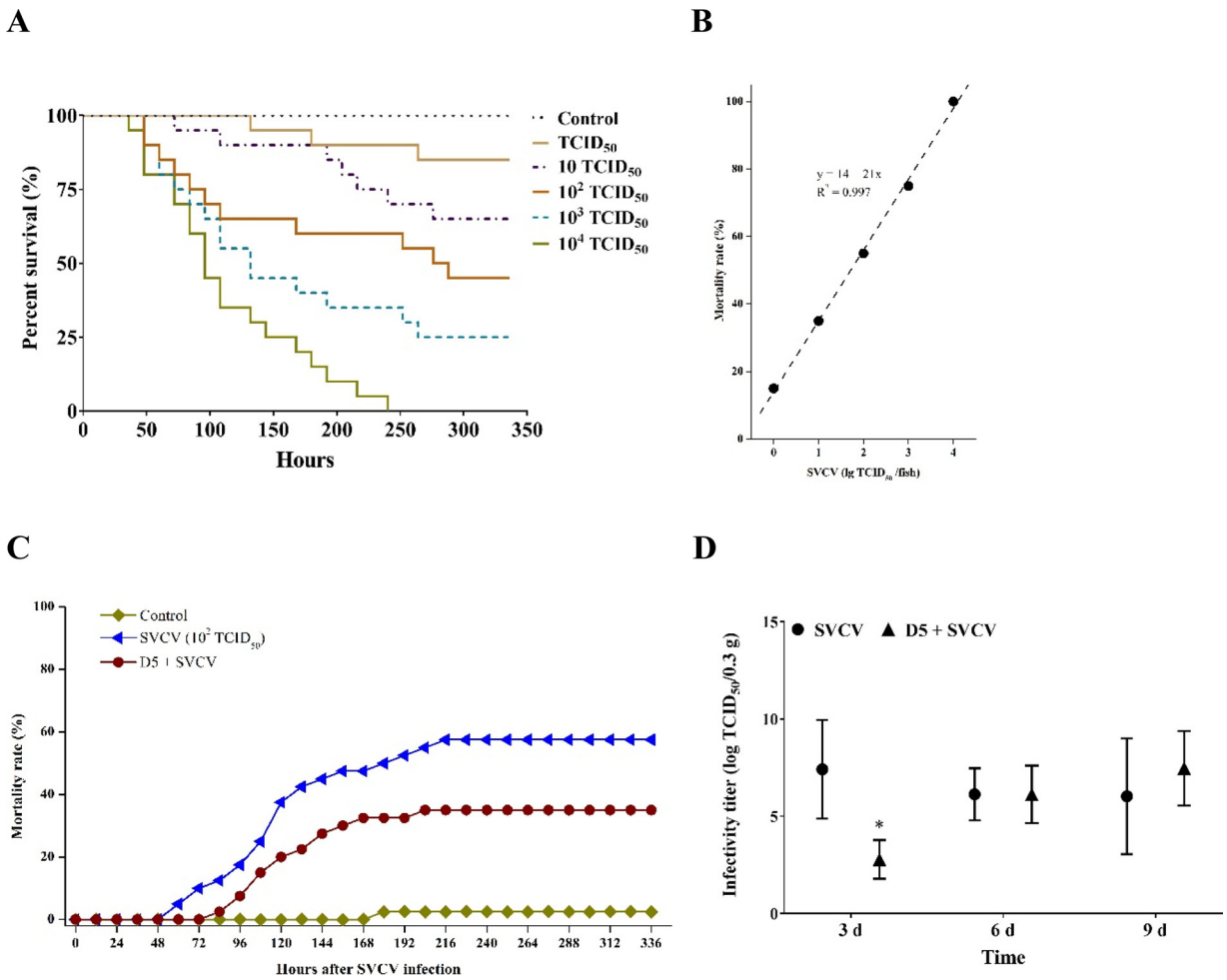


**Fig. 2.** D5 exhibited anti-SVCV infection in zebrafish. (A) Survivorship curves of fish intraperitoneally injected with SVCV at $10^{0-4}$ $TCID_{50}$ $fish^{-1}$. (B) Correlation of fish mortalities and infected SVCV doses. The broken line indicates a regression line for mortality rate and infected SVCV dose. (C) Survivorship curves of fish intraperitoneally injected with $10^2$ $TCID_{50}$ SVCV and 1.0 mg/L D5 $fish^{-1}$. (D) SVCV infectivity titer in zebrafish which reared at 18 °C were infected with SVCV at $10^2$ $TCID_{50}$ $fish^{-1}$ and 1.0 mg/L D5 by the intraperitoneal injection.

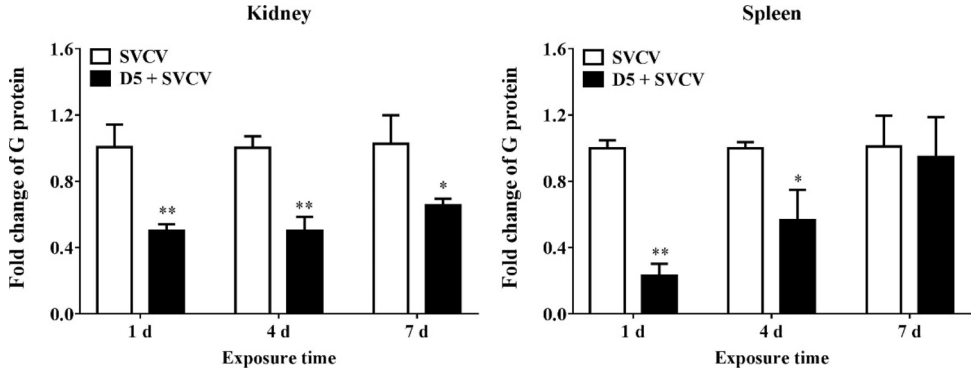


**Fig. 3.** The expression of SVCV G protein in zebrafish kidney and spleen after SVCV or SVCV/D5 injection. Each value was represented as mean ± SD of three fish. The *p* value for each study was determined by Student's *t* tests (two-tailed assuming equal variances). $^{**}p < 0.01$; $^{*}p < 0.05$.

transcribed by using HiScript Q Select RT SuperMix for qPCR (+gDNA wiper) (Vazyme, China). The qRT-PCR was performed with CFX96 Real-Time PCR Detection System (Bio-Rad, USA) by using AceQ® qPCR SYBR® Green Master Mix (Vazyme, China) with the parameters based on the previous study (Shen et al., 2018b). The sequences of primers are listed in Table 1 (Garcíavaltanen et al., 2014; Gotesman et al., 2015; Varela et al., 2014; Cobbina et al., 2015). Relative mRNA expression was calculated using $2^{-\triangle\triangle Ct}$ method with the formula (Livak and Schmittgen, 2001). All qRT-PCR reactions were performed in triplicates.

### 2.8. Antioxidant enzyme activity assays

The total protein from kidney and spleen was extracted by a glass tissue homogenizers under ice-bath cooling. The homogenates were centrifuged at 10,000 g, 4 °C for 15 min and the supernatants were analyzed for superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) activities according to the manufacturer's protocol

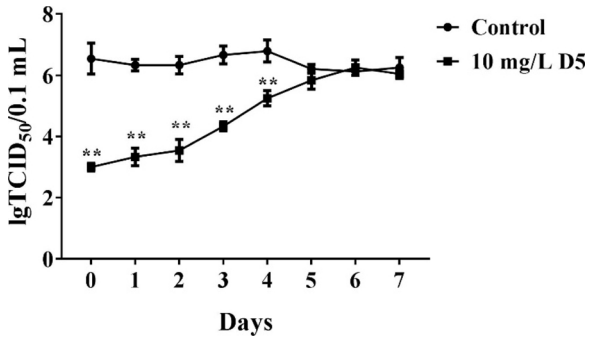


**Fig. 4.** Coumarin D5 is fairly stable in aquatic water at 17 °C. Data represent mean SVCV titers ± SD (n = 3). The *p* value for each study was determined by Student's *t* tests (two-tailed assuming equal variances). $^{**}p < 0.01$, $^{*}p < 0.05$.

(Jiancheng, Nanjing, China). All measurements were performed by using a microplate reader (ELX800, Gene, Hong Kong, China).

### 2.9. Statistical analysis

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. D5 blocked SVCV infection in vitro

As shown in Fig. S1B). Additionally, CCK-8 on cell viability indicated no cytotoxicity and protection of D5 for fish cells after SVCV infection.

### 3.2. D5 blocked SVCV infection in vivo

As shown in Fig. 2, the morality of zebrafish infected with SVCV at $10^{2-4} \times TCID_{50}$ occurred in the 1st to 2nd days, and their final cumulative mortalities were 55%, 75% and 100%, respectively; while the fish infected with SVCV at $10^{0-1} \times TCID_{50}$ began to die ranging from 3rd to 6th day, and the final cumulative mortalities were 15% and 35%, respectively (Fig. 2A). Often we observed the typical clinical symptoms including exophthalmia, haemorrhages on the skin and base of the fins, and abdominal distension in 72 h after SVCV injection. Our results suggested that there existed a closely correlated regression line "$y = 21x + 14$, $R^2 = 0.997$" in a semi-logarithm graph between fish mortalities and virus doses (Fig. 2B). It was calculated that 50% lethal dose ($LD_{50}$) of the viral isolate to zebrafish was approximately $10^{1.7} \times TCID_{50}$ SVCV $fish^{-1}$. The survival rate was greatly improved by zebrafish treated with D5 in which 22.5% reduction (65% survival rate in D5 treatment compared to 42.5% in SVCV treatment) was detected (Fig. 2C). In addition, D5 significantly reduced SVCV replication in zebrafish at the 3rd day; while viral titer of SVCV was the highest on the 3rd day, and we hypothesized that SVCV could rapidly expand, reach peak and remain a plateau in terms of replication, whereas the viral titer in D5 treatment was detected on the 9th day. As shown in Fig. 3, there was also a significant inhibition of SVCV G gene expression in kidney and spleen by D5 treatment at the 1st and 4th days. It should be noted that the expression of SVCV G gene was substantial inhibition at 1 mg/L D5 in all timepoints for kidney, but slightly reduction in spleen at the 7th day.

### 3.3. D5 kept relative stability in aquaculture environments

As shown in Fig. 4, the coumarin D5 was fairly stable in aquatic water at 17 °C in the early stage of virus infection (1–4 days), with virus titer decreasing significantly. Also we observed few amounts of organic material present in sample water. D5 had a calculated inhibitory half-life of 3.3 days ($R^2 = 0.95$) when a nonlinear regression was applied (data not shown).

### 3.4. D5 induced antioxidant protection in zebrafish

As shown in Fig. 5A, SOD (Mn), SOD (Cu/Zn), GPx and CAT expressions were enhanced in kidney and spleen when zebrafish were stimulated with SVCV infection and D5 treatment 1 day later. For those genes with a rapid response, D5 injection enhanced gene expression virus-infected fish over the non-stimulated D5 group. For longer times with the virus, SVCV induced a lower antioxidant genes expression than that in the untreated virus. In contrast, D5 induced a significant up-regulation of gene expressions, implying that D5 maintained the balance of oxidative system in virus-infected zebrafish. As shown in Fig. 5B, we observed a stimulatory effect on SOD/CAT activities and GSH content in zebrafish after D5 administration after 1 day SVCV infection. Despite antioxidant capacity dropping at the 4$^{th}$ day of SVCV infection in some extent, a significant enhancement of antioxidant enzyme activities was found in zebrafish under D5 exposure.

### 3.5. D5 elevated innate immune gene expression

The qRT-PCR was performed to detect the induction of selected genes related to innate immune response in kidney and spleen of zebrafish (Fig. 6). At the 1$^{st}$ day after application of D5, the expressions of IFNγ, IFNφ1, IFNφ2 and RIG-1 genes were significantly up-regulated in D5-treated fish. In kidney, the expressions of four genes were elevated more than 10-fold over compared to the control group, especially the most enhancement of IFNφ1 up to 39.1-fold. Interestingly, the change of immune-related genes in kidney was much greater than that in spleen, similar with the result of SVCV G protein in kidney. These findings replied that SVCV replication may be seriously suppressed by D5 in kidney. After D5 treatment for 4 days, the expressions of IFNγ, IFNφ1, IFNφ2 genes were not statistically significant difference.

## 4. Discussion

Two imidazole coumarin derivatives exhibited anti-SVCV activity in EPC cells, and the structure-activity relationship suggested that methylimidazole or benzimidazole play a key role in antiviral activity (Liu et al., 2017a). Thus, we synthesized coumarin D5 to evaluate its antiviral capacity in EPC cells. The data indicated that D5 not only directly affected the function of SVCV G protein, but also significantly suppressed SVCV-activated autophagy higher phosphorylation levels of Akt-mTOR to possess a better antiviral activity (Chen et al., 2018).

Based on the studies that performed an efficacy comparison between bath infection and intraperitoneal injection (Sanders et al., 2003; Encinas et al., 2013), herein we chose the intraperitoneal injection as the way of SVCV infection. In addition to viral titer reaching to the highest peak ($10^{7.4}$) at the 3$^{rd}$ day, SVCV replication sharply expanded in zebrafish within a shorter period. In a water-based environment, fish mortality rate was usually gradually improved by aquatic viruses because viral spread was exponentially enhanced (Workenhe et al., 2010). It was important to emphasize that D5 reduced SVCV replication in fish body to delay virus infection during the early stage, exhibiting that viral titer was reduced to $10^{2.8}$ at the 3$^{rd}$ day. Thus, D5 as an antiviral agent showed an efficient protection on SVCV-infected zebrafish.

The imbalance of intracellular redox-cycling occurs in cells when the cellular endogenous antioxidant defenses expired and ROS generation was triggered by viruses. Indeed, a major pathogenic

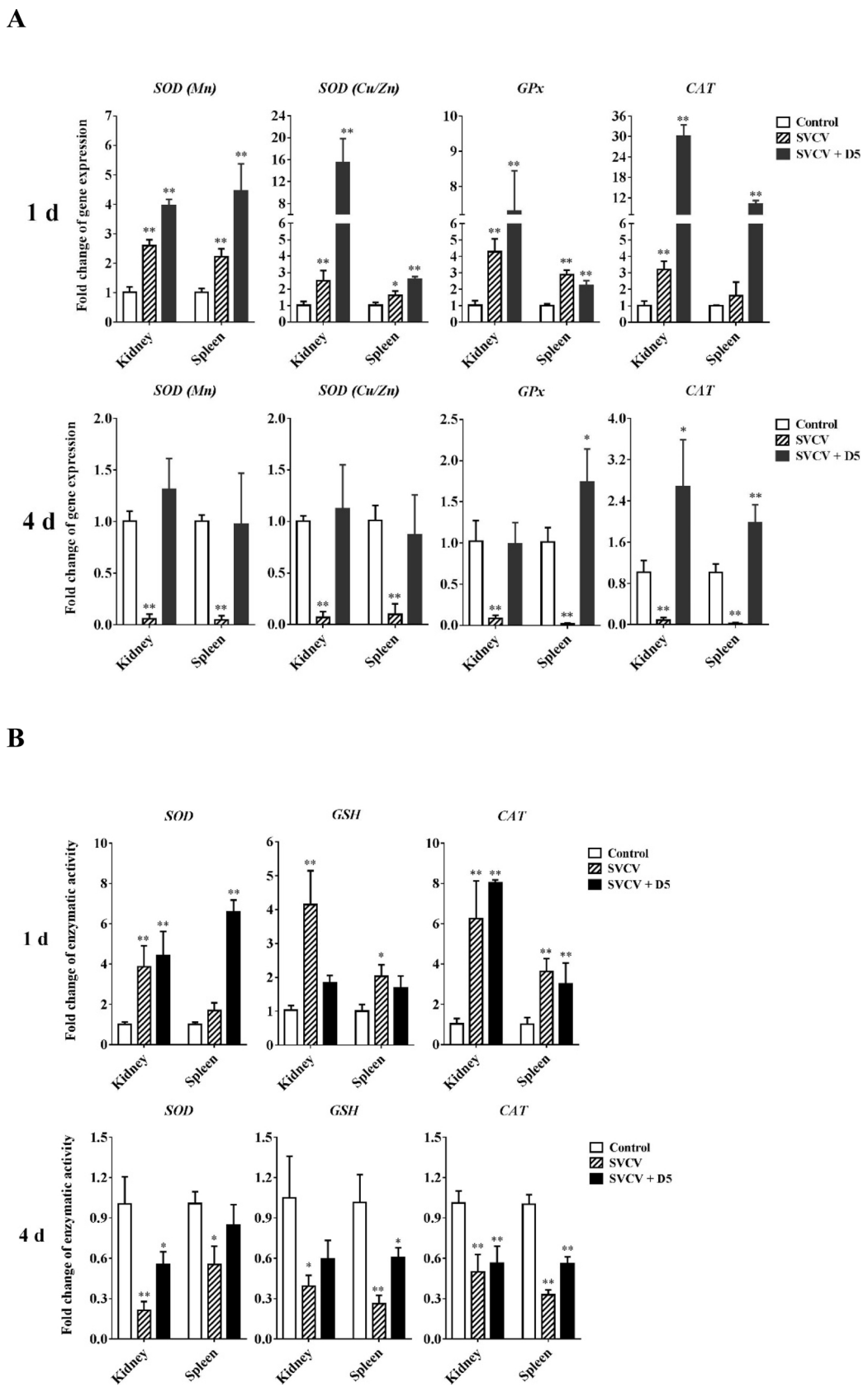


**Fig. 5.** Effect of coumarin D5 on the expression of antioxidant-related genes, enzyme activities and GSH content in SVCV-infected zebrafish. (A) The expression of antioxidant-related genes in kidney and spleen after treatment with SVCV/D5. Each value was represented as mean ± SD of four fish. (B) The change in GSH content and enzyme activities in zebrafish after injection with SVCV/D5. The $p$ value for each study was determined by Student's $t$ tests (two-tailed assuming equal variances). $^{**}p < 0.01$, $^{*}p < 0.05$.

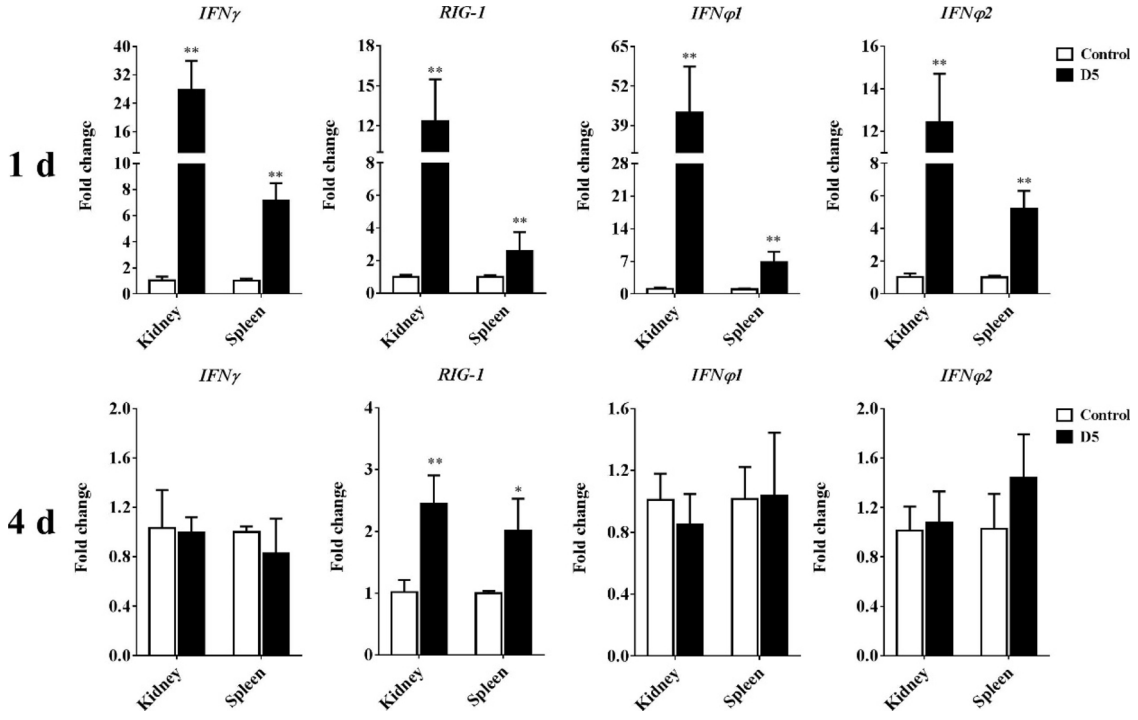


**Fig. 6.** D5 enhanced the expression of IFN-related genes. Zebrafish were injected with 10 μL 1 mg/L D5. After rearing for 1 and 4 d, the expression of IFN-related genes in kidney and spleen were measured. Each value was represented as mean ± SD of four fish. The *p* value for each study was determined by Student's *t* tests (two-tailed assuming equal variances). $^{**}p < 0.01$, $^{*}p < 0.05$.

mechanism that SVCV induced oxidative stress has been found, and is contributed to inflammatory responses and tissue injury in the host body (Yuan et al., 2012, 2014). During the infection period in EPC cells, SVCV disrupted the balance of intracellular redox-cycling, including the changed activities of antioxidant enzymes (SOD, CAT and POD etc.) and the levels of 8−OHdG and protein carbonyls (Liu et al., 2017b; Shao et al., 2016; Yuan et al., 2014). In accordance with some previous studies (Liu et al., 2017b; Shao et al., 2016), we demonstrated that antioxidant system produced multiple responses to virus infection in different periods, exhibiting antioxidant-related genes and enzyme activities activated by SVCV at the 1st day, but suppressed at the 4th day. For these results, we hypothesized that a degree of SVCV multiplication may mount a specific destructive effect on the antioxidant protective response in a time-dependent manner. Generally, hydroxyl coumarins were regarded as antioxidant drugs on the formation and scavenging of ROS, and influenced the processes involving free radical-mediated injury (Peng et al., 2013). In our study, the antioxdant activity showed that D5 treatment not only stimulated fish antioxidant capacity, but also maintained the balance of the redox state in the SVCV-infected fish. Therefore, D5 may prevent fish from virus-induced oxidative damage to enhance the fish survival rate.

Similar to mammals, fish IFNs played an important role in the innate immune system and triggered the expression of massive IFN-stimulated genes (Zou and Secombes, 2011). As the key resistance in the process of pathogenic infection, IFNγ, IFNφ1 and IFNφ2 had been shown to mediate resistance against virus infections in zebrafish (Lópezmuñoz et al., 2009; Sieger et al., 2009). Moreover, other IFN productions in zebrafish embryo induced significant up-regulation of viperin and a strong antiviral activity against infectious hematopoietic necrosis virus (IHNV) (Aggad and Mazel, 2009). Oppositely, RNA viruses depending on structural proteins antagonize the IFN response by different strategies to resist the host defense system (Min and Krug, 2006). The previous study replied that the N protein of SVCV inhibited zebrafish IFNφ1 expression by degrading MAVS (Lu et al., 2016), and the P protein as a substrate of fish TBK1 competed with IRF3 for phosphorylation, leading to the reduction of IRF3 phosphorylation and IFN transcription (Li et al., 2016). *In vivo* examination on the antiviral effect of D5 revealed that an IFN response was triggered in kidney and spleen by activating the transcription of genes. In this study, D5 directly initiated IFN transcription in fish tissues in addition to up-regulation of RIG-1 which preferentially binded to viral RNA and transmitted signaling to induce IFN production (Biacchesi et al., 2009). Therefore, these results suggeted that D5 contributed to positive regulation on SVCV replication and proliferation in zebrafish.

In conclusion, coumarin D5 played a positive protection against SVCV infection and showed an efficient protection on virus-infected zebrafish. These data provided a new insight into the therapeutic strategy of D5 for the devastating fish rhabdovirus infections.

## Acknowledgement

Thanks for Dr Yunfei Dang's help in language modification. This work was supported by the K.C. Wong Magna Fund in Ningbo University and General Scientific Research Project of Education of Zhejiang Province (Y201839187), and the National Natural Science Foundation of China (No. 31772873).

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.01.009.

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