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RESEARCH ARTICLE

α-Lipoic Acid Exerts Its Antiviral Effect against Viral Hemorrhagic Septicemia Virus (VHSV) by Promoting Upregulation of Antiviral Genes and Suppressing VHSV-Induced Oxidative Stress

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Abstract

Viral hemorrhagic septicemia virus (VHSV), belonging to the genus *Novirhabdovirus*, *Rhabdoviridae* family, is a causative agent of high mortality in fish and has caused significant losses to the aquaculture industry. Currently, no effective vaccines, Food and Drug Administration-approved inhibitors, or other therapeutic intervention options are available against VHSV. α-Lipoic Acid (LA), a potent antioxidant, has been proposed to have antiviral effects against different viruses. In this study, LA (CC_{50} = 472.6 μmol/L) was repurposed to exhibit antiviral activity against VHSV. In fathead minnow cells, LA significantly increased the cell viability post-VHSV infection (EC_{50} = 42.7 μmol/L), and exerted a dose-dependent inhibitory effect on VHSV induced-plaque, cytopathic effects, and VHSV glycoprotein expression. The time-of-addition assay suggested that the antiviral activity of LA occurred at viral replication stage. Survival assay revealed that LA could significantly upregulated the survival rate of VHSV-infected largemouth bass in both co-injection (38.095% vs. 1.887%, $P < 0.01$) and post-injection manner (38.813% vs. 8.696%, $P < 0.01$) compared with the control group. Additional comparative transcriptome and qRT-PCR analysis revealed LA treatment upregulated the expression of several antiviral genes, such as *IRF7*, *Viperin*, and *ISG15*. Moreover, LA treatment reduced VHSV-induced reactive oxygen species production in addition to *Nrf2* and *SOD1* expression. Taken together, these data demonstrated that LA suppressed VHSV replication by inducing antiviral genes expression and reducing VHSV-induced oxidative stress. These results suggest a new direction in the development of potential antiviral candidate drugs against VHSV infection.

Keywords Viral hemorrhagic septicemia virus (VHSV) · α-Lipoic acid (LA) · Antiviral · IFN response · Oxidative stress

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Introduction

Viral hemorrhagic septicemia (VHS) is one of the most economically fish infectious diseases worldwide and is characterized by high morbidity and mortality. VHS has been reported to cause severe hemorrhages in fish eyes, mouth, fin and live tissues in a wide range of sea or fresh water cultured fishes, including rainbow trout (Hørlyck *et al*. 1984), turbot (Ross *et al*. 1995), and largemouth bass (Zhang *et al*. 2019). The aetiologic agent of VHS is viral hemorrhagic septicemia virus (VHSV), which is a negative-sense, single-stranded RNA enveloped virus, belonging to the genus *Novirhabdovirus*, *Rhabdoviridae* family (Schütze *et al*. 1999). The genome of VHSV contains six genes, encoding nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), nonvirion protein (Nv), and polymerase (L). According to the OIE, VHSV

has been listed as a reportable fish virus (http://www.oie.int/index.php?xml:id=171&L=1&htmfile=chapitre_diseases_listed.htm), and no effective strategies against VHSV have yet to be developed.

Antiviral drugs and vaccines are two general strategies to combat viral infection. At present, various types of vaccines, such as whole-virus inactivated vaccines, attenuated vaccines, recombinant G protein-based vaccines, and DNA vaccines, have been developed to prevent VHSV dissemination in laboratory tests, but they have not been used commercially for several reasons, including their safety, production costs, and mass production difficulty (Pereiro *et al.* 2016; Gomez-Casado *et al.* 2011). To overcome these drawbacks, antiviral drugs are highly desired in combating the threat of VHSV. Currently, some compounds have been demonstrated to have anti-VHSV activities in vitro or in vivo, such as EICAR (Moya *et al.* 2000), ribavirin (Marroqui *et al.* 2007), LJ001 (Balmer *et al.* 2018) and VER-155008 (Pham *et al.* 2019). Furthermore, many bioactive compounds showed various degrees of antiviral efficacy to VHSV, such as flavonoids (Kang *et al.* 2012), curcumin (Jeong *et al.* 2015), extracts of Celosia cristata, Raphanus sativus roots (Park *et al.* 2017) and algal Ecklonia cava (Yang *et al.* 2018).

α-Lipoic acid (LA) is a disulfide-containing compound and acts as a cofactor for several enzymes participating in dehydrogenation and decarboxylation. As an essential biological antioxidant, LA has been reported to be involved in reactive oxygen species (ROS) scavenging, antioxidant recycling and metal chelating (Reed 1974; Hiller *et al.* 2016; Tibullo *et al.* 2017). For example, LA improves embryo development and protects against oxidative stress (Linck *et al.* 2007). Besides, LA also possesses many other biochemical functions, such as anti-inflammatory (Zhang and Frei 2001), anti-obesity (Miao *et al.* 2013), anticancer (Novotny *et al.* 2008), and antiproliferative effects (Selvakumar and Hsieh 2008). Due to its outstanding antioxidant capacity, LA has been used as an aquatic feed additive to enhance the detoxification and antioxidant capacity, thereby protecting the organism against oxidative stress in corydoras paleatus (Monserrat *et al.* 2008), pompano (Kütter *et al.* 2012), and pacu (Park *et al.* 2006).

Drug repositioning, also known as drug repurposing, is the process of assigning new indications for commercially available drugs other than the one(s) that they are originally intended (Pushpakom *et al.* 2019). Compared to traditional drug development, drug repurposing possesses multiple advantages, such as good safety and well-known pharmacokinetic and pharmacodynamics properties, which reduce the risk of failure to an extreme (Bloom 2015). Thus, drug repurposing has been considered to be a practical and economical approach for drug development since emerging in the early 1990s (Kesselheim *et al.* 2015; Hernandez *et al.* 2017; Novac 2013). Especially, drug repurposing played an important role in combating rapidly spreading viral infectious diseases. Up to now, several clinically approved drugs have been proved to be potential broad-spectrum antivirals through drug repurposing. For example, rilpivirine, a clinically approved human immunodeficiency virus (HIV) drug, was identified as a Zika virus (ZIKV) inhibitor by targeting ZIKV RdRp (Sariyer *et al.* 2019). Chlorpromazine hydrochloride, a widely used antipsychotic, was found to be effective at inhibiting either Middle East respiratory syndrome coronavirus or severe acute respiratory syndrome coronavirus (SARS-CoV) infection (Dyall *et al.* 2014).

To identify potential compounds with anti-VHSV activity, in this study, a library of 50 kinds of Food and Drug Administration (FDA) approved drugs, having antiviral activity against a series of other viruses, was screened for antiviral activity against VHSV. We found that LA was effective at inhibiting VHSV infection. Furthermore, we characterized the mechanism by which LA affected VHSV infection. Our results showed that LA had the potential to be developed as an effective anti-VHSV drug.

Materials and Methods

Cells, Compounds and Virus

Fathead minnow (FHM) cells were grown in Medium 199 (M199, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA). The cell culture was maintained in an incubator at 28 °C without CO_2 supplement.

An antiviral compound library consisted of 50 antiviral compounds (Supplementary Table S1), including LA (Cat #HY-N0492), was purchased from Med Chem Express (MCE, USA). Each compound was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and stored at − 80 °C. The stock solution was further diluted with M199 (FBS free), with the final concentration of DMSO less than 0.1%. Elesclomol, an oxidative stress inducer, was purchased from MCE (Cat #HY-12040) and dissolved in DMSO.

VHSV-LB2018 (IVa) was isolated from a wild largemouth bass in the Pearl River and kept in our laboratory (Zhang *et al.* 2019). VHSV was propagated in FHM cells at 28 °C. The virus titers, detected by 50% tissue culture infective dose ($TCID_{50}$), reached $10^{7.5}$ $TCID_{50}$/mL.

Antiviral Activity Screening

FHM cells were seeded in 96-well plates, at a density of 2×10^4 cells per well 24 h prior to the experiment. Firstly, the cytotoxicity of compounds was tested in FHM cells. Cells were incubated with serial diluted compounds or DMSO at 28 °C for 48 h. The maximum safe dosage, which did not cause cellular damage, was chosen for further screening. Then, VHSV and compounds were inoculated to FHM monolayers simultaneously in triplicate wells. After 48 h, the antiviral activity was evaluated by cytopathic effect (CPE).

Cell Viability Assay

Cell viability assay was performed to determine 50% cytotoxicity (CC_{50}) of LA and the median effective concentration (EC_{50}) of LA against VHSV by using Cell Counting Kit-8 reagent (CCK-8, MCE, Cat #HY-K0301). For CC_{50} calculation, FHM cells were seeded in 96-well plates and incubated with LA at concentrations of 10, 20, 30, 50, 100, 200, 400 or 800 μmol/L. After incubation for 48 h at 28 °C, the cells were washed with PBS for 3 times, then incubated with the fresh M199 (2% FBS) diluted CCK-8 solution (10 μL) for 2 h. The experiment was conducted three times in quadruplicate (4 wells in 96 wells) for each concentration of the compound. Cell viability was measured through the absorbance at 450 nm and calculated out using [Inhibitor] vs. response -Variable slope (four parameters) algorithms in GraphPad Prism 8.0.2.

For the cell protection assay, FHM monolayers were inoculated with M199 (2% FBS) containing VHSV and predetermined concentration of compounds or DMSO for 48 h. The experiment was conducted three times in quadruplicate. The EC_{50} was calculated out using [Agonist] vs. response-Variable slope (four parameters) algorithms in GraphPad Prism 8.0.2.

Cell Plaque Assay

FHM cells were seeded into 24-well plates and cultured at 28 °C to obtain 90% confluence. Then the growth media was replaced with fresh M199 (2% FBS) and treated with VHSV and different concentrations of LA for 36 h. Finally, the cells were fixed and stained using 0.1% crystal violet solution, the plaque induced by VHSV was observed using an inverted microscope.

Antiviral Activity Assay

A time-of-addition experiment was performed by treating FHM cells with VHSV ($10^{1.5}$ $TCID_{50}$) and LA (50 μmol/L) in different manners. Ribavirin (8 μmol/L, MCE, Cat #ICN-1229) was used as the positive control, DMSO was the negative control. In the co-exposure experiment, compound and VHSV were both incubated with cells. In the pre-exposure experiment, the cells were pretreated with compound for 4 h followed by VHSV infection. In the post-exposure experiment, the compound was added at 4 h post-infection (hpi). The above incubations were carried out at 28 °C. Cells were collected at 24 and 36 hpi for qRT-PCR analysis.

RNA Extraction and qRT-PCR Analysis

Total RNA was extracted using Trizol reagents (Invitrogen, Carlsbad, CA, USA), and reverse-transcribed into first-strand cDNA using a reverse transcription Mix (Promega, Madison, WI, USA). Viral gene expression level in FHM cells was determined by qRT-PCR targeting a partial region of VHSV *G* or *N* gene. Primers are presented in Supplementary Table S2. qRT-PCR amplification was carried out using the LightCycler 480 II (Roche, Mannheim, Germany) with cycling conditions as follows: 95 °C for 30 s, 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s. A melting curve analysis was conducted to verify the specificity of the amplified product. All experiments were conducted at least three times. Data from qRT-PCR were analyzed using $2^{-\Delta\Delta Ct}$ method and shown as mean ± standard deviation.

Virus Binding Assay

FHM cells in 12-well plates were incubated with M199 containing VHSV ($10^{1.5}$ $TCID_{50}$) in the presence of LA (50 μmol/L), ribavirin (8 μmol/L) or DMSO at 4 °C for 2 h to complete virus binding but not internalization. Unbound virus particles were removed by washing cells with PBS three times and renewing M199. The cells were cultured at 28 °C for another 22 h, the viral gene expression in cells was analyzed by qRT-PCR subsequently.

Virus Internalization Assay

FHM cells were incubated with M199 containing VHSV at 4 °C for 2 h. After removing the unbound virus by three times of washing with PBS, the cells were incubated in fresh M199 with LA, ribavirin or DMSO at 28 °C for 2 h to facilitate virus internalization. Then the cells were washed with PBS to remove the compounds and further cultured at 28 °C for another 20 h. The samples were analyzed by qRT-PCR subsequently.

Viral Gene Replication Assay

FHM cells were infected with VHSV at 4 °C for 2 h. After washing with PBS three times, the cells were replenished with fresh M199. After 2 h culture, the cells were incubated with LA, ribavirin or DMSO for 4 h, followed by collection for qRT-PCR analysis.

Survival Assay

Largemouth bass (*Micropterus salmoides*), approximately 5 cm in body length, was purchased from a fish farm in Zhuhai city, Guangdong, China. Fish was acclimated for 7 days before the experiment.

The survival rate was calculated in healthy largemouth bass. Largemouth bass was divided into five groups: the PBS negative control, VHSV co-exposed with DMSO, VHSV co-exposed with LA (25 μmol/L), VHSV post-exposed with DMSO at 4 hpi, and VHSV post-exposed with LA (25 μmol/L) at 4 hpi (n = 50). Each largemouth bass was injected with 20 μL of VHSV ($10^{1.5}$ $TCID_{50}$) with intraperitoneal injection, separately. The survival rate of each group was recorded every half-day by counting the numbers of largemouth bass. The differences between groups were analyzed with the log-rank test method using GraphPad Prism 8.0.2.

Transcriptome Analysis

FHM cells were grown in 25 cm^2 dishes. LA or DMSO was inoculated into FHM monolayers with VHSV ($10^{2.8}$$TCID_{50}$) for 24 h at 28 °C. Total RNAs of FHM cells were extracted and determined using NanoDrop 2000 UV–Vis Spectrophotometer for RNA-sequencing and transcriptome analysis by GENE DENOVO corporation.

Measurement of Intracellular ROS Production

The intracellular ROS level was measured using a Reactive Oxygen Species Assay Kit (Beyotime Biotechnology, Guangzhou, China). Briefly, FHM cells in 12-well plates were treated with VHSV ($10^{1.5}$ $TCID_{50}$) or PBS (negative control). The mock group was untreated, the positive control group was treated with Rosup according to the manufacturer's instructions. Following the treatment, cells were washed with PBS and incubated with 10 μmol/L oxidation-sensitive fluorescent probe (DCFH-DA) at 37 °C for 30 min in dark to detect the intracellular ROS production. The green fluorescence was observed using fluorescence microscopy (Olympus, Hamburg, Germany) under the following band-pass filters: 488-nm excitation and 515-nm long-pass filter.

To detect the effect of LA on VHSV-induced ROS production, FHM cells were infected with VHSV and treated with DMSO or LA (50 μmol/L) in pre-, co- or post-exposure manner for 24 h. The intracellular ROS production was detected as above.

Data Analysis

Statistical significance was determined by one-way ANOVA algorithm using Graphpad Prism. $P < 0.05$ was considered to be statistically significant.

Results

Initial Antiviral Screening of 50 FDA-Approved Drugs against VHSV

To identify potential antiviral compounds against VHSV, the anti-VHSV activity of 50 FDA-approved drugs was evaluated at a fixed concentration of 30 μmol/L using CPE-based screening assay in FHM cells. Ribavirin, a previously identified antiviral compound, was used as a positive control (Kamiyama *et al.* 2017). As shown in Fig. 1, LA showed strong inhibitory activity against VHSV-induced CPE at the concentration of 30 μmol/L without significant cytotoxicity (CC_{50} values of 472.6 μmol/L for LA) (Fig. 1). These results indicated that LA had a potential anti-VHSV activity, thus was selected for the following studies.

Antiviral Efficacy of LA against VHSV

To further confirm and evaluate the antiviral activity of LA, the cell viability, virus-induced plaque, CPEs and expression level of viral genes were assessed. First, the EC_{50} values for LA were inferred by calculating the percentage of virus-infected cells. As shown in Fig. 2A, LA dose-dependently increased the cell viability of VHSV infected FHM cells, resulting in an EC_{50} value of 42.7 μmol/L. Moreover, crystal violet staining indicated VHSV-induced plaques were significantly decreased at 36 h post LA treatment in a dose-dependent manner at concentrations ranging from 30 to 50 μmol/L (Fig. 2B). These results suggested that LA was a potential inhibitor of VHSV *in vitro*.

In parallel to the inhibition efficiency, the anti-VHSV activity of LA was assessed in more detail through a time-of-drug-addition experiment. LA was added to FHM cells at 4 h before infection (pre-exposure), following the virus inoculation (co-exposure) or 4 h after infection (post-exposure), respectively (Fig. 2C). As shown in Fig. 2D, typical severe CPEs, consisting of rounder clusters, were

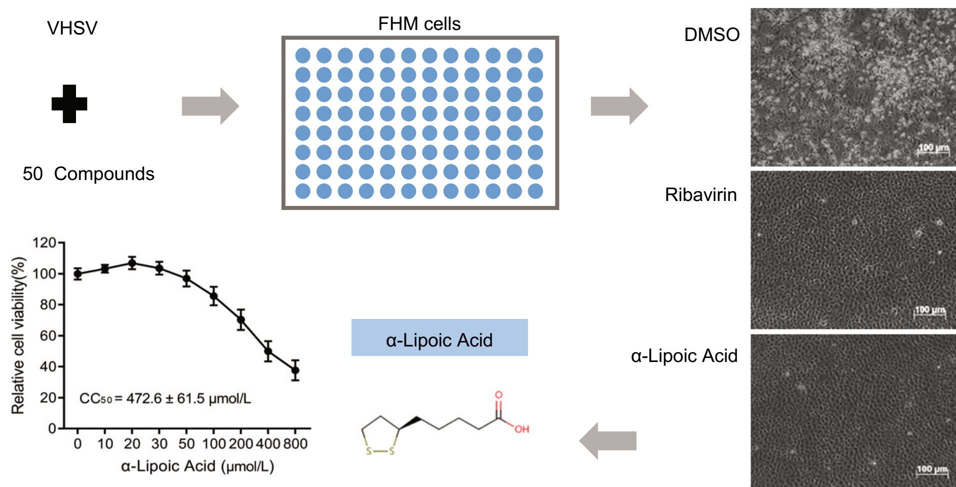

Fig. 1 Compound screening model and cytotoxicity of α-Lipoic Acid (LA) on FHM cells. The cytotoxicity of LA was examined in FHM cells using Cell Counting Kit-8 (CCK-8). The median cytotoxic concentration (CC_{50}) was calculated in GraphPad Prism 8.0.2.

observed in DMSO-treated cells under all experimental conditions. Notably, LA significantly reduced the CPEs induced by VHSV infection at 36 hpi. Moreover, the antiviral activity of LA was further verified by detecting the expression of VHSV *G* mRNA. As shown in Fig. 2E, LA significantly inhibited the increase of *G* mRNA levels in comparison with DMSO treated and VHSV infected FHM cells in pre-exposure, co-exposure and post-exposure assays, and LA showed an antiviral effect similar to the known antiviral drug ribavirin. Collectively, these results confirmed LA could reduce the infectivity of VHSV at different treatment times.

LA Inhibits Viral Replication but Not Entry

To determine which step of the VHSV infection cycle was affected by LA, we explored the antiviral effect of LA on virus attachment, internalization, and gene replication stages by a time-of-drug-addition assay. FHM cells were infected with VHSV with the presence of LA at indicated times, respectively (Fig. 3A). As shown in Fig. 3B and 3C, the attachment and internalization of VHSV were not affected by LA treatment. LA significantly decreased the mRNA level of VHSV *G* gene in FHM cells treated with LA at 4 hpi, indicating the replication of VHSV was inhibited by LA treatment (Fig. 3D). Moreover, the survival rate of largemouth bass co-injected with LA and VHSV was significantly higher than that of the DMSO and VHSV injected group (38.095% vs. 1.887%, $P < 0.01$) (Fig. 3E). In addition, the survival percent in largemouth bass injected with VHSV for 4 h prior to injection of LA showed a highly significant enhancement in comparison with that of the control group (38.813% vs. 8.696%, $P < 0.01$) (Fig. 3F).

LA Treatment Induces Upregulation of Antiviral Genes

To dissect the biological mechanism of LA-mediated VHSV inhibition, we examined the effect of LA on cell immune response through RNA-sequencing assay on DMSO and LA-treated FHM cells with VHSV infection (NCBI Sequence Read Archive under BioProject number: PRJNA715306). Comparative transcriptome analysis showed that 2262 up-regulated genes and 3257 down-regulated genes were identified in LA-treated cells compared with DMSO treatment. GO enrichment assigned 2994 differentially expressed genes (DEGs) to biological process (BP), 1574 to cellular component (CC) and 951 to molecular function (MF). For BP, 340 unigenes belonging to the cellular process were up-regulated in LA-treatment group. For CC, the most up-regulated genes were assigned to cell and cell part with 224 unigenes. For MF, 272 unigenes associated with binding were highly up-regulated (Fig. 4A). Among which, a majority of DEGs involved in antiviral response were significantly up-regulated, such as *PKR*, *Trim25*, *IFI27 2a/b*, *IRF2/3/5/7*, *Mx2*, *IFN1*, *ISG15*, *Viperin*, *Mxra7*, *DDX21/46*, *TRIM8*, *STAT1/2*, *TBK1*, *TRAF2/3/4*, *TLR18*, *JAK2* and so on (Fig. 4B).

Furthermore, the transcriptional levels of *IFN1*, *IRF3*, *IRF7*, *ISG15*, *Viperin* and *PKR* were analyzed by qRT-PCR. As shown in Fig. 4C–4H, *IRF3*, *IRF7*, *Viperin*, *ISG15*, *IFN1* and *PKR* exhibited 2.5-, 22-, 47-, 21-, 2.3- and 1.5-fold increases under LA treatment compared with DMSO control. Meanwhile, post LA and VHSV co-stimulation, those genes also exhibited 1.5-, 6-, 18-, 16-, 1.7- and 1.4-fold increases compared with the control group, which was almost in accordance with the high throughput sequencing data. These results indicated that the anti-VHSV effect of LA was partial dependent of the activation of antiviral genes.

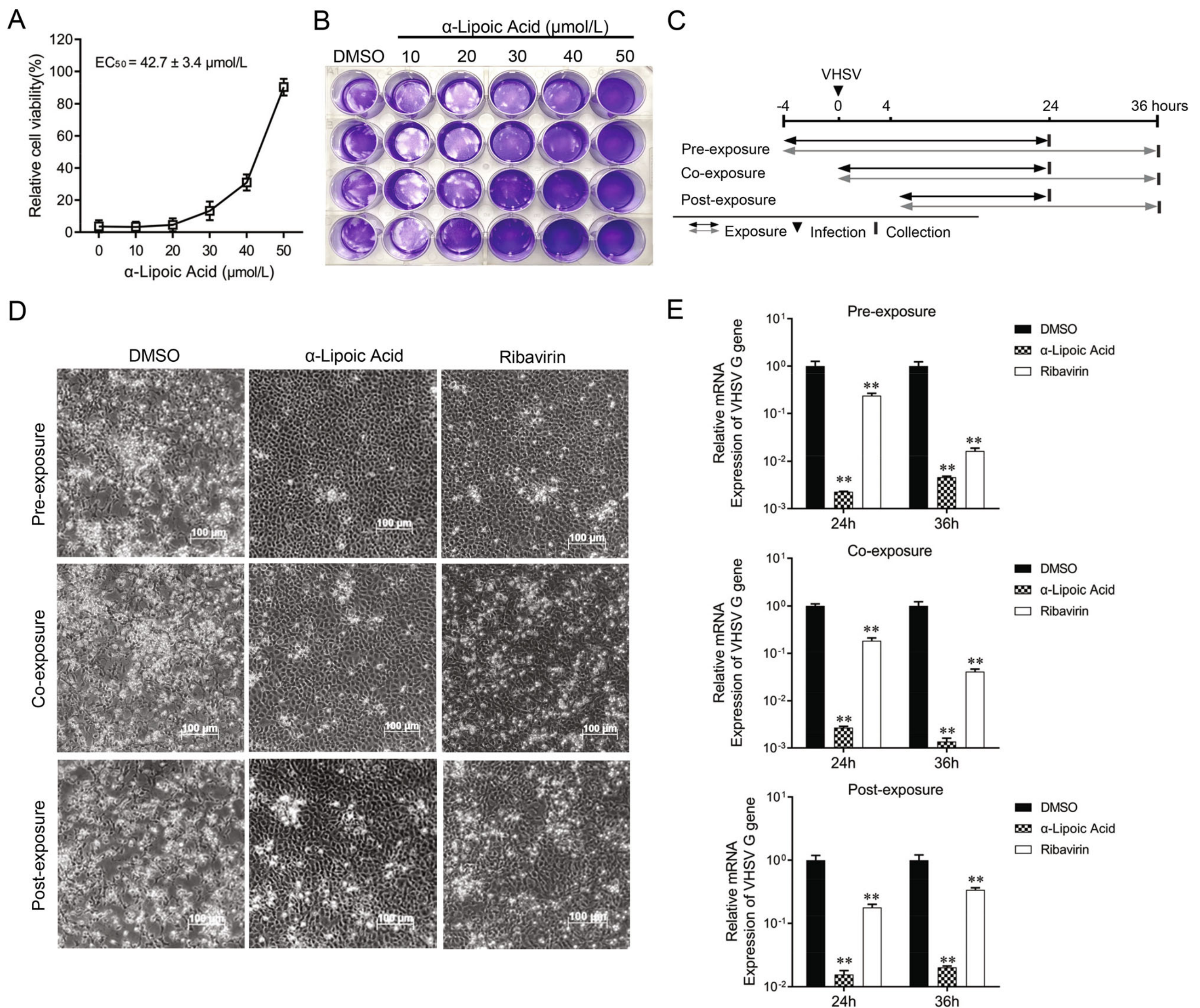

Fig. 2 The antiviral activity of LA against VHSV. **A** The median effective concentration (EC_{50}) of LA was measured using CCK-8 under VHSV infection and LA treatment at 48 hpi, and calculated using GraphPad Prism 8.0.2. **B** The plaque staining of VHSV infected cells was conducted under LA treatment at 36 hpi with the concentration of 10, 20, 30, 40 and 50 μmol/L. DMSO treated cells were used as the negative control. **C** LA exposure schemes in time-of-addition experiment. FHM cells were infected with $10^{1.5}$ $TCID_{50}$ VHSV and treated with LA at indicated time-points, then the cells were harvested at 24 hpi or 36 hpi for qRT-PCR analysis. Horizontal arrows represent the compound treatment period, triangles represent the addition of VHSV at 0 h, vertical bars represent the end of treatments and cell collection. **D** The cytopathic effect (CPE) of FHM cells under DMSO, LA or ribavirin treatment at 36 h post VHSV infection. **E** The relative mRNA expression of VHSV *G* gene in VHSV-infected FHM cells under DMSO, LA or ribavirin treatment at 24 and 36 hpi was analyzed by qRT-PCR and normalized to the level of the *β-actin* housekeeping gene. $^{*}P < 0.05$; $^{**}P < 0.01$.

LA Treatment Reduces VHSV-Induced Oxidative Stress

Oxidative stress plays a pivotal role in the pathogenesis of viral diseases. Multiple viruses triggered oxidative stress in infected cells to cause cell death. LA is a potent antioxidant and can reduce oxidative stress. We speculated that LA might enhance antioxidant levels in host cells to exert its antiviral effect. Firstly, we investigated whether VHSV infection could induce host oxidative stress in FHM cells. As shown in Fig. 5A, in VHSV-infected cells at 24 hpi, the ROS, characterized by the green luciferase activity, appeared with a higher percentage than that in the negative control group, and the gene expression level of *Nrf2* and *SOD1* had a great upregulation (Fig. 5E and 5F), suggesting VHSV infection could induce oxidative stress. Thus, we examined the effect of oxidative stress on VHSV infection. As shown in Fig. 5C and 5D, ROS production induced by Elesclomol, an oxidative stress inducer, potentiated the replication of VHSV significantly. Furthermore, the effect of LA on VHSV-induced oxidative stress was investigated and found pre-, co-, or post-

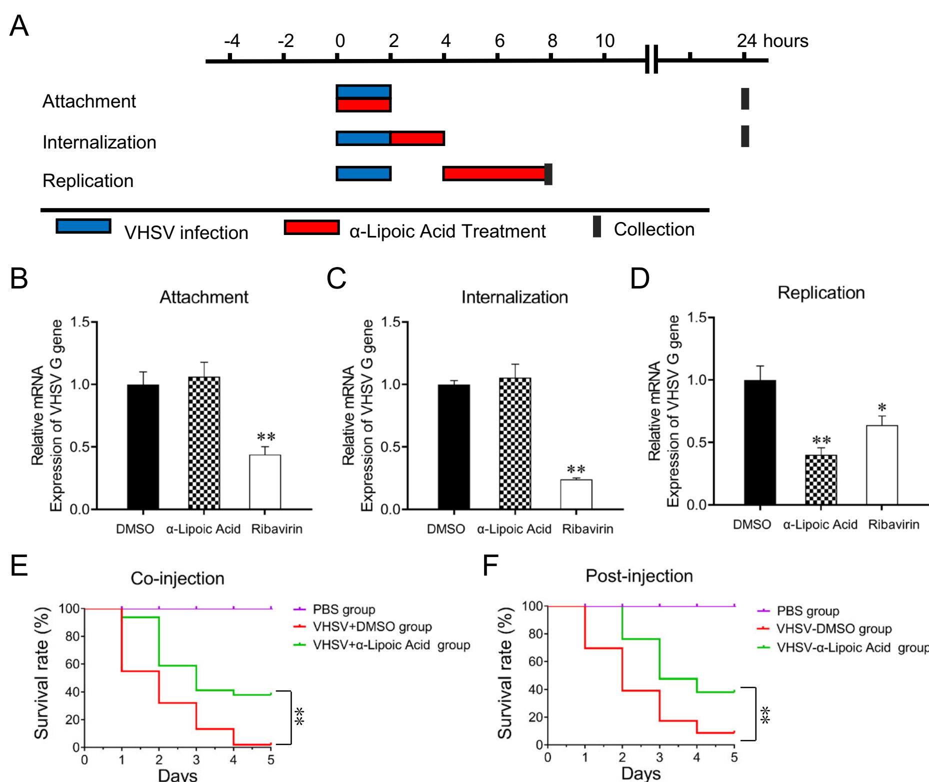

Fig. 3 Effect of LA treatment on VHSV infection cycle. **A** LA treatment schemes. Blue bars represent VHSV infection, red bars represent LA treatment, vertical bars represent the cell collection. **B–D** FHM cells were infected with VHSV and treated with LA at indicated time-points (**A**), which represented the stage of viral attachment (**B**), internalization (**C**) or replication (**D**), respectively. The relative mRNA expression of VHSV *G* gene was subsequently analyzed by qRT-PCR and normalized to the level of the *β-actin* housekeeping gene. **E–F** Survival rate of VHSV-infected largemouth bass with LA (25 μmol/L) treatment was conducted through intraperitoneal injection, LA or DMSO was co-injected with 20 μL of VHSV ($10^{1.5}$ $TCID_{50}$) into largemouth bass (**E**) or injected post VHSV injection at 4 hpi (**F**), respectively. The largemouth bass injected with the same volume of PBS was used as the negative control. The survival rate of each group was calculated and the differences between the two groups were analyzed with log-rank test using the software of GraphPad Prism 8.0.2. $^{*}P < 0.05$; $^{**}P < 0.01$.

exposure of LA inhibited VHSV-induced ROS production apparently (Fig. 5B) and suppressed the upregulation of *Nrf2* and *SOD1* at mRNA levels (Fig. 5E and 5F). These results revealed that LA might inhibit VHSV infection by functioning as an antioxidant to reduce VHSV-induced oxidative stress.

Discussion

Drug repurposing, also referred to as drug repositioning, reinvestigate the drugs for a new application against other diseases, has generated substantial attention in recent years (Trivedi and Mohan 2020). The drugs being investigated have already been deemed with eliminated toxicity, so that drug repurposing drastically reduces the failure rate compared with designing novel drugs (Pushpakom *et al*. 2019). Here, 50 compounds were screened for inhibiting properties against VHSV in FHM cells, among which, LA stood out as a novel small molecular inhibitor with low cytotoxicity (CC_{50} = 472.6 μmol/L) and potent anti-VHSV activity (EC_{50} = 42.7 μmol/L).

LA, acting as a cellular coenzyme, was routinely used to treat diabetic polyneuropathy and hepatic disorders (Sachse and Willms 1980). Apart from that, LA has been found to exhibit antiviral effects against some viruses. Schmidt *et al*. found LA supplementation promoted the immune response of steers to have a more rapid recovery from IBRV challenge than others (Schmidt *et al*. 2006). Addition of LA attenuated the susceptibility of human cells to HCoV 229E infection (Wu *et al.* 2008). Herein, LA showed a dose-dependent inhibition on VHSV-induced plaque, CPEs and viral gene expression in FHM cells, indicating LA had a protective effect against VHSV infection and could be repurposed as an antiviral drug. Furthermore, we tried to identify which step in VHSV growth cycle was inhibited by LA. Our results showed the addition of LA significantly decreased virus replication. The inhibitory effect of LA on the replication was also found for VACV in several cell lines, since LA significantly reduced the expression of

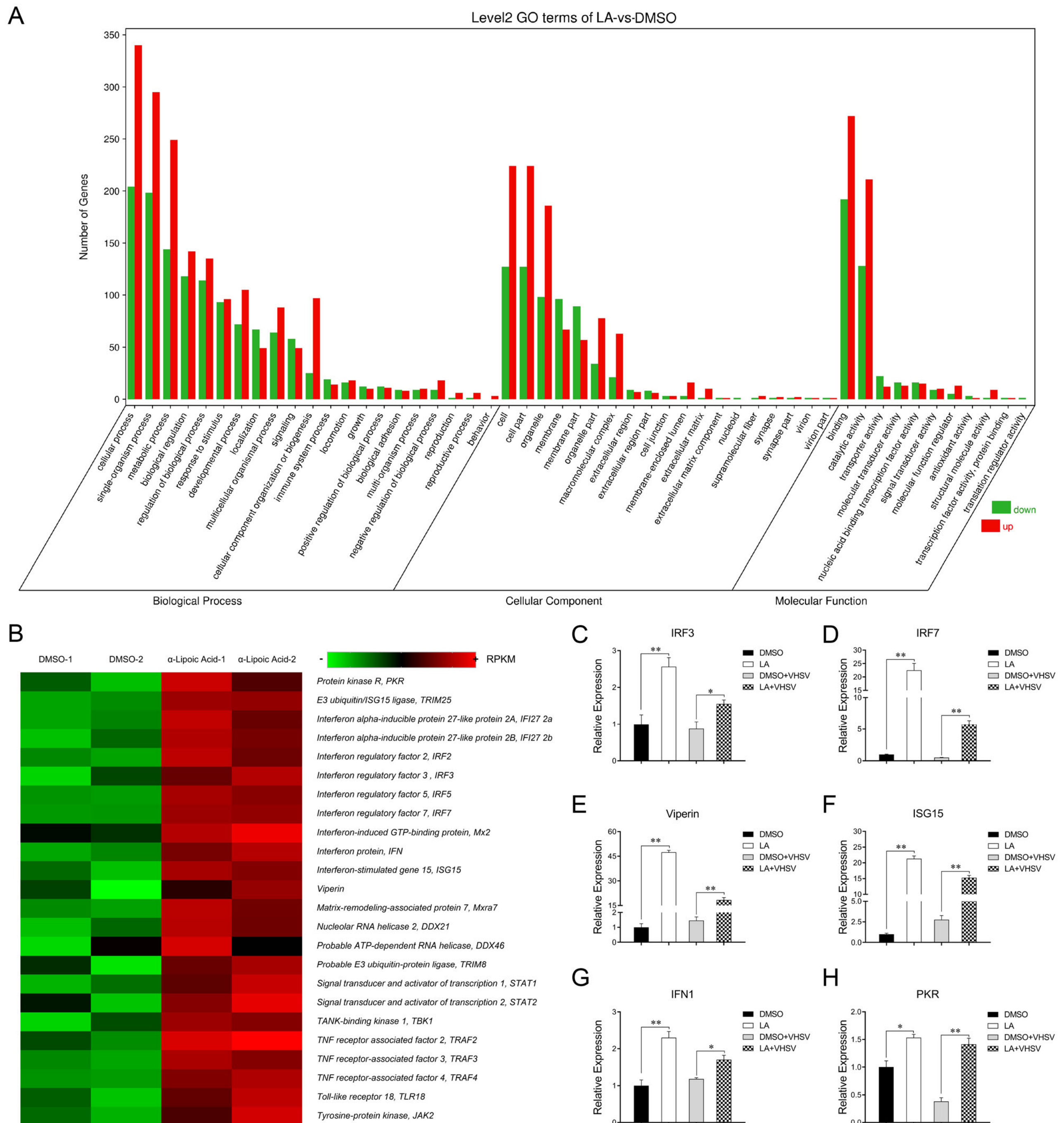

Fig. 4 Effect of LA treatment on antiviral genes expression in FHM cells. **A** Gene Ontology function classification of differentially expressed genes (DEGs) in transcriptomic analysis on DMSO or LA treated FHM cells with VHSV infection. **B** The heatmap showed some upregulated antiviral genes in LA treated FHM cells compared with the DMSO treated group, according to the fold change value of the robust rank aggregation analysis. Red indicates that DEGs are upregulated in LA samples compared with the DMSO samples; green indicates the opposite. **C–H** FHM cells were treated with DMSO or LA with or without VHSV infection, respectively. The expression level of antiviral genes, including *IRF3* (**C**), *IRF7* (**D**), *Viperin* (**E**), *ISG15* (**F**), *IFN1* (**G**) and *PKR* (**H**), was analyzed by qRT-PCR and normalized to the level of the *β-actin* housekeeping gene. $^{*}P < 0.05$; $^{**}P < 0.01$.

VACV late genes but did not inhibit VACV entry or early VACV DNA synthesis (Spisakova *et al.* 2009). Baur *et al.* found the replication of HIV in T-cell lines was inhibited by LA treatment post HIV-infection acutely or chronically (Baur *et al.* 1991). In this study, LA did not affect VHSV *G* gene expression at the entry stage of VHSV, suggesting the inhibition of LA was irrelevant with virus entry. While, as for COVID-19, LA interfered with the entry of SARS-

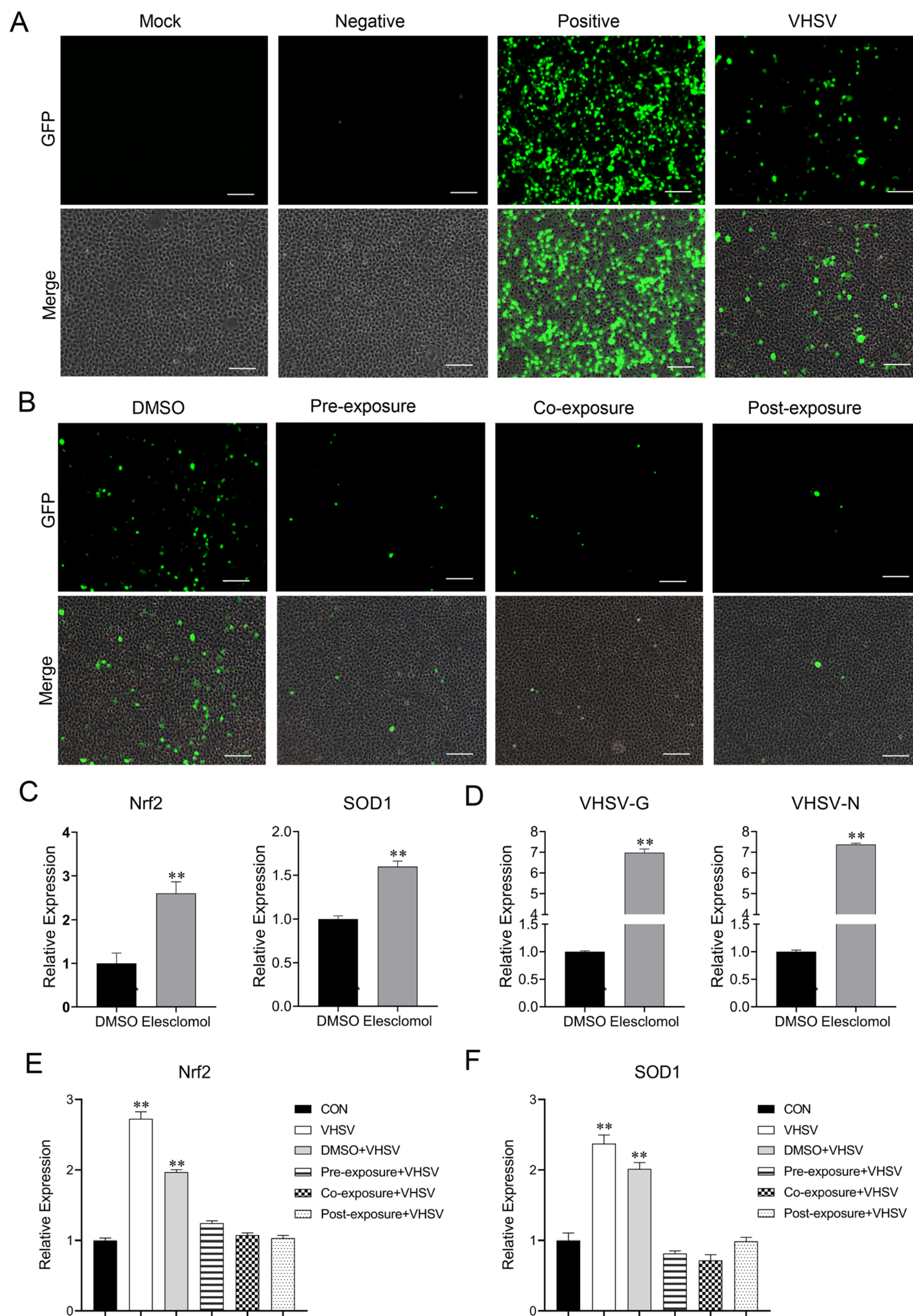

Fig. 5 LA exerted anti-oxidants role to reduce VHSV-induced oxidative stress. **A** FHM cells were untreated (Mock) or treated with PBS (negative), Rosup (positive), or VHSV for 24 h, respectively. The intracellular ROS was measured with the oxidation-sensitive fluorescent probe (DCFH-DA) and observed under fluorescent microscope. The ROS production positive cells were showed with green fluorescence. GFP: green fluorescence images; Merge: the fusion image of green fluorescence signals and white-light visualization; Scale bar = 20 mm. **B** FHM cells infected with VHSV were treated with DMSO or LA in pre-exposure, co-exposure, post-exposure manners for 24 h, then the intracellular ROS was measured as above. **C–D** FHM cells were infected with VHSV for 4 h, and treated with Elesclomol to induce ROS production. The cells were further collected at 24 hpi for analysis of the expression level of *Nrf2* and *SOD1* (**C**), VHSV *G* and *N* (**D**) by qRT-PCR. **E–F** The expression level of *Nrf2* (**E**) and *SOD1* (**F**) of FHM cells at different conditions were analyzed by qRT-PCR. CON, mock treatment; VHSV, VHSV infection; DMSO + VHSV, VHSV infection with DMSO treatment; pre-exposure + VHSV, VHSV infection with LA treatment in pre-exposure manner; co-exposure + VHSV, VHSV infection with LA treatment in co-exposure manner; post-exposure + VHSV, VHSV infection with LA treatment in post-exposure manners. $*P < 0.05$; $**P < 0.01$.

CoV-2 into cells by raising the intracellular pH through activating ATP-dependent K^+ channels, which further strengthened the human host defense (Cure and Cumhur Cure 2020). These findings suggested the antiviral mechanism of LA differed among various viruses, and it exerted an inhibitory effect on VHSV by interfering with the replication of VHSV. Furthermore, *in vivo* experiment showed that LA significantly enhanced the survival rate of VHSV-infected largemouth bass in both co-injection and post-injection manners, indicating that LA might be a promising candidate for the treatment of VHSV infection in fish. It was known that many factors might influence the antiviral effect of LA in live fish, such as administration methods (oral or injectable administration), administration dosage and so on. Thus, the relationship between the antiviral effect of LA and contributing factors will be analyzed in our future study.

Type I IFNs are an important part of the innate immune response against virus infection (Zhang and Gui 2012). Pre-activation of the IFN system could significantly inhibit the replication of fish Rhabdoviruses (Purcell *et al*. 2012). Considering the inhibitory effect of LA on VHSV replication, we speculated that LA might be involved in the IFN response. Additional comparative transcriptome and qRT-PCR analysis revealed that several antiviral genes showed enhanced expression in LA-treated cells. IRF3 and IRF7 were characterized as antiviral factors, and they could activate the *IFN* gene promoter and upregulate IFN and ISGs (Cui *et al*. 2011; Holland *et al*. 2008). ISG15 was a ubiquitin homolog induced by IFN or viral infection. Knockout of ISG15 in EPC cells revealed the important role of ISG15 against VHSV infection (Kim and Kim 2019). Viperin is another IFN-stimulated antiviral protein, functioning in inhibiting virus replication (Mattijssen and Pruijn 2012). Poyntera *et al.* reported that enhanced Viperin and ISG15 resulted in a significant limit on VHSV infection in fathead minnow skin cells (Poynter *et al.* 2019). Herein, the antiviral genes stimulated by LA treatment may strengthen the FHM cell host defense against VHSV and suppress viral replication. Thus, we speculated that LA could inhibit VHSV infection in part through activating the expression of antiviral genes.

Oxidative stress was an important factor for virus infectivity, oxidative damage to cellular components would only occur when the production of ROS exceeded the cell's antioxidant capacity (Krapfenbauer *et al*. 2003). Many viruses could trigger oxidative stress and induce ROS-mediated cell death to facilitate virus replication, including red-spotted grouper nervous necrosis virus (Chang *et al.* 2011), HBV (Vierucci *et al*. 1983) and HIV (Müller 1992). Here, we found that VHSV infection induced ROS production and upregulated the expression of *Nrf2* and *SOD1*, which were antioxidant transcription factors and could protect against the deleterious effects of oxidative stress via regulating the transcriptional activation of various antioxidant biomolecules (Kaspar *et al*. 2009). Correspondingly, ROS production induced by Elesclomol significantly increased the replication of VHSV, suggesting VHSV infection triggered the oxidative stress in FHM cells to facilitate its replication. LA, as a universal antioxidant, was involved in scavenging free radicals to protect against oxidative damage in some diseases (Tibullo *et al.* 2017). By reducing oxidative stress, LA exerted an antiviral effect against several viruses, for example, the addition of LA modulated total glutathione (GSH + GSSG) levels and ameliorated HIV-induced redox-stress in HIV-infected subjects (Baur *et al*. 1991). Here, LA treatment markedly reduced VHSV-generated ROS and suppressed VHSV-induced expression of *Nrf2* and *SOD1* in pre-, co- or post-exposure manner, indicating the protective effect of LA against VHSV could be due to its antioxidant role in reducing oxidative stress.

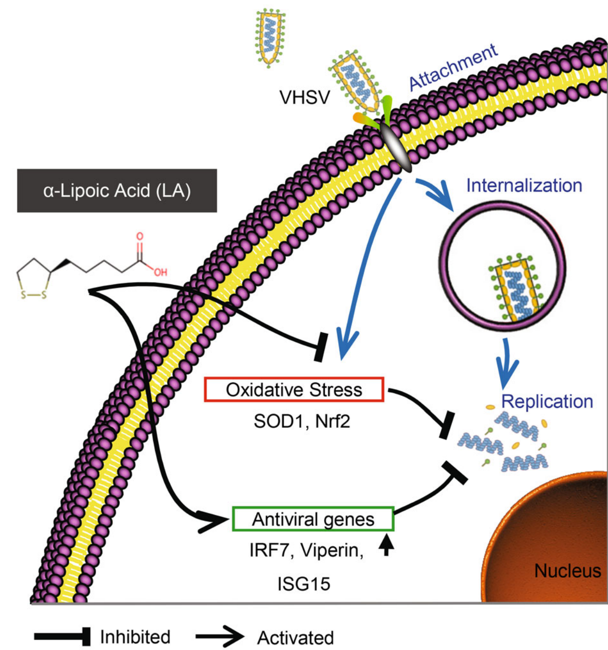

Fig. 6 Scheme summarizing the inhibitory effect of LA on VHSV replication via activation of antiviral genes and suppression of VHSV-induced oxidative stress.

In conclusion, the present study identifies LA as a promising candidate of the anti-VHSV compound, activating antiviral genes and suppressing host cellular oxidative stress to inhibit VHSV infection (Fig. 6). This finding offers a new potent antiviral compound for combating infections caused by VHSV.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and Animal Rights All animal experiments procedures were approved by the Ethics Committee of Sun Yat-Sen University and were carried out following the approved guidelines.

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