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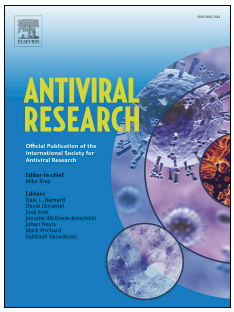

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VER-155008 induced Hsp70 proteins expression in fish cell cultures while impeding replication of two RNA viruses

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ABSTRACT

The heat-shock protein 70 (Hsp70) inhibitor, VER-155008 (VER), was explored as a potential antiviral agent for two RNA viruses important to fish aquaculture, viral hemorrhagic septicemia virus (VHSV) and infectious pancreatic necrosis virus (IPNV). Studies were done at a temperature of 14 °C, and with cell lines commonly used to propagate these viruses. These were respectively EPC from fathead minnow for VHSV and CHSE-214 from Chinook salmon embryo for IPNV. Additionally, both viruses were studied with the Atlantic salmon heart endothelial cell line ASHe. For both VHSV and IPNV, 25 μM VER impeded replication. This was evidenced by delays in the development of cytopathic effect (CPE) and the expression of viral proteins, N for VHSV and VP2 for IPNV, and by less production of viral RNA and of viral titre. As VER inhibits the activity of Hsp70 family members, these results suggest that VHSV and IPNV utilize one or more Hsp70s in their life cycles. Yet neither virus induced Hsp70. Surprisingly VER alone induced Hsp70, but whether this induction modulated VER's antiviral effects is unknown. Exploring this apparent paradox in the future should improve the usefulness of VER as an antiviral agent.

Key words: VER-155008, Hsp70, IPNV, VHSV, fish, viruses

1.0 INTRODUCTION

Inhibitors that target the heat-shock protein (Hsp) 70 family have increasingly become a focus of antiviral strategies for viruses important to human health (Howe and Haystead, 2015; Mayer, 2005; Santoro, 1994) but relatively less explored for fish viruses, with the one exception being a virus important to Chinese aquaculture (Shan et al., 2018). Hsps or chaperones are a diverse group of highly conserved proteins that regulate the proper folding of proteins and are organized into four main Hsp families (small Hsps, Hsp60, Hsp70 and Hsp90) (Bar-Lavan et al., 2016). The human Hsp70 family has at least 8 members, with Hsp70-1 (Hsp70, Hsp72, or Hsp1A1) being the most studied stress-inducible member, whereas the heat-shock cognate 70 (Hsc70) (Hsp73 or HspA8) is constitutively expressed and glucose-regulated protein 78 (Grp78, Bip or HspA5) is inducible by endoplasmic reticulum (ER) stress (Murphy, 2013). The salmon Hsp70 family also has a major stress-inducible form, Hsp70, the heat-shock cognate 70, Hsc70 and ER-stress inducible Grp78 (Ojima et al., 2005; Yamashita et al., 2010). In the context of virology, several compounds that inhibit Hsp70 family members have been explored, including HS-72 (Howe et al., 2016) and VER-155008 or VER (Taguwa et al., 2015). VER is an adenosine analogue and is perhaps most studied. VER binds to Hsp70, Hsc70 and Grp78, with the highest affinity for Hsp70 (Massey et al., 2010; Williamson et al., 2009) but best inhibits the refolding of a model substrate by Hsc70 (Schlecht et al., 2013).

The effect of VER on virus replication has been studied for several viral groups. This includes at least two DNA viruses, a human herpes virus (Baquero-Pérez and Whitehouse, 2015) and an insect baculovirus (Lyupina et al., 2014), and on RNA viruses from several viral families. These include at least one virus from a positive sense (+), single-stranded RNA (ssRNA) family, Dengue virus (DENV) from the *Flaviviridae* (Taguwa et al., 2015), and several from negative

sense (-), ssRNA families, including Ebola virus from the *Filoviridae* (García-Dorival et al., 2016), mumps virus (MuV) from the *Paramyxoviridae* (Katoh et al., 2017), human respiratory syncytial virus (HRSV) from the *Pneumoviridae* (Munday et al., 2015), and enterovirus A71 (EV-A71) from the *Picornaviridae* (Dong et al., 2018). The only fish virus studied was grass carp reovirus, a member of the double-stranded (ds) RNA *Reoviridae* family (Shan et al., 2018). Generally, VER has been found to impair the replication of these viruses, indicating positive role(s) for Hsp70 family member(s) in the life cycle of these viruses.

Two of the most economically important viruses in salmonid aquaculture are viral hemorrhagic septicemia virus (VHSV) and infectious pancreatic necrosis virus (IPNV). VHSV causes viral hemorrhagic septicemia (VHS) disease, which has been destructive to the rainbow trout aquaculture industry for over five decades and continues to threaten future economic performance (Cieslak et al., 2016). VHSV is a negative sense (-) ssRNA virus with an envelope (Basurco and Benmansour, 1995). The virus belongs to the *Novirhabdovirus* genus of the *Rhabdoviridae* family (Basurco and Benmansour, 1995). IPNV causes infectious pancreatic necrosis (IPN) disease, which has devastated fish farms in South America (Tapia et al., 2015; Escobar-Dodero et al., 2018), Europe (Ariel and Olesen, 2002) and Asia (Zhu et al., 2017). IPNV has a dsRNA genome, comprised of two segments and contained within a protein capsid without an envelope (Dobos, 1995). IPNV belongs to the *Aquabirnavirus* genus of the *Birnaviridae* family (Dobos, 1995). Whether Hsp70s have positive or negative effects on the life cycle of IPNV and VHSV is unknown, but VER provides an opportunity to study this and to determine whether antivirals for these viruses and their diseases should target Hsp70s.

In the current study, fish cell lines that are known to support VHSV and IPNV replication were shown to remain viable in VER for up to 5 days. Over 1 to 5 day-exposures, VER inhibited

the replication of VHSV and IPNV as measured by multiple endpoints of the viral life cycles. This suggests that Hsp70s have roles in the replication and production of VHSV and IPNV. However, neither virus induced Hsp70, and surprisingly VER itself strongly induced Hsp70. Thus, the interactions of VER and Hsp70s during virus replication might be complex and contradictory, with the activity of Hsp70s being inhibited by VER on one hand and Hsp70 levels being elevated by VER on the other.

2.0 MATERIALS AND METHODS

2.1 Cell lines and their routine propagation

Materials for culturing cells were from several commercial suppliers. ThermoFisher was the source of most cell culture reagents and vessels, but the manufacturers of these varied. Hyclone prepared the basal medium, Leibovitz's L15 (L15), penicillin-streptomycin (P/S), Dulbecco's phosphate-buffered saline (DPBS) and Trypsin-EDTA whereas fetal bovine serum (FBS) was from Gibco. BioLite was the manufacturer of 6-well, 24-well and 96-well plates and T25 cm^2 and T75 cm^2 flasks.

Three cell lines were used. ASHe is an Atlantic salmon heart endothelial cell line, which was developed in this laboratory (Pham et al., 2017b). The other two were Chinook salmon embryo (CHSE-214) and fathead minnow *epithelioma papulosum cyprini* (EPC) whose origins in this laboratory have been described previously (Pham et al., 2011). The growth medium was L15 supplemented with 10% FBS and 1% P/S (10% FBS/L15). ASHe was grown in T25 cm^2 flasks while CHSE-214 and EPC were grown in T75 cm^2 flasks. For subcultivation, cells were washed with DPBS and then detached using Trypsin-EDTA. After detachment, cells were resuspended in enough growth medium and then added into new flasks in a ratio of 1:2 or 1:3. The total growth volume was 6 mL for T25 cm^2 flasks and 12 mL for T75 cm^2 flasks. Subcultivation was done every 1-2 weeks. All cell lines were cultured at room temperature, which hereafter will be referred to as 22 °C.

2.2 Viruses and their routine propagation and titration

The two viruses were viral hemorrhagic septicemia virus (VHSV) genotype IVa and infectious pancreatic necrosis virus (IPNV). The way how these were obtained has been

described previously (Pham et al., 2014, 2013). Monolayers of EPC and CHSE-214 in T25 cm^2 flasks were used to propagate stocks of respectively VHSV and IPNV. Viral propagation and titration were done in L15 with 2 % FBS and 1 % PS (2% FBS/L15) at 14 °C. The cell lines, FBS concentration and the temperature are those that have been recommended for these viruses in several editions of the OIE Manual of Diagnostic Tests for Aquatic Animals (2017) (OIE - World Organisation for Animal Health, 2017). Upon 7 to 10 days post infection (d.p.i.), cytopathic effects (CPE) were prominent and the monolayers had almost completely deteriorated. At this point, the flask contents were collected and centrifuged at 4,500 x g for 5 minutes. The supernatants were stored at -80 °C. The viral titres of these stock preparations and of preparations from experimental cultures were determined in 96-well plates with monolayers of EPC for VHSV and of CHSE-214 for IPNV. The titres were expressed as the 50 % tissue culture infectious dose ($TCID_{50}$/mL) as determined by the Karber method and as described in more detail previously (Pham et al., 2011).

2.3 Establishing fish cell monolayers

Cells in 10% FBS/L15 were seeded into various culture vessels and incubated at room temperature for 3 to 5 days until the cells formed confluent monolayers over the vessel surfaces. The average number of cells per well for confluent monolayer of each cell line in each of the culture vessels were as follows. ASHe was approximately 19 million in T25 cm^2 flasks, 7 million in 6-well plates, and 1.3 million in 24-well plates. EPC was approximately 25 million in T25 cm^2 flasks, 9 million in 6-well plates, and 2.3 million in 24-well plates. CHSE-214 was approximately 5.2 million in 6-well plates, and 1.3 million in 24-well plates. These monolayer cultures were then used to study VER -155008 (VER). Sigma-Aldrich was the source of VER

and of cell culture grade dimethyl sulfoxide (DMSO) in which a stock solution of VER was prepared. Virus infections with and without VER were set up and monitored as described in the sections below.

2.4 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting of cell lysates

The preparation of cell lysates and their analysis by western blotting was done as documented previously (Pham et al., 2017a) with a few additional reagents and the antibodies described here. Preparation began by scraping cells off the plastic vessel surface into 15 mL conical centrifuge tubes. After centrifugation at 4000 xg for 5 minutes, the supernatants were discarded and the pellets with cells were washed with 2 mL DPBS followed by a second round of centrifugation at the same speed for another 5 min. After removal of the wash solution, the pellet was resuspended in 200 µL radio immunoprecipitation assay (RIPA) buffer and incubated at 4 °C for 30 min before being centrifuged at 10,000 xg at 4 °C for 15 min. The supernatants were collected and stored at -20 ^{o}C. Upon use of these, protein amounts were determined with the Pierce^{TM} BCA Protein Assay Kit (ThermoFisher) according to the manufacturer's instructions. Equal amounts of proteins from each sample in each experiment were denatured in 1x Laemmli sample buffer (0.0005% bromophenol blue, 10% glycerol, 2% SDS, 63mM Tris-HCl, pH: 6.8, 0.1% β-mercaptoethanol) by boiling for 5 min. Protein amounts varied for each experiment but ranged between 20 to 50 µg. After being boiled and cooled, the samples were resolved on a 12% SDS-PAGE gel at 120 V until the dye front approached close to the bottom of the gel.

All antibodies except one were from commercial suppliers. Rabbit polyclonal antibody against salmon Hsp70 (SPC-314B) was from StressMarq (Victoria BC, Canada). Sigma-Aldrich

was the source of rabbit polyclonal antibodies against actin. A mouse monoclonal antibody (FM-130CY-5) to IPNV VP2 protein was manufactured by Austral Biologicals and obtained from Cedarlane (Burlington, ON, Canada). Dr Niels Lorenzen of the Technical University of Denmark (Lyngby) provided a mouse monoclonal antibody (IP5B11) to VHSV N protein. For secondary antibodies, goat anti-rabbit IgG and anti-mouse IgG conjugated with horseradish peroxidase by Biorad were used.

After separation on SDS-PAGE, polypeptides in cell lysates were transferred from gels to a nitrocellulose membrane (Biorad Life Sciences) using either the Biorad Mini-PROTEAN Tetra Cell system with 1x Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol) or the Biorad Trans-Blot Turbo Transfer System with 1x Bjerrum Schafer-Nielsen buffer (48 mM Tris, 39 mM glycine, 20% methanol). After transfer, the membranes were blocked with 5% skim milk in 1× TBS-T (10 mM Tris-HCl, 100 mM NaCl, 0.1% Tween 20) for 1 h. All primary antibodies were diluted at their respective concentration in 5% skim milk/TBS-T, and incubations were done overnight at 4 ^{o}C. The primary antibodies used and their concentrations are listed in Table 1. The membranes were washed 3x with 1x TBS-T after primary incubation followed by incubation in secondary antibodies for up to 3 h. The secondary antibodies are either HRP-conjugated goat anti-rabbit or anti-mouse secondary antibody diluted to 1:5000 in 5% skim milk/TBS-T. After secondary incubation, membranes were washed 3x again before exposure to SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher). Protein bands were imaged using a BioRad ChemiDoc MP Imaging System (Image Lab 5.2.1 software).

Table 1. Primary antibodies used in western blots

Antibody	**Species**	**Dilution**

Anti-VHSV N protein (IP5B11)	Mouse	1:600
Anti-IPNV Vp2 protein (FM-130CY-5)	Mouse	1:1000
Anti-actin (A2066)	Rabbit	1:1000
Anti-Hsp70 (SPC-314B)	Rabbit	1:1000

2.5 Co-immunoprecipitation (Co-IP)

A Co-IP assay was done with anti-N protein antibodies as the bait antibody bound to Dynabeads to investigate a possible interaction between Hsp70 and VHSV N protein. The sheep anti-mouse M-280 Dynabeads were from Thermofisher. Cell lysates from T25 cm^2 flask cultures were collected as described previously for control ASHe and EPC cultures and from ASHe and EPC cultures that had been infected with VHSV for 2 days. Cell lysates were centrifuged (21,100 xg for 10 min at 4 °C) to remove cell debris. The supernatant was collected, and the protein quantified as described previously. Immunoprecipitation was performed using conjugated sheep anti-mouse M-280 Dynabeads. All steps were performed on ice. Beads were rinsed thoroughly in wash buffer (DPBS with 5 mg/mL bovine serum albumin) and then incubated with 0.5 μL of anti-N protein antibody in 200 μL of wash buffer overnight with end-over-end rotation at 4 °C. Beads were pulled down, rinsed with wash buffer to remove unbound antibody, equilibrated in RIPA buffer, and incubated with equal concentrations of infected and uninfected lysates for 1 h at 4 °C with end-over-end rotation. Beads were pulled down using a magnetic particle concentrator, and the supernatant fraction (SUP) was collected, quantitated by BCA assay, and saved. Beads were rinsed with wash buffer several times and then rinsed with DPBS. Finally, beads containing bound proteins were resuspended in 40 μL of DPBS; this is the

immunoprecipitated (IP) fraction. Whole cell extract (WCE), SUP, and IP fractions were boiled in Laemmli's buffer for 10 minutes, and 50 μg of WCE and SUP fractions and 10 μL of the boiled beads were loaded onto gel lanes. SDS-PAGE and western blotting were done as described above.

2.6 Effect of VER on fish cell energy metabolism and the maintenance of fish cell monolayers

The ASHe cultures were exposed to VER at final concentrations between 6.25 and 50 μM with control cultures receiving DMSO between 0.2% to 0.25%, which is not cytotoxic to fish cells (Schnell et al., 2009). CHSE-214 and EPC cultures were exposed to VER at the same concentration range in experiments that measured metabolic activity, while exposed to VER at only 25 μM in the experiments that evaluated monolayer integrity. The experiments were done in either 6-well plate or 24-well plate. The exposures were carried out for up to 8 days under conditions meant to mimic the temperature regimen that would be used in viral infections. The cultures were in 2% FBS/L15, held at 22 °C for the first day, and then dropped down to 14-15 °C for the duration of each experiment. At days 2, 4 and 8, cellular capabilities were assessed with the fluorescent indicator dyes, Alamar Blue (AB) for energy metabolism. At day 0, before treatments commenced, cellular capabilities were also assessed on a replicate set of culture to determine the initial level of energy metabolism. The AB assay was carried out and analyzed as described previously in step by step detail (Dayeh et al., 2013) and relative fluorescence units (RFUs) were measured with an EnSpire Multimode Plate Reader. The results were recorded as RFUs, and RFUs at days 2, 4 and 8 were expressed as percentages of the RFUs in cultures at day 0. The continued adherence of cells to the plastic surface of vessels during VER exposure was

monitored by daily observations of cultures under a phase contrast microscope. After the end of some experiments, cell adherence was illustrated by staining cultures with crystal violet as documented previously (Pham et al., 2017a).

2.7 Pre-exposure of cells to VER before infection with viruses or mock infection

Briefly, the cell monolayers in 2% FBS/L15 were exposed to VER and incubated at 22 °C for 1 day. At this point the media were removed from cultures and replaced with 2% FBS/L15 that either had the virus alone, virus plus VER, no virus, and no virus plus VER. The cultures were then shifted to 14 °C and incubated for up to 7 days, which should be enough time for the viral life cycle to be completed in at least the controls without VER. The VER concentrations were in the 3.125 to 50 μM range and specific concentrations are noted in the Results and Figures. Control cultures received just DMSO instead of VER or just medium instead of virus. The virus dose depended on the culture vessel, which in turn depended on the endpoint and so are mentioned in the sections below under each endpoint.

2.7.1 Effect of VER on the development of cytopathic effect (CPE)

Cultures in 24-well plates were exposed to VER at various concentrations for 1 day followed by infection with VHSV at $10^{5.5}$ $TCID_{50}$/mL ($10^{5.2}$ $TCID_{50}$/well) or IPNV at $10^{5.6}$ $TCID_{50}$/mL ($10^{5.3}$ $TCID_{50}$/well) as described in Section 2.7. Cultures were examined daily under a phase contrast microscope and changes in the appearance of the cells and monolayers were noted. The gross integrity of the monolayers p.i. were assessed by crystal violet staining of ASHe cultures at 3 d.p.i., EPC cultures at 4 d.p.i. and CHSE-214 cultures at 5 d.p.i.

2.7.2 Effect of VER on the expression of two viral proteins

Cultures in 6-well plates were exposed to 25 μM VER for 1 day followed by infection with VHSV at $10^{5.5}$ $TCID_{50}$/mL ($10^{5.8}$ $TCID_{50}$/well) or IPNV at $10^{5.6}$ $TCID_{50}$/mL ($10^{5.9}$ $TCID_{50}$/well) as described in Section 2.7. Cell lysates were collected at 1, 2, and 3 d.p.i. for ASHe and at 1, 3, and 5 d.p.i. for CHSE-214 and EPC cultures. The cell lysates were subject to western blotting as described in section 2.4. VHSV N protein and IPNV Vp2 protein were detected respectively with the mouse monoclonal antibodies IP5B11 and FM-130CY-5.

2.7.3 Effect of VER on viral RNA levels

RNA extraction was done with an RNeasy Mini Kit from Qiagen and a one-step quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay was done with Roche LightCycler 480 Master Hydrolysis Probes (Roche Applied Science) as documented previously (Pham et al., 2017a). Cultures in 6-well plates were exposed to 25 μM VER for 1 day followed by infection with VHSV at $10^{5.5}$ $TCID_{50}$/mL ($10^{5.8}$ $TCID_{50}$/well) or IPNV at $10^{5.6}$ $TCID_{50}$/mL ($10^{5.9}$ $TCID_{50}$/well) as described in Section 2.7. Samples were collected at 1 and 2 d.p.i. for RNA extraction in ASHe culture and at 1 and 3 d.p.i in CHSE-214 and EPC cultures. RNA was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. A one-step qRT-PCR assay was used to amplify target sequences targeting the N gene of VHSV (Al-Hussinee et al., 2016), and the VP2 gene in IPNV (Hoferer et al., 2017). The PCR reaction was performed in a total volume of 10 μL containing 100 ng of template RNA using Roche LightCycler 480 Master Hydrolysis Probes (Roche Applied Science) and following standard protocol. For VHSV, the cycling conditions were: reverse transcription at 63 °C for 3 min; denaturation, 95 °C for 30 s; and amplification (37 cycles) 95 °C for 12 s, 60 °C for 45 s at, and

72 °C for 1 s. For IPNV, the RNA was heated to 99 °C for 10 min then frozen directly in liquid nitrogen for 20 seconds and transferred to ice. The master mix was added on the frozen RNA template. Similar cycling conditions were used except the annealing temperature was adjusted to 58 °C. The primer sets used for VHSV and IPNV are listed in Table 2.

Table 2: List of primers and probes used in detection of VHSV and IPNV by qRT-PCR

Oligonucleotide	**Sequence (5' → 3')**	**Reference**
IPN(1)-F	GGCGTATCCAACTATGAGCTGAT	(Hoferer et al., 2017)
IPN(1)-R	GGACAGGATCATCTTGGCATAGTT	
IPN(1)-Probe	FAM-AACCCAGACCTCCTGAAGAACATGGTCAC-BHQ1	
VHSV-FWD	ACCTCATGGACATCGTCAAGG	(Hope et al., 2010)
VHSV-REV	CTCCCCAAGCTTCTTGGTGA	
VHSV-PROBE	FAM-CCCTGATGACGTGTTCCCTTCTGACC-BHQ1	

2.7.4 Effect of VER on viral titre

Cultures in 6-well plates were exposed to 25 μM VER for 1 day followed by infection with VHSV at $10^{5.5}$ $TCID_{50}$/mL ($10^{5.8}$ $TCID_{50}$/well) or IPNV at $10^{5.6}$ $TCID_{50}$/mL ($10^{5.9}$ $TCID_{50}$/well) as described in Section 2.7 but with the following modifications. At 2 h.p.i., the media were removed, and the wells were washed 3x with 2 mL of DPBS. After washing, 2 mL of fresh virus-free medium (either DMSO control or containing VER) were added to the wells. Immediately upon the addition of this media, 200 μL samples were collected from each well and were used to determine day zero virus titre. Afterwards, 200 μL of virus-free medium was added back to each well to bring the total volume back to 2 mL. Samples of 200 μL were collected again at 3 and 7 d.p.i. Viral titres in these samples were determined as described in section 2.2.

2.8 Effect of VER and virus infections on Hsp70

For studying stress protein levels through western blotting, monolayer cell cultures in 6-well plates with 2 ml of 2% FBS/L15 per well were used. Viruses and VER exposed independently to cell lines were studied. For viruses only, the wells were infected with either VHSV at $10^{5.5}$ $TCID_{50}$/mL ($10^{5.8}$ $TCID_{50}$/well) or IPNV at $10^{5.6}$ $TCID_{50}$/mL ($10^{5.9}$ $TCID_{50}$/well) and the plates incubated at 14 °C. At 2, 8, and 24 hours (h.p.i.), cell lysates were collected from wells as described in section 2.4. For VER alone, the Hsp70 inhibitor was added to wells to final concentrations of 25 μM and plates were incubated at 14 °C for 24 h at which point cell lysates were collected. Cell lysates were subjected to SDS-PAGE and western blotting as described in section 2.4. Hsp70 was detected with rabbit polyclonal antibodies to Hsp70 (StressMarq, SPC-314B).

3.0 Results

3.1 Effect of VER on fish cell energy metabolism and the maintenance of fish cell monolayers

VER reduced energy metabolism, as measured by AB assay, in fish cells but the magnitude of reduction varies with each cell line. The reduction was most severe in ASHe but less so in CHSE-241 and EPC. In ASHe, VER at 25 and 50 μM reduced energy metabolism to about 62.45% and 65.92% by day 2 relative to day 0, respectively (Fig. 1a). By day 4 and 8, at 50 μM VER, energy metabolism dropped to 17.57% and 9.91%, respectively; however, at 25 μM VER, over the same period, energy metabolism was at 60.77% by day 4 and 49.19% by day 8 (Fig. 1a). VER at concentrations of 12.5 μM and lower, did not show less energy metabolism than in 0 μM VER-DMSO control cultures over the eight days period (Fig. 1a). For CHSE-214, even after eight days of VER exposure, energy metabolism at 50 μM VER was 71.32% and at 25 μM VER was 82.50% relative to day 0 (Fig. 1b). At 12.5 μM VER and lower, energy metabolism in CHSE-214 was not significantly different from 0 μM VER-DMSO control cultures (Fig. 1b). In EPC, on day 8, energy metabolism at 50 μM VER was 82.41% and at 25 μM VER was 93.74% relative to day 0 (Fig. 1c).

At 12.5 μM VER, energy metabolism was higher than 98%, while at 6.25 μM VER and 0 μM-DMSO control, energy metabolism was higher than 100% relative to day 0 (Fig. 1c), suggesting potential cell growth in those cultures. Overall, VER at 25 μM, the concentration that is used in subsequent experiments with viruses, had major impact on ASHe energy metabolism but minor impact on CHSE-214 and EPC energy metabolism over eight days.

Monolayers of ASHe, EPC, and CHSE-214 cells in 2 % FBS/L15 remained adherent to the culture surface in the presence of VER. Adherence was observed by phase contrast microscopy

as illustrated in Fig. 2 for ASHe after 5 days exposure to VER concentrations ranging from 6.25 to 50 μM, and for CHSE-214 and EPC after 5 days exposure to 25 μM VER. ASHe monolayer developed regions of openings, exposing the underlying plastic surface, after 5 days post exposure to VER at concentrations of 12.5, 25 and 50 μM, with the number of openings increased as the concentration increased. The same holes were not observed in the 6.25 μM VER, 3.125 μM VER (data not shown), and DMSO control cultures. While ASHe monolayer appeared sensitive to VER at 25 μM, most of the monolayer remained attached even after 5 days post exposure. In both CHSE-214 and EPC cultures exposed to 25 μM VER, the monolayers also remained intact after 5 days post exposure.

3.2 Likelihood of VER acting through fish Hsp70s

As VER was developed for targeting mammalian Hsp70s, a bioinformatic analysis of fish Hsp70 homolog/identity with human Hsp70 was undertaken. Comparisons were done of the Salmon Hsp70 protein vs human Hsp70 and the fathead minnow Hsc70 vs human Hsc70. For Fathead minnow, Hsc70 was chosen because there was no Hsp70 protein sequence available and VER can also target human Hsc70 ATP binding pocket. When looking at the entire Hsp70 protein, an alignment in Clustal Omega showed that both Atlantic salmon [UniProtKB - B5X4Z3 (B5X4Z3_SALSA)] and Chinook salmon [UniProtKB - Q91233 (HSP70_ONCTS)] Hsp70 have approximately 85% amino acid identity to human Hsp70 [UniProtKB - P0DMV8 (HS71A_HUMAN)]. Furthermore, when looking only at the nucleotide (ATP) binding domain (NBD) of Hsp70, the amino acid identity is approximately 90%. In human Hsp70, as annotated in the UniProtKB database, ATP interactions with the Hsp70 NBD occur at amino acid residues 12-15, 202-204, 268-275, and 339-342; these amino acids remained unchanged between the

human and salmon Hsp70 NBD. For Fathead minnow (from which the EPC cell line was derived), the Hsc70 NBD [UniProtKB Q6QIS4 (Q6QIS4_PIMPR)] has almost 95% amino acid identity to the human Hsc70 NBD [UniProtKB - P11142 (HSP7C_HUMAN)] and again the ATP interaction residues remains unchanged. This analysis suggests that the ATP binding function of Hsp70/Hsc70 is highly conserved between fish and human and that VER, as an ATP analogue, could interact with the Hsp70 NBD of fish and as well as humans.

3.3 VER delayed development of IPNV infections

The effects of inhibiting Hsp70/Hsc70 function in ASHe and CHSE-214 with VER on IPNV replication was determined by examining four parameters: development of CPE, expression of viral proteins and viral RNA levels, and production of viral titre. From 3.125 to 12.5 µM of VER, no difference in CPE was observed between IPNV infected cultures treated with either VER or DMSO as cultures in both conditions were completed destroyed by IPNV (Fig. 3a). However, at 25 to 50 µM of VER, IPNV-induced CPE was inhibited in ASHe cells at 3 d.p.i (Fig. 3a). Similarly, treatment of CHSE-214 cells with 25 µM VER reduced IPNV-induced CPE development in CHSE-214 cells up to at least 5 d.p.i. (Fig. 3b) when compared to infected cultures treated with DMSO control.

The effects of inhibiting Hsp70/Hsc70 function in ASHe and CHSE-214 on the expression of IPNV proteins in these infected cells was determined by looking at IPNV VP2 proteins. For ASHe, 25 µM VER treatment delayed IPNV VP2 protein expression by at least two days, as the VP2 proteins were not present in VER-treated culture but was seen in DMSO-treated cultures (Fig. 3c). For CHSE-214, infected DMSO-treated cultures produced detectable VP2 proteins by

day 5 whereas, these proteins were not detectable in 25 µM VER-treated cultures even by day 5 (Fig. 3d).

The effects of inhibiting Hsp70/Hsc70 function in ASHe and CHSE-214 on IPNV RNA levels in these infected cells was determined using qRT-PCR. For ASHe and CHSE-214, 25 µM VER treatment significantly reduced the IPNV RNA levels (higher average Ct values) relative to the DMSO treatment (Fig. 3e, f); this reduction was significant at 2 d.p.i for ASHe (Fig. 3e) and at 1 and 3 d.p.i. for CHSE-214 (Fig. 3f).

The effects of inhibiting Hsp70/Hsc70 function in ASHe and CHSE-214 on the capacity of IPNV to produce infectious virions was determined by measuring infectious titre ($TCID_{50}$/mL). In ASHe, VER treatment significantly reduced IPNV titre at 3 d.p.i. when compared to DMSO-treated cultures; however, by 7 d.p.i, the difference in the level of virus between VER-treated and DMSO-treated cells reduced and lost significance (Fig. 3g). A similar result was observed for CHSE-214 and IPNV where VER-treated cultures produced significantly lower IPNV titre at 3 and 7 d.p.i. than DMSO-treated cultures, but once again, the magnitude of the difference was lower at 7 d.p.i. than 3 d.p.i. (Fig. 3h).

3.4 VER delayed development of VHSV infections

The effects of inhibiting Hsp70/Hsc70 function in ASHe and EPC with VER on VHSV replication was determined by examining four parameters: development of CPE, expression of viral proteins and viral RNA levels, and production of viral titre. VHSV completely destroyed ASHe monolayer in culture treated with either DMSO or 3.125 to 12.5 µM VER at 3 d.p.i. (Fig. 4a); however, at 25 to 50 µM of VER, adherent and viable culture monolayer remained (Fig. 4a). The inhibitory effect of VER on CPE development was further confirmed in EPC cells where

cultures treated with DMSO was completed destroyed but cultures treated with 25 µM of VER retained many viable cells up to 4 d.p.i. (Fig. 4b).

The effects of inhibiting Hsp70/Hsc70 function in ASHe and EPC on the expression of VHSV proteins in these infected cells was determined by looking at VHSV N proteins. Infected DMSO-treated ASHe and EPC cultures showed N protein expression at 1 d.p.i., but for infected VER-treated cultures, N protein was absent at 1 d.p.i. but appeared by 3 d.p.i. (Fig. 4c, d).

The effects of inhibiting Hsp70/Hsc70 function in ASHe and EPC on VHSV RNA levels in these infected cells was determined using qRT-PCR. VHSV expressed higher relative RNA levels (lower average Ct value) in DMSO-treated cultures than VER-treated cultures in both ASHe and EPC (Fig. 4e, f); this was significant at 2 d.p.i. for ASHe and at 1 and 3 d.p.i. for EPC.

The effects of inhibiting Hsp70/Hsc70 function in ASHe and EPC on the capacity of VHSV to produce infectious virions was determined by measuring infectious titre ($TCID_{50}$/mL). VER reduced VHSV titre in both ASHe (Fig. 4g) and EPC (Fig. 4h) cultures at 3 d.p.i. relative to the infected DMSO-treated cultures. The difference in titre between DMSO- and VER-treated culture disappeared in ASHe at 7 d.p.i. but was retained in EPC, albeit at a lower magnitude than at 3 d.p.i..

3.5 Hsp70 levels were upregulated by VER but not by IPNV and VHSV infections

Since VER is known to inhibit Hsp70/Hsc70 function rather than expression, the effect of VER on Hsp70 expression was evaluated by western blotting. At a concentration of 25 µM, VER induced Hsp70 in both ASHe (Fig. 5a) and CHSE-214 (Fig. 5b) cultures at day 1 post exposure, and the induction persisted for at least 5 days in the continuous presence of VER. The anti-Hsp70 antibody used in this work was designed to target salmonid Hsp70 so it inconsistently

cross-reacted with protein lysates from the fathead minnow EPC cells, making interpretation inconclusive (data not shown). While 1 day exposure of ASHe to 25 μM VER resulted in strong induction of Hsp70 protein (Fig. 5a), infection of ASHe with either IPNV or VHSV during the first 24 h.p.i. showed little change in Hsp70 levels (Fig. 5c).

3.6 A co-immunoprecipitation assay revealed no Hsp70/VHSV N interaction

Inasmuch as the VER experiments indicated positive regulation of VHSV replication by Hsp70, Hsp70 and VHSV N protein were examined for a possible interaction using a Co-IP assay. Anti-N protein primary antibodies were bound to Dynabeads and used as the bait antibody. The Co-IP assay successfully bound and captured a small amount of total VHSV N proteins in infected cultures of ASHe, as shown in the IP fraction; however, Hsp70 was not detected in the IP fraction (Fig. 6), suggesting that Hsp70 did not directly interact with VHSV N protein. Both Hsp70 and VHSV N proteins were detected in the unbound SUP fraction of the Co-IP assay and in the WCE (Fig. 6), which indicated that the initial collected whole cell lysate contained both Hsp70 and VHSV N proteins. The two proteins however showed no detectable interaction.

4.0 DISCUSSION

The heat-shock protein 70 (Hsp70) inhibitor, VER-155008 (VER), impeded the replication of viral hemorrhagic septicemia virus (VHSV) and infectious pancreatic necrosis virus (IPNV) in three fish cell lines: EPC, CHSE-214 and ASHe. These results suggest that IPNV and VHSV require Hsp70 activity for optimal completion of their life cycles. Yet, neither virus enhanced Hsp70 levels, but unexpectedly VER alone did. Therefore, as an antiviral, VER is a paradox, inhibiting Hsp70 activity but increasing Hsp70 levels.

4.1 Effect of VER on fish cell energy metabolism and the maintenance of fish cell monolayers

VER appeared less cytotoxic to the fish cell lines than has been reported for mammalian cell cultures. This could reflect a difference between piscine and mammalian cells but more likely is due to the exposure conditions. The mammalian cells have been exposed at their optimal temperature (37 °C) at cell densities that permit more proliferation (Chatterjee et al., 2013). By contrast, the fish cells were incubated with VER under conditions that were limiting to cell proliferation but were optimal for replication of IPNV and VHSV (OIE - World Organisation for Animal Health, 2017). These conditions were cells as monolayers in 2 % FBS incubated for the most part at 14 °C, which is a temperature that supports only slow fish cell proliferation (Bols et al., 1992). Despite this, exposures of 5 to 8 days at VER concentrations of 25 µM did change fish cell morphology and impaired energy metabolism substantially in ASHe and minorly in CHSE-214 and EPC, while during the same exposures the cells were sufficiently viable to remain adherent to the plastic growth surface. Thus, the diminishment of IPNV and VHSV replication in VER treated cultures is unlikely due to the outright death of the cells. Instead, VER likely acts either directly by disrupting Hsp70/viral protein interaction(s) specific for

replication or indirectly by causing the accumulation misfolded host proteins that impair cellular processes generally necessary for viral replication. An example of a cellular process involving Hsp70 might be energy metabolism. Hsp70 activity has been found to support mitochondrial functions in the mammalian brain (Belenichev et al., 2011) and Hsp70 defects cause mitochondrial stress in yeast (Ruan et al., 2017). The responses of the three cell lines to VER alone and to viral infections after VER treatments will make them and VER useful in the future for exploring more precisely the role(s) of Hsp70s in the IPNV and VHSV life cycles.

4.2 VER delayed development of IPNV and VHSV infections

VER inhibited the development of IPNV and VHSV infections in fish cells and did so presumably through Hsp70s. This was seen as delays in the appearance of CPE and in the expression of viral proteins, VP2 for IPNV and N for VHSV, and as diminishments in viral RNA levels and of viral titre. Viral replication was impeded by VER in the Atlantic salmon parr heart endothelial cell line ASHe (Pham et al., 2017b), in the Chinook salmon embryonic epithelial cell line, CHSE-214, which for decades has been used for detecting and propagating IPNV (McAllister, 1997), and in the fathead minnow epithelial cell line, EPC, which is a recommended cell line for detecting VHSV (OIE - World Organisation for Animal Health, 2017; Winton et al., 2010). Thus, VER might be expected to impede replication of these viruses in most cells of susceptible fish. VER likely acted by targeting the fish Hsp70s because the Hsp70 family is a highly conserved group of chaperones (Radons, 2016; Yamashita et al., 2010), although inhibitors that have been developed for mammalian cells can occasionally have off-target actions in fish cells (Zeng et al., 2016). Based on the affinity of VER for Hsp70 family members, VER is expected to preferentially target Hsp70 followed by Hsc70 and GRP78 (Massey et al., 2010).

Whether VER acts on IPNV and VHSV infections by inhibiting specifically one or all of these Hsp70s is a matter of speculation.

VER could be acting at multiple steps in the IPNV and VHSV life cycles. When considering RNA viruses broadly, examples of Hsp70s involvement have demonstrated for most life cycles stages, including viral receptor/entry (Chuang et al., 2015; Reyes-Del Valle et al., 2005), uncoating (Alam and Rochon, 2017; Ivanovic et al., 2007), genome replication (Gao et al., 2014), morphogenesis (Macejak and Sarnow, 1992), and release (Watanabe et al., 2006). Whether VER inhibits the IPNV at specific or multiple life cycle stages requires further research. One approach might be to monitor virus replication after adding VER at different times after infection. On the other hand, delineating a specific stage in this manner is made difficult by the asynchronous development of infections and the likelihood of Hsp70s being involved at multiple life cycle stages. However, adding VER before the virus as in the current experiments clearly shows the dependence of IPNV and life cycle on Hsp70s. As to specific Hsp70s, co-immunoprecipitation experiments with a mammalian rhabdovirus, rabies virus (RABV), have demonstrated an association between N protein and Hsp70 (Lahaye et al., 2012), but such an association could not be demonstrated in the current study with VHSV. Whether this means that a Hsp70/VHSV N protein interaction is absent and thus not a target for VER or has been missed for technical reasons requires further research.

4.3 Hsp70 levels after VHSV IVa and IPNV infections

As judged by western blotting, Hsp70 remained steady in fish cell cultures during at least the first 24 h after infection with VHSV or IPNV. In the past, protein level changes in Hsp70 family members have been noted after infection with VHSV and two other fish rhabdoviruses, spring viremia of carp (SCV) and infectious hematopoietic necrotic virus (IHNV). However, the

changes have been modest, and a pattern of change has yet to emerge. Infection of fathead minnow (FHM) with VHSV led to Hcs71 levels being elevated 1.4-fold at 2 h but appearing to return to near control levels by 4 h (Jeong et al., 2015). After 48 h of SCV infection, EPC had less Hsc70 (Liu et al., 2013). In fish, Hsp70 was elevated 20 and 60 days after exposure of Chinook salmon to IHNV (Eder et al., 2007). At the mRNA level, modulation of Hsp70 expression has been noted frequently. VHSV induced Hsp70 mRNA 4.5-fold at 48 h in a flounder cell line (Kong et al., 2009), but in flounder leucocytes, Hsp70 mRNA was down-regulated at 24 h and up-regulated at 72 h (Matsuyama et al., 2011). IHNV down-regulated Hsp70 transcripts at 24 h and up regulated them at 72 h in the rainbow trout head kidney (MacKenzie et al., 2008), and depending on IHNV strain, down regulated them in fry (Purcell et al., 2011). For IPNV, Hsp70 was not induced in current study, but IPNV was found by others to induce Grp78 6 and 12 h after infection of CHSE-214 (Huang et al., 2011). Despite the lack of Hsp70 induction in the current study, the literature suggests a complex regulation of the Hsp70 gene family by fish rhabdoviruses and birnaviruses, hinting at their importance during infections.

4.4 Hsp70 levels after VER treatments

VER at 25 μM was found to elevate Hsp70 protein levels in fish cell cultures. Whether this is a common response of animal cells to VER remains to be determined because the actions of VER on Hsp70 levels have only begun to be examined. VER at 50 μM for 24 h induced Hsp70 and Grp78 in canine osteosarcoma cell lines (Asling et al., 2016). On the other hand, VER treatments of 20 and 25 μM for 48 to 72 h failed to induce Hsp70 in several human cancer cell lines (Kim et al., 2014a; Wen et al., 2014), while Hsc70 declined in anaplastic thyroid carcinoma (ATC) cells (Kim et al., 2014b). Interestingly, the Hsp70 inhibitor, 2-phenylethynesulfonamide (PES), was shown to induce Hsp70 in a rainbow trout gill cell line (Zeng et al., 2014). How

VER elevates Hsp70 levels is a matter of speculation but actions through misfolded proteins, protein aggregates, or Hsp90 are three possibilities to consider. By inhibiting Hsp70 at ATP-binding site, VER will prevent misfolded proteins from being corrected, causing them to accumulate and to induce more Hsp70 synthesis. Similarly, Hsp70 inhibitors often lead to protein aggregates (Howe et al., 2014), and these could be the possible trigger for Hsp70 induction. Finally, because VER has some weak binding affinity for Hsp90 (Massey et al., 2010), inhibition of Hsp90 activity might have occurred and led to Hsp70 induction. In a variety of mammalian cancer cells, Hsp90 inhibitors have been found to induce Hsp70 (Chatterjee et al., 2013; Schaefer et al., 2017).

4.5 Summary and future research

In the current study, 25 μM VER impeded viral infections by IPNV and VHSV but also induced Hsp70 in the fish cell lines. The impairment of infections implies that Hsp70 activity is essential for the life cycles of these two viruses. Confirmation of this can be sought in the future using other approaches. One is to test the effect of anti-Hsp70 siRNA on virus replication. When expression of grouper heat shock cognate protein 70 (GHSC70) was knocked down by this method, nervous necrosis virus (NNV) entry into grouper cells was impaired (Chang & Chi, 2015).

Whether the Hsp70 induction compromised the antiviral action of VER is interesting to consider. Several scenarios can be envisioned under which VER maintained some antiviral effects, despite inducing Hsp70. For example, 25 μM VER might be sufficient to inhibit both the constitutive and the induced Hsp70 ATPase activity so that the antiviral action remains uncompromised. Alternatively, the antiviral action of VER might be mediated by other Hsp70 family members, such as Hsc70 or GRP78, so that the antiviral activity continues despite Hsp70

induction. On the other hand, several scenarios can be envisioned by which Hsp70 induction might be detrimental to VER's antiviral activity. For example, Hsp70 might accumulate over time to levels that overwhelm VER inhibition, and as a result, sufficient Hsp70 activity becomes available to support viral life cycles. A more speculative scenario is suggested by the recent observations that independent of its ATPase activity Hsp70 binds RNA and stabilizes mRNA (Kishor et al., 2017). If viruses were to exploit this RNA-binding activity of Hsp70 in their life cycle, Hsp70 elevation by VER might possibly be pro-viral. These will be interesting scenarios to distinguish experimentally in the future in order to improve the utility of VER as an antiviral agent and as a tool to investigate viral life cycles.

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Figure 1. Effect of VER on fish cell energy metabolism

The potential toxic effect of VER on fish cells was investigated with ASHe, CHSE-214, and EPC cultures. In panel (a), (b) and (c), respectively ASHe, CHSE-214 and EPC were exposed to either the 0 μM VER-DMSO control or VER at concentrations ranging from 6.25 to 50 μM, and energy metabolism was measured at days 0 (before VER exposure) and days 2, 4 and 8 post exposure (n=4, except for EPC day 8 with n=3). Percent metabolic activity was calculated relative to day 0. Statistical analyses were performed using the student's t test to compare between 0 μM VER-DMSO control and VER treatments within each time point with level of significance indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data shown represent one of two independent experiments.

Figure 2. Effect of VER on fish cell culture monolayers

The potential disruptive effect of VER on fish cell monolayers was investigated with ASHe, CHSE-214, and EPC cultures. In panel (a), ASHe monolayers were exposed to either the DMSO control or VER at concentrations ranging from 6.25 to 50 μM and monitored for 5 days. In panels (b) and (c), CHSE-214 and EPC monolayers, respectively, were exposed to either DMSO control or VER at 25 μM and monitored for 5 days. Each scale bar represents 200 microns.

Figure 3. Effect of VER on IPNV infections

The effect of VER on IPNV infections was determined by examining four endpoints: development of cytopathic effects (CPE), expression of viral proteins and viral RNA levels, and production of viral titre. Panels (a) and (b), respectively show crystal violet assay of CPE development in control and IPNV-infected ASHe and CHSE-214 monolayer treated with either

DMSO control or VER at various concentrations. Crystal violet assays were performed at 3 d.p.i. for ASHe and at 5 d.p.i. for CHSE-214 cultures. Panels (c) and (d), respectively show IPNV VP2 and actin proteins expression in IPNV-infected ASHe and CHSE-214 cultures treated with either DMSO control or VER at 25 μM. Protein lysates were collected at days (D) 1, 2, and 3 p.i. for ASHe and at days (D) 1, 3, and 5 p.i. for CHSE-214 cultures.

Panels (e) and (f), respectively show relative viral RNA levels (average real time PCR Ct values, n=3) of IPNV-infected ASHe and CHSE-214 cultures treated with either DMSO control or VER at 25 μM. Samples were collected for RNA extraction at 1 and 2 d.p.i. for ASHe and at 1 and 3 d.p.i. for CHSE-214. Panels (g) and (f), respectively show level of IPNV titre (average log $TCID_{50}$/mL, n=6) produced in IPNV-infected ASHe and CHSE-214 cultures treated with either DMSO control or VER at 25 μM. Samples were collected from both ASHe and CHSE-214 cultures at 0, 3, and 7 d.p.i. Statistical analyses were performed using the student's t test to compare between DMSO and VER treatments within each time point with level of significance indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 4. Effect of VER on VHSV infections

The effect of VER on VHSV infections was determined by examining four endpoints: development of cytopathic effects (CPE), expression of viral proteins and viral RNA levels, and production of viral titre. Panel (a) show crystal violet assay of CPE development in VHSV-infected ASHe monolayer treated with either DMSO control or VER at various concentrations; mock-infected control ASHe monolayer is shown in Fig. 3, panel (a). Panel (b) show crystal violet assay of CPE development in VHSV-infected EPC monolayer treated with either DMSO control or VER at 25 μM. Crystal violet assays were performed at 3 d.p.i. for ASHe and at 4

d.p.i. for EPC cultures. Panels (c) and (d), respectively show VHSV N and actin proteins expression in VHSV-infected ASHe and EPC cultures treated with either DMSO control or VER at 25 μM. Protein lysates were collected at days (D) 1, 2, and 3 p.i. for ASHe and at days (D) 1, 3, and 5 p.i. for EPC cultures. Panels (e) and (f), respectively show relative viral RNA levels (average real time PCR Ct values, n=3) of VHSV-infected ASHe and EPC cultures treated with either DMSO control or VER at 25 μM. Samples were collected for RNA extraction at 1 and 2 d.p.i. for ASHe and at 1 and 3 d.p.i. for EPC. Panels (g) and (f), respectively show level of VHSV titre (average log $TCID_{50}$/mL, n=6) produced in VHSV-infected ASHe and EPC cultures treated with either DMSO control or VER at 25 μM. Samples were collected from both ASHe and EPC cultures at 0, 3, and 7 d.p.i. Statistical analyses were performed using the student's t test to compare between DMSO and VER treatments within each time point with level of significance indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

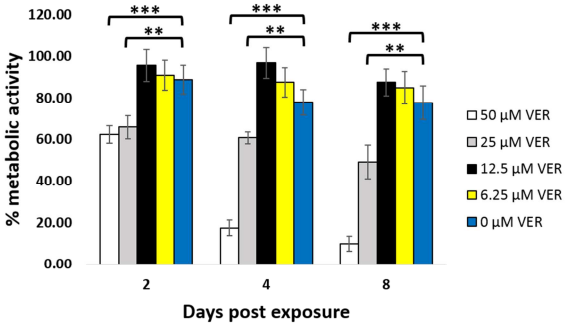
Figure 5. Effects of VER treatment and virus infections on Hsp70 protein expression in fish cell cultures

Panels (a) and (b) respectively show Hsp70 and actin proteins expression in ASHe and CHSE-214 cultures treated with either DMSO control or 25 μM VER. Protein lysates were collected at days 1, 3, and 5 post exposure for both cell lines. Panel (c) shows Hsp70 and actin proteins expression in ASHe cultures mock-infected (control), infected with IPNV, or infected with VHSV in two independent experiments. Protein lysates were collected at 2, 8, and 24 h.p.i.

Figure 6. Lack of co-immunoprecipitation of VHSV N protein and Hsp70

ASHe was infected with either control medium (-) or VHSV (+) for 2 days. Whole cell extract (WCE) fraction was collected using modified RIPA buffer at 2 d.p.i. The supernatant of the WCE fraction was incubated with VHSV N antibody-bound M-280 Dynabeads as the bait antibody. After incubation with the VHSV N antibody-bound M-280 Dynabeads, the supernatant (containing leftover proteins that did not interacted with the bait VHSV N antibody) was collected and saved for western blotting to detect the amount of leftover N protein. This is labeled as the SUP fraction. The IP fraction is the immunoprecipitated fraction containing Dynabeads and bound with VHSV N proteins. The top panel shows a western blot of all three fractions (from control and VHSV infected cultures) probed with VHSV N antibody. Strong N protein bands were detected in the WCE and SUP fraction of infected cultures while a visible but weaker N protein band was seen in the IP fraction. The bottom panel shows a western blot of the WCE, SUP and IP fractions probed with Hsp70 and actin antibodies. Both Hsp70 and actin were detected in the WCE and SUP fractions but were not present in the IP fraction.

(a) ASHe



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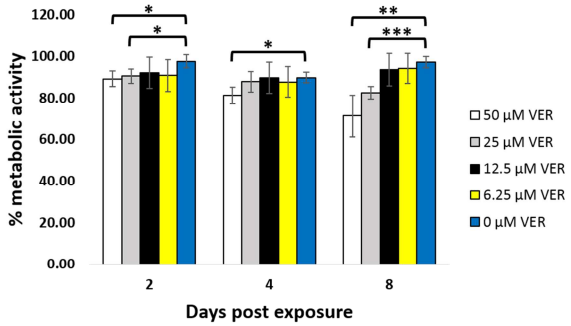
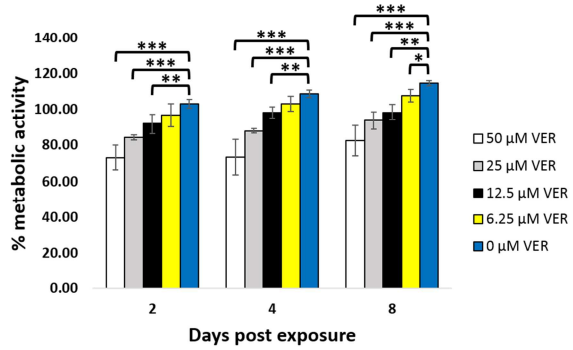
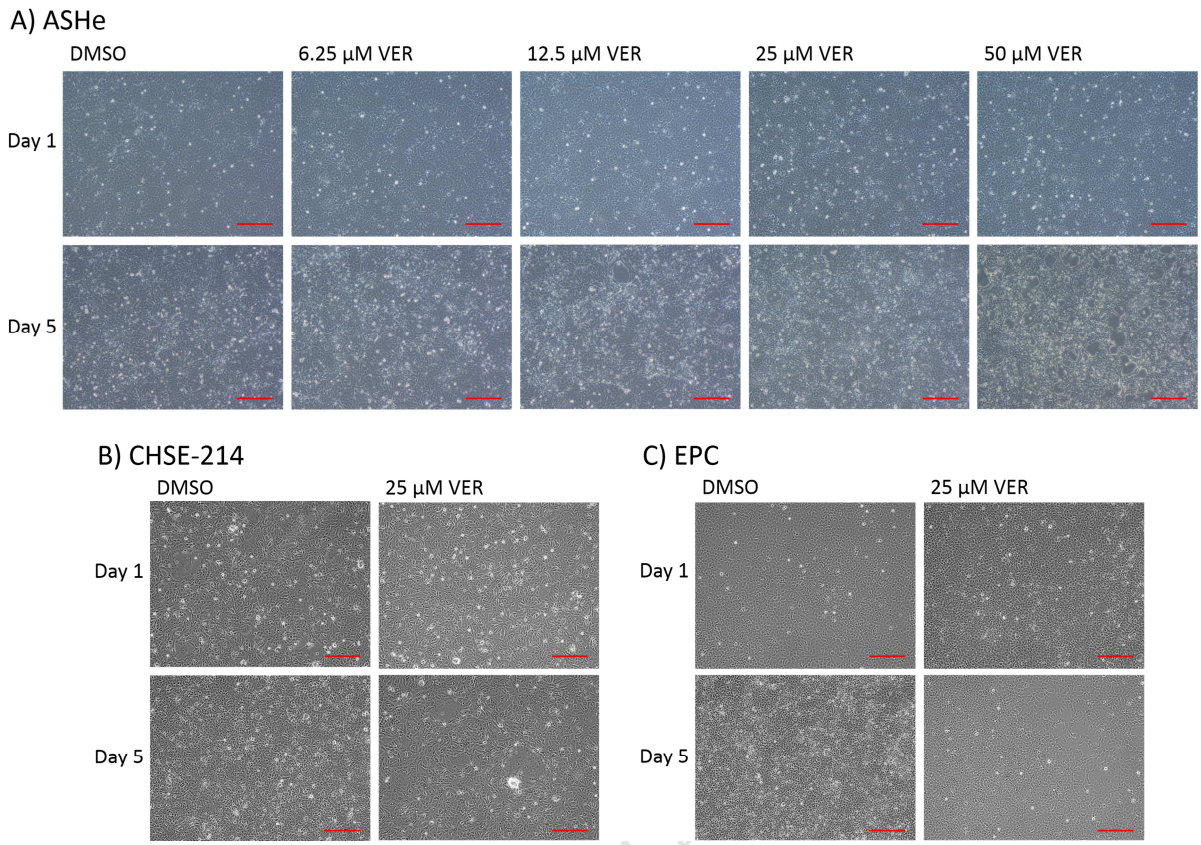
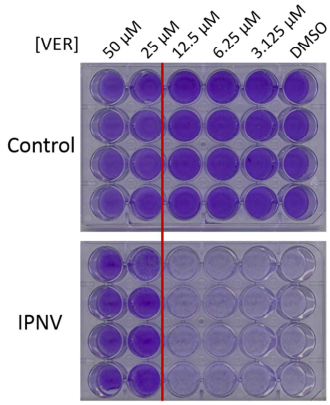


(c) EPC





(a) ASHe and IPNV



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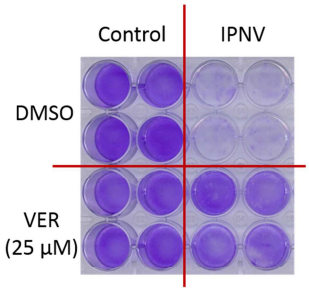

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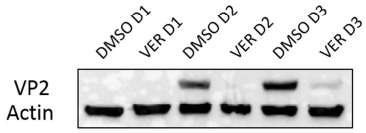
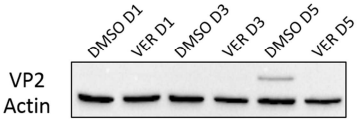


(d) CHSE-214 and IPNV



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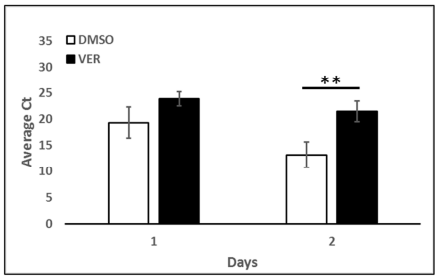

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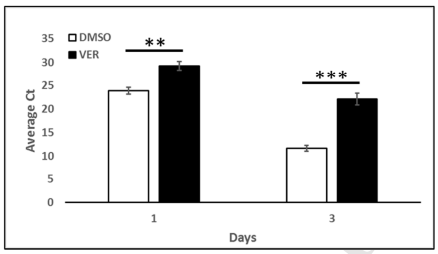
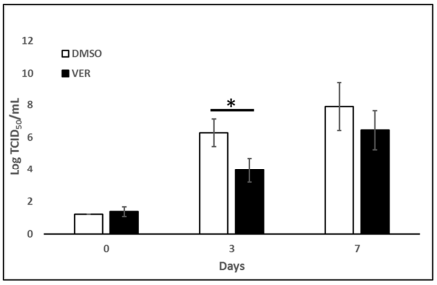
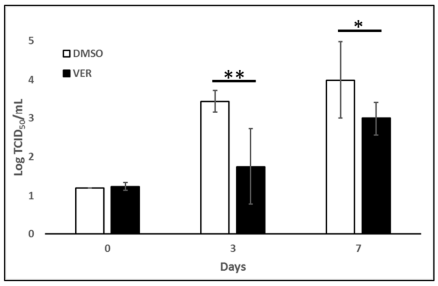


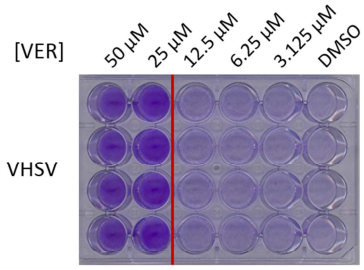
(g) ASHe and IPNV



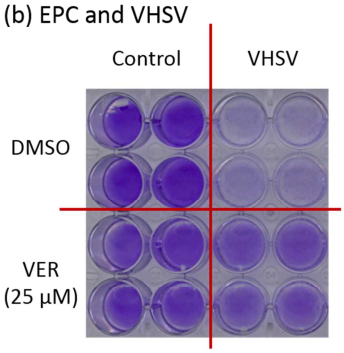
(h) CHSE-214 and IPNV



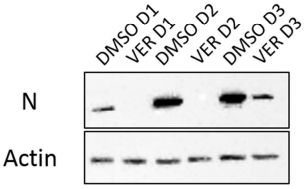
(a) ASHe and VHSV



(b) EPC and VHSV



(c) ASHe and VHSV



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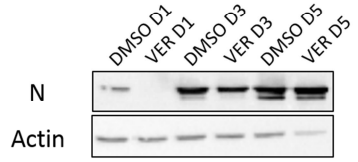

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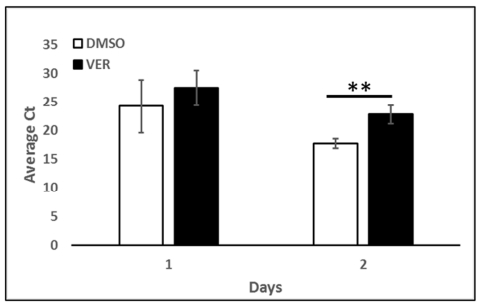
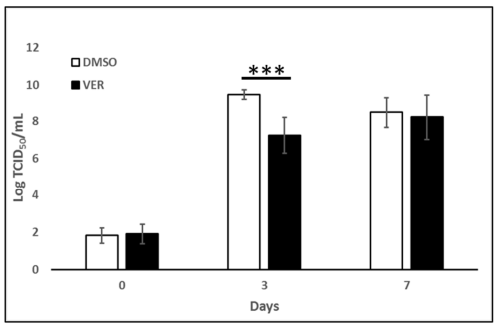


(g) ASHe and VHSV



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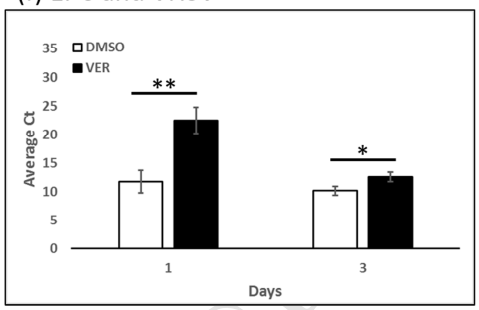
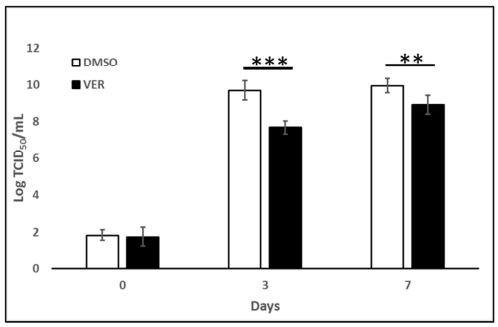


(h) EPC and VHSV



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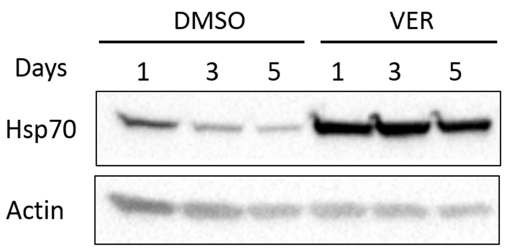

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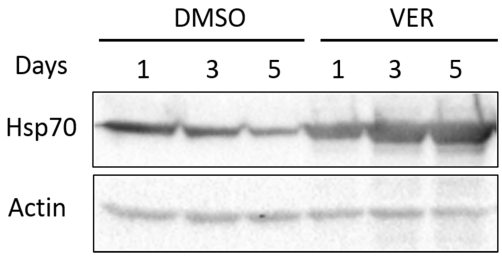

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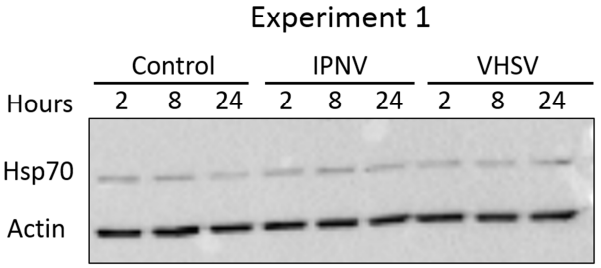

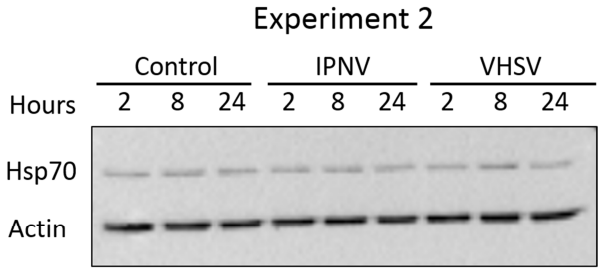

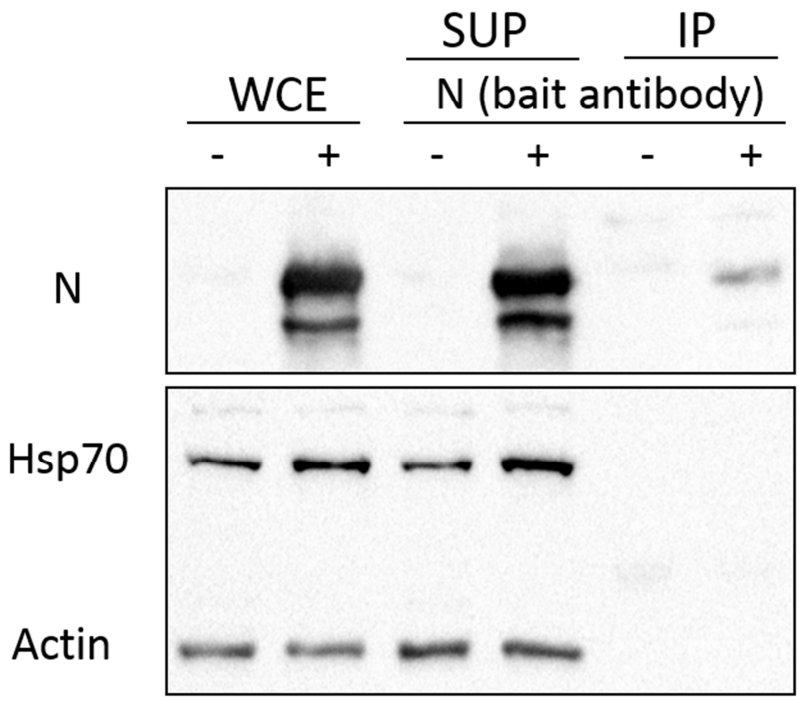

Highlights

- VER-155008 at 25 µM induces Hsp70 proteins in fish cell lines
- IPNV and VHSV did not induce Hsp70 proteins in fish cell lines during early infection
- VER-155008 at 25 µM reduces replication of both IPNV and VHSV in fish cell lines