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Z-FA-FMK demonstrates differential inhibition of aquatic orthoreovirus (PRV), aquareovirus (CSRV), and rhabdovirus (IHNV) replication

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ABSTRACT

Benzyloxycarbonyl-phenylalanyl-alanyl-fluoromethyl ketone (Z-FA-FMK) is a protease inhibitor that has been shown to strongly inhibit mammalian orthoreovirus replication. Here we explore the ability of Z-FA-FMK to inhibit three important yet genetically discrete aquatic fish viruses: chum salmon aquareovirus (CSRV), piscine orthoreovirus (PRV), and the rhabdovirus infectious hematopoietic necrosis virus (IHNV). Z-FA-FMK significantly attenuated CSRV *in vitro* transcription and infectious yield following low-dose (2–20 μM) exposure, yet a relatively high dose (200 μM) was required to completely block CSRV replication. For PRV and IHNV, no significant attenuation of *in vitro* viral transcription was observed following low-dose (2–20 μM) exposure; and although high dose (200 μM) exposure significantly attenuated both PRV and IHNV transcription, neither was completely inhibited. These transcriptional results were similarly reflected in IHNV infectious titre observed at 7 days post exposure. PRV titre is currently undeterminable *in vitro*; however, *in vivo* intra-peritoneal injection of PRV into juvenile Atlantic salmon (*Salmo salar*) in conjunction with 1.5 mg/kg Z-FA-FMK did not affect PRV replication as measured by blood associated viral transcripts at 14 days post challenge. These results indicate that aquatic ortho- and aqua-reoviruses appear to possess resilience to Z-FA-FMK relative to mammalian orthoreoviruses and suggest that environmental parameters or alternative mechanisms for viral replication may affect the efficacy of Z-FA-FMK as an antireoviral compound. Further, as Z-FA-FMK has been shown to irreversibly inhibit cysteine proteases such as cathepsins B and L *in vitro* at concentrations of ≤100 μM, continued replication of IHNV (and possibly PRV) at 200 μM Z-FA-FMK suggests that replication of these viruses can occur in a cathepsin-independent manner whereas CSRV likely requires cathepsins or similar cysteine proteases for successful replication.

Benzyloxycarbonyl-phenylalanyl-alanyl-fluoromethyl ketone (Z-FA-FMK) is a cell-permeable irreversible inhibitor of cysteine proteases including cathepsins B, S, and L (reviewed by Powers et al., 2002). It also inhibits effector caspases 2, 3, 6, and 7 (100 μM) without affecting the initiator caspases 8 and 10 (Lopez-Hernandez et al., 2003) and can inhibit T-cell proliferations and NF-κB activation in mammalian cells at a similar (100 μM) concentration (Lawrence et al., 2006). As many viruses require cysteine proteases for entry and/or maturation, Z-FA-FMK presents an attractive target for use as an antiviral compound. At 20 μM, Z-FA-FMK has been shown to block replication of mammalian orthoreovirus (MRV) serotype 3 – Dearing, which appeared to act through both cathepsin-dependent and -independent mechanisms (Kim et al., 2010). Nevertheless, the universality of Z-FA-FMK antiviral effects remain questionable as Kim et al. (2010) report that influenza A virus (orthomyxoviridae) and adenovirus (adenoviridae) remain unaffected. Furthermore it remains unknown as to whether Z-FA-FMK antiviral effects are limited to orthoreoviruses or whether efficacy extends to other reovirus members and/or other viral families. Thus, the primary purpose of this study was to determine if Z-FA-FMK could inhibit transcription and/or viral particle formation for three important aquatic viruses and if inhibition of virus replication from Z-FA-FMK may help to elucidate uncharacterized steps involved in the life cycle or pathogenesis of these viruses. Here we chose chum salmon aquareovirus (CSRV), piscine orthoreovirus (PRV), and the novirhabdovirus infection hematopoietic necrosis virus (IHNV) as important viruses of fish that infect a wide range of host species yet could each be evaluated in salmonid cells under relatively uniform temperature and culture conditions.

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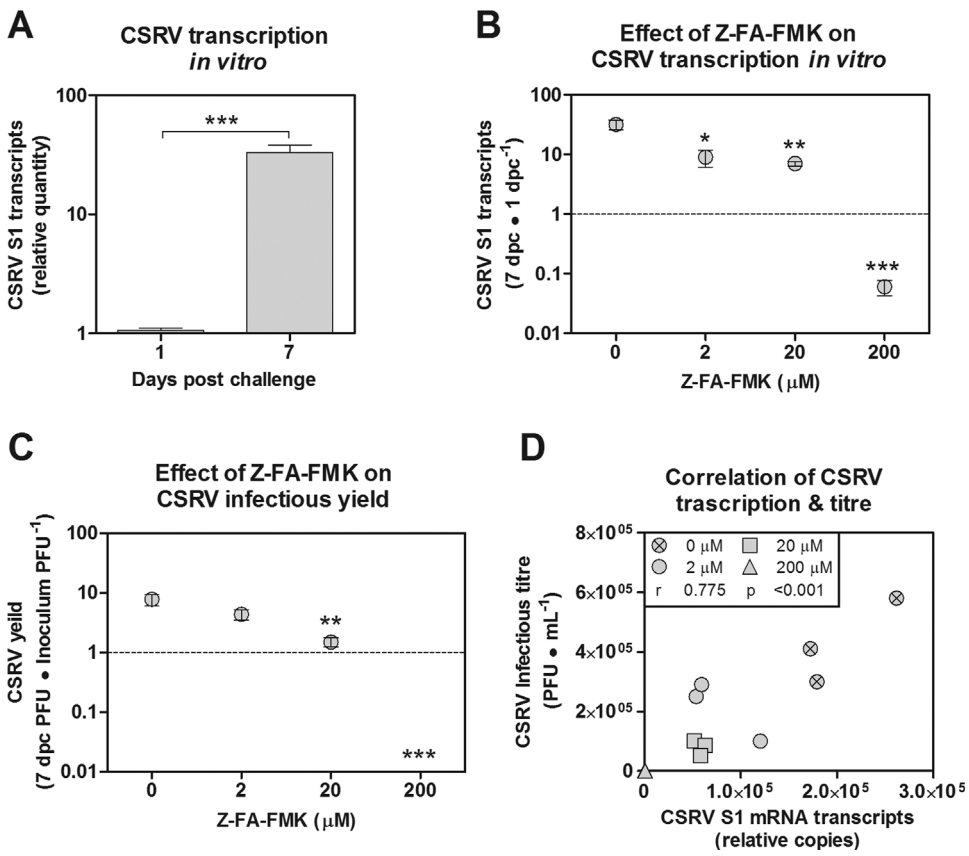

Fig. 1. Influence of Z-FA-FMK on chum salmon reovirus (CSRV) replication and infectivity. **(A)** Mean (± SEM) relative CSRV S1 transcripts at 1 and 7 days post-challenge (dpc) in absence of Z-FA-FMK. Asterisk indicates a significant increase ($p < 0.001$) at 7 dpc as determined by a one-tailed Student's *t*-test on the log-transformed values. **(B)** Mean (± SEM) relative fold change in CSRV S1 transcripts at 7 dpc relative to 1 dpc quantities under varying concentrations of Z-FA-FMK. **(C)** Mean (± SEM) increase in viral titre following 7 dpc in the presence of varying concentrations of Z-FA-FMK. Significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) reduction relative to Z-FA-FMK control (0 μM) cultures is presented in each figure as determined by one-way ANOVA followed by a Dunnett post hoc test on log-transformed data. ND: none (transcripts or plaques) detected. **(D)** Correlation between CSRV transcripts and titre at varying concentrations of Z-FA-FMK. Pearson r (r) and associated p-value are provided.

CSRV infection is typically nonlethal in salmon, although transient focal liver necrosis has been observed in both chum and Chinook salmon following infection (Winton et al., 1989, 1981) and the virus can cause significant cytopathic effects in multiple fish cell lines (DeWitte-Orr and Bols, 2007; Winton et al., 1981). Other aquareoviruses however, such as grass carp hemorrhage virus and Atlantic halibut reovirus, can be highly pathogenic and have contributed to significant fish mortality and economic loss (Blindheim et al., 2015; Rangel et al., 1999). To evaluate the potential for Z-FA-FMK to inhibit aquareovirus replication, we propagated CSRV (ATCC® CRL-1681) on monolayers of CHSE-214 cells (ATCC® VR1395) and infected with CSRV at a multiplicity of infection (MOI) of approximately 0.1 in the presence of either 2, 20, or 200 μM Z-FA-FMK (for detailed materials and methods see Supplement 1). A significant decrease in CSRV transcription (total RNA replication) was observed at all concentrations of Z-FA-FMK at 7 days post-challenge (dpc) relative to untreated controls (Fig. 1). CSRV segment 1 (S1) transcription was reduced (3–5 fold) in the presence of 2–20 μM Z-FA-FMK and at 200 μM a > 500 fold reduction in viral transcripts were observed indicating a net loss rather than a gain of viral transcripts relative to 1 dpc quantities (Fig. 1B). A similar pattern of inhibition was observed in the production of infectious particles. Viral titre at 7 dpc was significantly reduced (5 fold) at 20 μM Z-FA-FMK and undetectable at 200 μM concentration of the drug (Fig. 1C). Further, a high correlative agreement between viral mRNA transcription and infectious particle formation was observed in this study (Fig. 1D) which indicates that the major influence of Z-FA-FMK on CSRV replication occurred prior to transcription; most probably during viral disassembly. This conclusion is supported by previous studies demonstrating the potent inhibition of cathepsin B and L by Z-FA-FMK (Ahmed et al., 1992; Rasnick, 1985) and the requirement of these enzymes by MRV for effective disassembly (Ebert et al., 2002). Although Z-FA-FMK has also been hypothesized to affect MRV packaging and viral factory development (Kim et al., 2010), the close relationship between transcription and infective particle formation in this study suggests that this effect (if present) would appear secondary in consideration of Z-FA-FMK's effect on CSRV. Nevertheless, it should be noted that the concentration of Z-FA-FMK used to block CSRV replication in CHSE-214 cells at 15 °C in this study (200 μM) was ten-fold higher than that required to block MRV replication in human-derived HT 1080 cells at 37 °C (20 μM) (Kim et al., 2010), indicating that alternative reoviral life cycle strategies or host environmental conditions can dramatically affect Z-FA-FMK anti-reoviral efficacy.

PRV is ubiquitous in many salmon-producing countries and has been demonstrated to be the causative agent of a disease known as heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (Wessel et al., 2017). Studies have indicated that, despite sharing sequence similarity to both orthoreoviruses and aquareoviruses, PRV remains phylogenetically distinct, only loosely grouping with other orthoreoviruses (Key et al., 2013; Kibenge et al., 2013). PRV is also distinct from other known reoviruses with regard to its tropism for infecting erythrocytes (Finstad et al., 2014; Wessel et al., 2015) and to date has not been effectively cultured in any other cell type. As PRV currently cannot be cultured *in vitro* using typical cell lines, we adapted previous *ex vivo* culturing methods developed by Wessel et al. (2015) using purified Atlantic salmon erythrocytes to test if Z-FA-FMK could effect PRV transcription in a laboratory setting. At 200 μM Z-FA-FMK, a significant decrease in PRV transcription (3.5 fold) was observed 14 dpc relative to untreated controls (Fig. 2B). However, erythrocyte viability also significantly decreased (4 fold) 14 dpc at 200 μM Z-FA-FMK (Fig. 2C), suggesting the reduction in PRV transcription at 200 μM concentration may have occurred as an indirect effect of reduced cell viability and not as a direct consequence of Z-FA-FMK on viral entry, disassembly or transcription. The cytotoxicity of 200 μM Z-FA-FMK to Atlantic salmon erythrocytes in this study was somewhat unexpected, as no cytotoxicity, reduced viability, or reduction in total RNA expression was observed in CHSE-214 cells during this trial at similar concentrations (data not shown) and 100 μM Z-FA-FMK has previously been found not to decrease cell viability in human-derived Jurakat T-

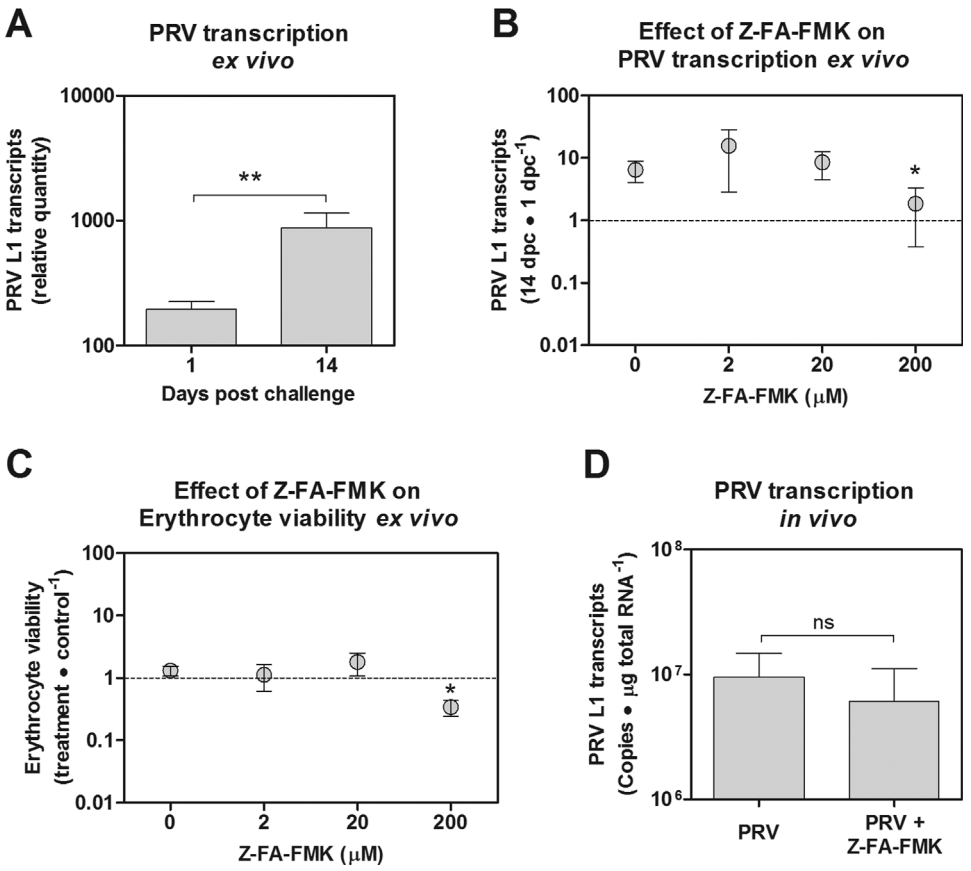

Fig. 2. Influence of Z-FA-FMK on piscine reovirus (PRV) replication. **(A)** Mean (± SEM) *ex vivo* relative PRV L1 transcripts at 1 and 14 days post-challenge (dpc) relative to the minimum value obtained in absence of Z-FA-FMK. Asterisks indicate a significant increase ($p < 0.001$) between 1 and 14 dpc as determined by a one-tailed paired Students *t*-test on the log-transformed values. **(B)** Mean (± SEM) relative fold change in *ex vivo* PRV L1 transcripts at 14 dpc relative to 1 dpc quantities under varying concentrations of Z-FA-FMK. **(C)** Mean (± SEM) relative fold change in *ex vivo* erythrocyte viability detected at 14 dpc relative to the untreated control under varying concentrations of Z-FA-FMK. **(D)** Mean (± SEM) change *in vivo* PRV L1 transcripts at 14 dpc with and without Z-FA-FMK. Significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) reduction relative to Z-FA-FMK control (0 μM) cultures is presented in each figure as determined by one-way ANOVA followed by a Dunnett post hoc test on log-transformed data. Ns: no significance.

cells at 37 °C (Liow and Chow, 2013). Further studies of Z-FA-FMK cytotoxicity in nucleated erythrocytes of fish are necessary; however, these results imply that while Z-FA-FMK may have inhibitory effects on orthoreoviruses such as MRV and, to a lesser degree, aquareoviruses such as CSRV, Z-FA-FMK appears to have only a minor effect on PRV entry, disassembly and transcription. This suggests that the discrete phylogeny and tropism of PRV for infecting erythrocytes has manifested unique strategies for early entry and replication of this virus relative to other orthoreoviruses. However, it should be noted that *ex vivo* PRV replication in this and previous studies has been relatively poor (approximately 10 fold increase over 14 days in this study; Fig. 2A) which may obscure the potential efficacy of Z-FA-FMK in this model system.

To see if Z-FA-FMK could inhibit infectious particle formation and haematogenous dissemination of PRV *in vivo*, Atlantic salmon smolts were administered an intra-peritoneal injection of 100 μL PRV inoculum which contained 2000 μM Z-FA-FMK (~1.5 mg/kg). No significant difference in PRV transcription was observed in PRV blood load between fish injected with Z-FA-FMK and those that were not at 14 dpc; all PRV injected fish developed substantial blood loads reaching approximately 10^7 PRV L1 transcripts per 1 μg total extracted RNA by 14 dpc (Fig. 2D). Thus, a single injection of 2000 μM Z-FA-FMK does not appear to be an effective treatment for blocking PRV replication and dissemination *in vivo*. Although it is possible that a higher dosage of Z-FA-FMK may prove more efficacious against PRV, the cytotoxicity of Z-FA-FMK to nucleated erythrocytes *ex vivo* observed in this study presents concern for experimenting with higher doses during *in vivo* challenges, particularly given the poor efficacy of this drug in blocking PRV *ex vivo*.

In contrast to the typical sub-lethal effects observed during CSRV and PRV infection in salmon, IHNV often causes severe disease resulting in high mortality and is a pathogen of significant regulatory importance to the World Organisation for Animal Health (OIE) (OIE, 2009). As a rhabdovirus, IHNV also employs distinctly different entry and replication strategies compared to reoviruses like CSRV and PRV. Specifically, there is currently no evidence to suggest rhabdoviruses require cysteine proteases such as cathepsins B or L during cell entry (reviewed by Albertini et al., 2012) nor do they produce “factory-like” inclusions during assembly—two factors considered to be the most likely mechanisms by which Z-FA-FMK inhibits MRV replication (Kim et al., 2010). Nevertheless, the possible contribution of cysteine proteases to rhabdoviral entry is unknown. In this study, experiments to assess the effect of Z-FA-FMK on IHNV replication were carried out in a similar manner to those described for CSRV where a virulent isolate of IHNV (BC93-057; Garver et al., 2013) was propagated on CHSE-214 cells at an MOI of 0.1 in the presence of 0, 2, 20, and 200 μM Z-FA-FMK. Surprisingly, a significant decrease in IHNV nucleoprotein (N) transcription (approximately 4 fold) was observed at 5 dpc relative to untreated controls at the 200 μM Z-FA-FMK (Fig. 3B). Although this reduction was relatively minor with regard to overall transcription—IHNV N transcription was still nearly 100 fold higher at 5 dpc than at 1 dpc—and may in part be due to Z-FA-FMK’s direct effect on CHSE-214 transcription (~2-fold; Fig. 3C), it nevertheless indicates that Z-FA-FMK can reduce early IHNV entry/replication, probably through its inhibition of cysteine proteases which may enhance virus entry. A similar pattern of inhibition was also seen for IHNV infectious particle production but appeared to be magnified; viral titre at 5 dpc was significantly reduced by more than 10 fold in the presence of 200 μM Z-FA-FMK (Fig. 3D). The lack of inhibition at 2 and 20 μM Z-FA_FMK is consistent with the previous observations that Z-FA-FMK does not affect viruses such as rhabdoviruses and orthomyxoviruses which can demonstrate cathepsin-independent viral disassembly (Albertini et al., 2012; Kim et al., 2010). Nevertheless, the significant reduction in IHNV transcription and infectious particle production afforded by 200 μM Z-FA-FMK also indicates that cysteine proteases such as cathepsins B and L, although not required, may enhance rhabdoviral entry. Alternatively, the inhibition of transcription and infectious

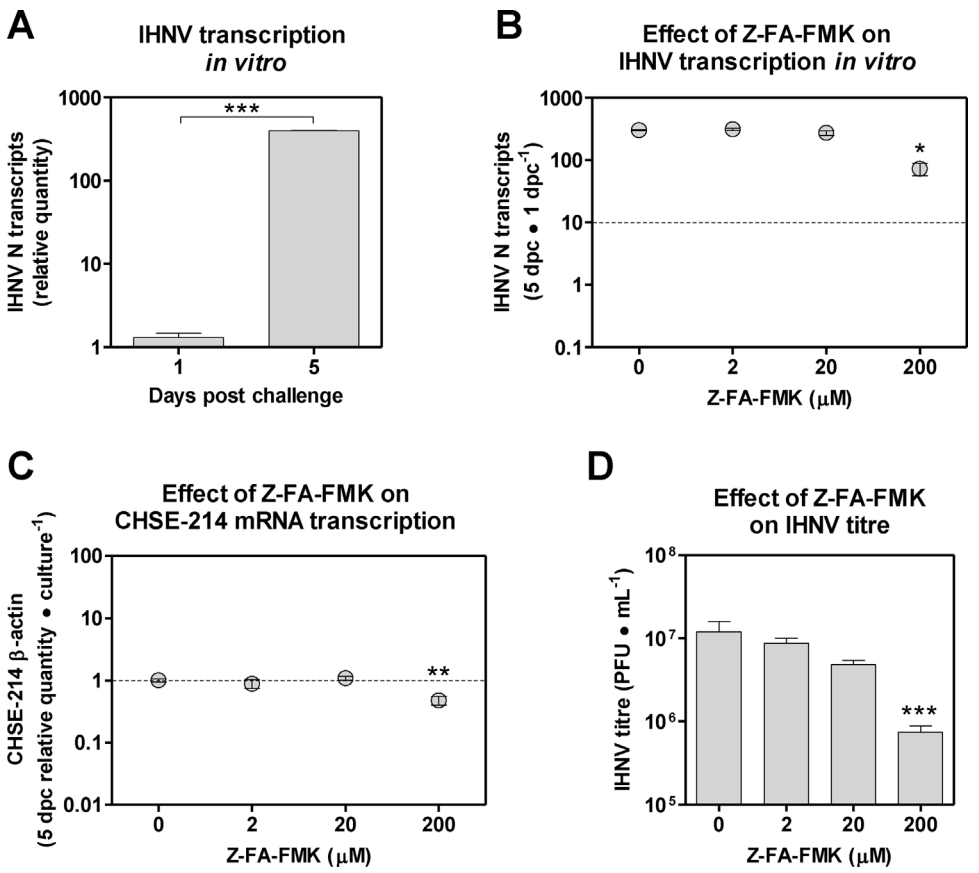

Fig. 3. Influence of Z-FA-FMK on infectious hematopoietic necrosis virus (IHNV) replication and infectivity. **(A)** Mean (± SEM) relative IHNV N gene transcripts at 1 and 5 days post-challenge (dpc) in absence of Z-FA-FMK. Asterisks indicate a significant increase ($p < 0.0001$) at 5 dpc as determined by a one-tailed unpaired Student's *t*-test on the log-transformed values. **(B)** Mean (± SEM) fold change in IHNV N gene transcripts at 5 dpc relative to 1 dpc quantities under varying concentrations of Z-FA-FMK. **(C)** Mean (± SEM) expression of CHSE-214 B-actin mRNA following 5 dpc in the presence of varying concentrations of Z-FA-FMK. **(D)** Mean (± SEM) IHNV yield following 5 dpc in the presence of varying concentrations of Z-FA-FMK. Significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) reduction relative to Z-FA-FMK control (0 μM) cultures is presented in each figure as determined by one-way ANOVA followed by a Dunnett post hoc test on log-transformed data.

particle formation at 200 μM Z-FA-FMK could indirectly be affecting host cell processes that favor rhabdoviral replication and further investigation will be needed. Nevertheless, these results indicate that Z-FA-FMK is not a potent inhibitor of IHNV transcription or viral particle formation *in vitro* and has limited utility as an anti-rhabdoviral compound.

In conclusion, Z-FA-FMK exhibited inhibitory effects on CSRV transcription and viral particle formation, but to a lesser degree than what has been previously demonstrated for MRV in homeothermic mammal cells (Kim et al., 2010). As both transcription and CSRV titre were correlatively inhibited by Z-FA-FMK, it is likely that Z-FA-FMK's main mechanism for aquareoviral inhibition focuses on processes upstream of transcription. Based on Z-FA-FMK's known inhibition of cysteine proteases such as cathepsins, it is likely that the affected process is viral disassembly. This data supports a potential similarity between ortho- (MRV) and aquareoviral (CSRV) disassembly mechanisms. However, PRV did not appear to be notably affected by Z-FA-FMK *ex vivo* or *in vivo*, which implies that PRV may be significantly divergent from other known ortho- and aquareoviruses, or alternatively, that the nucleated erythrocytes targeted by this virus present unique alternatives for reoviral entry and uncoating relative to other cell types typically targeted by viruses of this family. Lastly, inhibition of IHNV was not achieved by Z-FA-FMK in these experiments except to a limited degree at high (200 μM) concentrations of the drug, tentatively suggesting that cysteine proteases aid but are not required during IHNV disassembly. These findings indicate that Z-FA-FMK may be a viable treatment against typical aquatic reovirues such as CSRV, but have limited capacity for affecting other aquatic viral families such as rhabdoviruses like IHNV or reoviruses with high family divergence such as PRV.

Competing interests

The authors declare they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2017.11.024.

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