

DOI 10.2478/pjvs-2014-0108

Short communication

Small interfering RNA treatment can inhibit *Cyprinid herpesvirus 3* associated cell death *in vitro*

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Abstract

A *Cyprinid herpesvirus 3* infection of carp induces a disease which causes substantial losses in carp culture. Here we present the use of a possible strategy for the management of the virus infection RNA interference based on small interfering RNAs. As a result of *in vitro* studies, we found that a mixture of short interfering RNAs specific for viral DNA enzyme synthesis and capsid proteins of the CyHV-3 can be a potential inhibitor of virus replication in fibroblastic cells. This gives the basis for the development of a combinatorial RNA interference strategy to treat CyHV-3 infections.

Key words: RNA interference, small interfering RNA, *Cyprinid herpesvirus 3*, type I interferon

Introduction

An infection with the *Cyprinid herpesvirus 3* (CyHV-3) induces a disease which significantly harms carp aquaculture worldwide. Currently no treatment besides preventional vaccinations is available. One possible strategy for the management of a viral infection is the application of RNA interference (RNAi), a post-transcriptional gene silencing which is showing great potential in human and veterinary medicine. RNAi has also been successfully used in fish, especially against RNA viruses with a simple genome (Lima et al. 2013). However, the main challenge when designing a treatment against CyHV-3 is the large complex genome which encodes

for 155 possible proteins. Therefore, the selection of single genes as targets for the most effective disease management could be problematic. The aim of the presented work was to investigate a suitable treatment strategy and identify the potential of small interfering RNA (siRNA) for the treatment of CyHV-3 infections *in vitro*.

Materials and Methods

For gene silencing, 21 to 27 bp long siRNA oligonucleotides (sequences in Table 1) were designed to knock down mRNA expression of (i) the viral capsid proteins: capsid triplex protein (CTP) and ma-

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Table 1. Sequences of siRNA used in this study. The symbol * indicates phosphorothioate linkages, the lower case letters indicate 2'-O-methyl-modified nucleotides, dT indicates deoxythymidine.

Target	Length	5'-3' sequence sense/antisense
CyHV-3 CTP	21	GGCCGAGUUCCACAACUUUAAdTdT UAAAAGUUGUGGAACUCGGCCdTT
CyHV-3 DH	21	GUGUGUGAGGUUCAAGCUAG*A*dT*dT UCUAGCUUGAACCUCACACa*c*dT*dT
CyHV-3 DP	25	GCGACGUGCUGUUUGUCUUAACUAdTdT UAGUUGAAGACAAACAGCACGUCGdTT
CyHV-3 MCP	27	CACCAACCUCAACGACUUUCUGAGA*G*A UCUCUCAGAAAGUCGUUGAGGUUG*U*G

for capsid protein (MCP) or (ii) the viral DNA synthesis enzymes: DNA helicase (DH) and DNA polymerase (DP).

Common carp brain (CCB) fibroblasts were cultured and infected with CyHV-3 in 24 well plates as previously described (Adamek et al. 2012). After 1h of infection with 1×10^3 TCID₅₀ ml⁻¹ of CyHV-3, cell monolayers were washed twice with cell culture medium and subsequently incubated in triplicates with 100 µl of transfection solution consisting of culture medium without foetal calf serum (FCS), 3 µl HiPerFect transfection reagent (Qiagen, Germany) and 1.5 µl of 2 µM siRNA oligo targeting one specific CyHV-3 gene (see Table 1). Other monolayers were transfected with a mix of all four siRNAs, or 10 µg ml⁻¹ of polyinosinic:polycytidylic acid (poly I:C). For controls, cells were transfected with 6 µl of 2 µM nonsense siRNA (AllStars Negative Control, Qiagen, Germany). Non-infected controls were also included for each of the treatments. After 18h of incubation, all cell monolayers were washed twice with medium, then 1ml of fresh culture medium supplemented with FCS was added and the cells were incubated for 7 days at 25°C. The cells were then fixed with 4% formalin for 10min, washed with water and stained with 0.5% crystal violet (in 25% methanol) for 30min. After removal of all unbound dye by washing, the cells were air dried and digitally recorded. Next, the dye was extracted from the cells by the addition of 500 µl of 10% acetic acid per well and the optical density of the dye extracts was measured at 590 nm using a FLUOstar plate reader (BMG Labtech, Germany). A statistical analysis was performed by ANOVA with a Holm-Sidak test for multiple comparisons as post hoc test.

Results and Discussion

Microscopic analysis of the cell monolayers showed that at day 8 p.i., a severe cytopathic effect (CPE) was detected in most of the cell cultures (Fig. 1A). A CPE was not seen in uninfected controls. Interestingly, in cell cultures treated with the mix of all four siRNAs, as well as in cultures stimulated by poly I:C treatment for an induction of a type I interferon response, a lower level of CPE was observed. The crystal violet staining (Fig. 1B) confirmed that the treatment of cells with single target siRNA did not lower CPE formation (survival of 48% to 57% of cells), when compared to non-treated and nonsense siRNA treated cells (both destruction of 50% of cells). In contrast to our observations, another recent study showed that treatment of CyHV-3 infected CCB cells with siRNA targeting against a single gene, CyHV-3 DNA polymerase, could also be effective in inhibiting virus replication (Gotesman et al. 2014). However, as Gotesman et al. (2014) analysed the number of released virus particles and not the influence on cell viability, the results are difficult to compare with the observations presented here. Interestingly, when the virus was treated with a mixture of all four oligos, a statistically significant reduction in the number of killed cells was achieved (survival of 79% of cells). This indicates that mixing siRNAs which target different viral genes could be the most promising approach for a treatment of CyHV-3. However, the treatment of cells with siRNAs and its modifications may induce a type I IFN response in cells (Schyth et al. 2012), which can be responsible for the observed reduction in CyHV-3 replication rather than the silencing of CyHV-3 genes by the siRNA oligonucleotides. This concern is supported by our data (Figs. 1A, 1B), where we showed that the induction of type I IFN responses with poly I:C can limit the replication

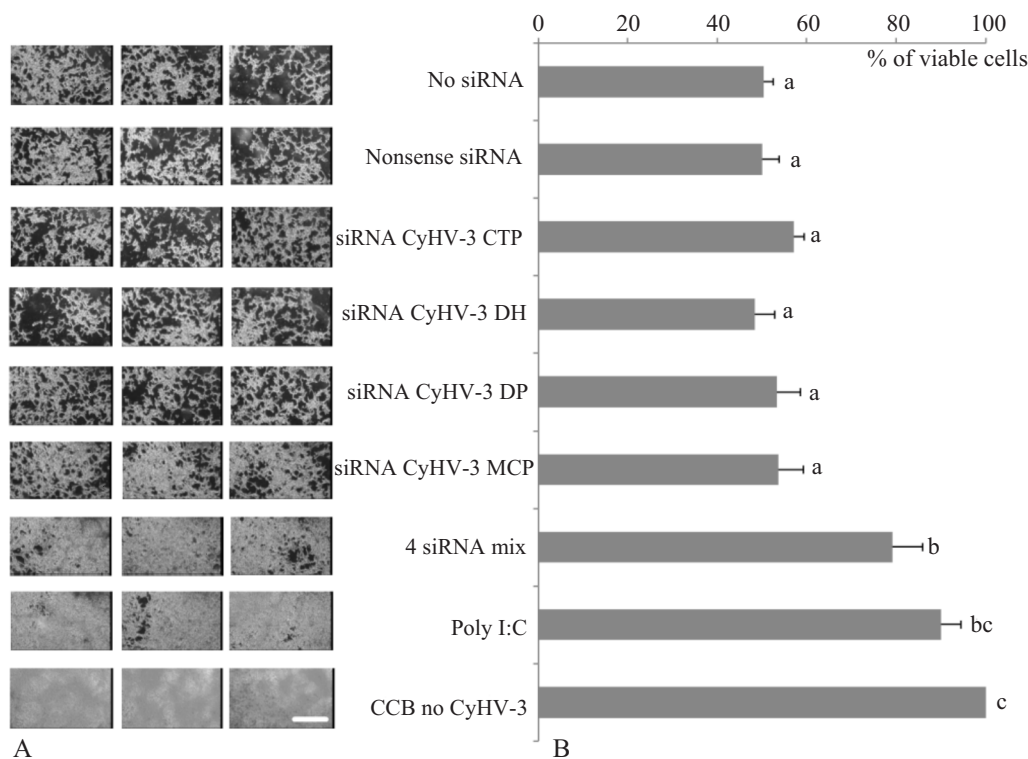


Fig. 1. (A) CCB cells infected with 1×10^3 TCID₅₀/ml of CyHV-3 for 1h followed by 18h of siRNA or poly I:C treatment, harvested at 8 d.p.i. and stained with crystal violet (0.5%). White bar indicates a size of 300 μ m. (B) Percentage (+SD) of viable cells based on optical density readings ($n=3$ cultures per treatment) of crystal violet bound to cells which survived infection. Letters a, b and c indicates statistically significant differences at $P \leq 0.05$.

of CyHV-3, leading to the survival of 90% of the cells from the infected monolayer. However, our nonsense siRNA control was used in the same concentration as the mix of four siRNAs, and the treatment with the nonsense mix gave the same results as in the non-treated cells (50% survival). Therefore we can conclude that a mixture of siRNAs specific for viral DNA enzyme synthesis and capsid proteins of the CyHV-3 can be a specific inhibitor of virus replication in carp cells. This conclusion makes mixes of siRNAs potentially interesting as candidates for a further treatment of the disease caused by CyHV-3 infection. However, before this can be widely applied, additional studies must be conducted to ascertain the underlying mechanisms. Further research also needs to be carried out to develop an improved combinatorial treatment based on multiple shRNAs expressed on one vector, which could be used in *in vivo* studies.

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