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Species specific inhibition of viral replication using dicer substrate siRNAs (DsiRNAs) targeting the viral nucleoprotein of the fish pathogenic rhabdovirus *viral hemorrhagic septicemia virus* (VHSV)

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ABSTRACT

Gene knock down by the use of small interfering RNAs (siRNAs) is widely used as a method for reducing the expression of specific genes in eukaryotic cells via the RNA interference pathway. But, the effectivity of siRNA induced gene knock down in cells from fish has in several studies been questioned and the specificity seems to be a general problem in cells originating from both lower and higher vertebrates. Here we show that we are able to reduce the level of viral gene expression and replication specifically in fish cells *in vitro*. We do so by using 27/25-mer DsiRNAs acting as substrates for dicer for the generation of siRNAs targeting the nucleoprotein N gene of *viral hemorrhagic septicemia virus* (VHSV). This rhabdovirus infects salmonid fish and is responsible for large yearly losses in aquaculture production. Specificity of the DsiRNA is assured in two ways: first, by using the conventional method of testing a control DsiRNA which should not target the gene of interest. Second, by assuring that replication of a heterologous virus of the same genus as the target virus was not inhibited by the DsiRNA. Target controls are, as we have previously highlighted, essential for verification of the specificity of siRNA-induced interference with virus multiplication, but they are still not in general use.

1. Introduction

The cellular mechanism known as RNA interference (RNAi) can be programmed for sequence-specific degradation of messenger RNA (mRNA) targets by double-stranded RNA molecules (dsRNA) with sequence identity to the target (Fire et al., 1998; Caplen et al., 2001). The mechanism is mediated by an RNase III enzyme known as Dicer which is responsible for cutting the dsRNA into 21–23 bp small interfering RNAs (siRNAs; Zamore et al., 2000) and delivering these into the RNA-induced silencing complex RISC (Liu et al., 2006). RISC binds the target mRNA by using one of the siRNA strands as a guide and degrades the target by its endonuclease activity (Zamore et al., 2000; Schwartz et al., 2004). The mechanism has been tested extensively as a means of targeting viral genes with the perspective of establishing the use of siRNAs as active compounds in antiviral medicine (Bitko et al., 2004; Li et al., 2005; Morissey et al., 2005; Palliser et al., 2006; DeVincenzo et al., 2010; Lyall et al., 2011). But the specificity of siRNA mediated viral inhibition is challenged by their immune stimulatory potential (Robbins et al., 2008). Furthermore, because stimulation of the interferon system has in some cases been shown to be dependent on the sequence of the siRNA (Hornung et al., 2005; Sioud, 2005) the use of a virus that is closely related to the target virus seems more relevant to use as a control than using a control siRNA with a mismatched or even non-relevant sequence. We have previously used a heterologous interferon sensitive virus as control to test the specificity of *in vitro* transcribed antiviral siRNAs (Schyth et al., 2006). This control was able to reveal a very potent but non-specific antiviral interferon response induced possibly through interaction between the *in vitro* transcribed siRNAs and the cytoplasmic receptor RIG-I like it has been shown in mammalian cells (Kim et al., 2004). Despite these findings, the use of target related viruses as controls is still not common in use. Regarding RNAi activity in fish cells, several studies have claimed a lack of both effectivity and specificity (reviewed in Schyth, 2008). We are currently seeking new strategies to overcome these problems. In this work we evaluate a 27/25-mer long chemically synthesized dsRNA intended as substrate for Dicer cleavage into 21-mer nt siRNAs, a type of siRNAs which have been shown effective for gene silencing in mammals (Kim et al., 2005; Rose et al., 2005). By using this strategy the generation of mature siRNAs takes place inside the cell thereby possibly linking siRNA production by Dicer with incorporation into RISC (Kim et al., 2005). We hoped that the resulting duplex and its loading into RISC via Dicer–RISC interaction would make this approach better adapted for functionality in the fish cells.

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Our target was the rainbow trout pathogenic virus *viral hemorrhagic septicemia virus* (VHSV). The viral negative sense RNA genome is 11 kb long and contains six open reading frames encoding the nucleoprotein N, phosphoprotein P, matrix protein M, glycoprotein G, non-structural protein NV and the polymerase L protein (Schutze et al., 1999). We designed our siRNAs to target the mRNA transcript encoding the viral nucleocapsid protein N. In rhabdoviruses this protein is used for packaging the viral genome in the nucleocapsid. It assures binding between the viral genome, the phosphoprotein P and the L polymerase in the ribonucleoprotein (RNP) complex required for transcription and replication of the viral genome and it has an important role in the shift from transcription to replication of the rhabdoviral genome (Patton et al., 1984; Finke and Conzelmann, 2005). For these reasons the N protein has previously been used as the target for siRNAs in rhabdoviruses (Brandão et al., 2007; Israsena et al., 2009). We test the specificity of our DsiRNA by the use of a heterologous virus as target control.

2. Materials and methods

2.1. Cells and virus

All studies were carried out using the fish cell line Chinook Salmon Embryo (CHSE-214; Lannan et al., 1984) grown at 20 °C in Eagle's minimum essential medium (MEM) (Invitrogen, Cat. No. 61100-103) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin and buffered with Tris–HCl buffer. The virus used was the VHSV strain DK-3592B which was freshly grown in CHSE-214 cells. The virus stock used for all studies was produced as 0.22 μm filtered supernatant from infected cell layers showing 80% cytopathogenic effect. The viral titer (Reed and Muench, 1938) of VHSV was $2.5 * 10^6$ $TCID_{50}$/ml. A stock of the VHSV related virus *infectious hematopoietic necrosis virus* (IHNV strain US220-90; Acc. No. GQ413939) was produced for testing the specificity of the VHSV specific DsiRNA. The batch of IHNV held a Titer of $1.3 * 10^6$ $TCID_{50}$/ml.

2.2. DsiRNA against VHSV DK-3592B nucleocapsid

A double stranded RNA Dicer substrate (DsiRNA) of 27/25-mer length (Kim et al., 2005; Rose et al., 2005) was designed to target the nucleocapsid (N) mRNA of VHSV strain DK-3592B (Acc. No. AF012093). For this purpose the bioinformatics tool RNAi Design from IDT SciTools (IDT, USA) was used. The DsiRNA with the highest scoring result obtained was selected corresponding to the duplex: 5′-CCA CAA UCA CCU UCC UGU CUU CCU CAC-3′ (sense strand) and 5′-GAG GAA GAC AGG AAG GUG AUU GUG G-3′ (antisense strand). As can be seen from the alignment in *Fig.* A1 (supplementary material) the target sequence we chose was not conserved for all VHSV genotypes. We found maximally two misaligning nucleotides when comparing target sequences among a selection of VHS viruses of the same genotype. A DsiRNA targeting human influenza H1N1-2009 nucleocapsid 5′-ACU CCA UUG CUA UUG UUC CAA CUC CUU-3′ (sense strand); 5′-GGA GUU GGA ACA AUA GCA AUG GAG T-3′ (antisense strand) was used as siRNA control.

2.3. Transfection method

Cells were trypsinized, diluted in growth medium, $4 * 10^5$ cells seeded into each well of a 24 well plate and incubated for 24 h before transfection at a cell confluence of approximately 80%. The cationic lipid-based transfection reagent Trifectin® (IDT, USA) was used for the transfection. Briefly, a 100 μl transfection mix was prepared in the following way. In one tube the desired amount of siRNA was mixed with the medium containing 4% FBS without antibiotics to a final volume of 50 μl and incubated for 10 min at room temperature. In another tube 4 μl of Trifectin was mixed with the medium to a final volume of 50 μl and subsequently combined with the siRNA solution, briefly centrifugated and incubated at room temperature for 10 min. The medium was removed from each cell culture well and 500 μl fresh medium was added. The Trifectin-siRNA solution was then added to each well followed by incubation at 15 °C. Successful transfection was shown using a fluorescein labeled random nucleotide control siRNA: 5′-UUC UCC GAA CGU GUC ACG UdTdT-3′ (sense strand); 5′-ACG UGA CAC GUU CGG AGA AdTdT-3′ (antisense strand; Qiagen, Germany, Cat. No. 1022079). The transfection efficiency was determined by fluorescence microscopy after 24 h incubation. Pictures of the cell cultures were taken at 20× magnification using a Leica DMIL light microscope equipped with a Leica DC300 camera and analyzed using the Software TotalLab TL120 (Nonlinear dynamics, USA), evaluating the fluorescence emitting area with respect to the total area of cells in the picture.

2.4. Effect of nucleocapsid targeting DsiRNA at the mRNA level

We established an assay for testing the efficiency of DsiRNA inhibition of viral replication. Briefly, antiviral and control siRNAs formulated in Trifectin were transfected into CHSE-214 cells grown in 96 well plates (2.6×10^5 cells/well) directly following infection of the same cell layers with VHS virus. Following infection and transfection with DsiRNAs cells were incubated for 24 h at 15 °C upon which they were assayed for the level of VHSV-N mRNA by qPCR. Four different concentrations of DsiRNA against the nucleocapsid of VHSV (1 nM, 4 nM, 15 nM and 30 nM) were tested. Controls included wells transfected with DsiRNA targeting human influenza H1N1-2009 nucleocapsid and negative cells without virus added but transfected with DsiRNA against VHSV-N. The set-up was tested with four different dilutions of the VHSV batch (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). Each combination of DsiRNA concentration and viral dilution was initially tested with two replicates ($n = 2$) and then repeated with six replicates ($n = 6$). Cells were lysed using the TRK lysis buffer (Omega-Biotek, USA) and total RNA was purified using E.Z.N.A.® Total RNA Kit I (Omega-Biotek, USA). Reverse transcription of RNA into cDNA and subsequent qPCR were made in one step mode using Superscript™ Platinum® One-Step Quantitative RT-PCR Kit (Invitrogen, USA) in an Mx3000p Real-Time PCR Machine (Stratagene, La Jolla, USA). The levels of VHSV nucleocapsid mRNA was detected by hydrolysis probe real time QRT-PCR using probes containing fluorochrome and quencher as shown in Fig. 1. The primer set and probe VHSVN_F (5′-TCG TGT ATG TTG GTG GAT TTG G-3′), VHSVN_R (5′-TCC TGT GTG TTC CCT TGG AG-3′) and VHSV_P (FAM/5′-AGA AGC ACG GAC AAC GCC TGT AC-3′/BHQ-1) respectively were designed using the software AlleleID v6.0 (PremierBioSoft, USA) and purchased from Biosearch Technologies. The DsiRNA targeting site was localized between the forward primer and the antisense probe (Fig. 1A and B). Salmonid elongation factor (ELF-1α) was used as an endogenous control (Snow et al., 2006). The thermal profile was: RT (15 min; 50 °C), *Taq* polymerase activation (2 min; 95 °C) and 45 cycles of denaturation (15 s; 95 °C) and annealing (30 s; 55 °C). The final concentrations of each primer set were 900 nM and each probe was 250 nM. The probe for nucleocapsid was labeled with 6-FAM and the quencher BHQ-1. The probe for ELF-1a was labeled with Cy5 and the quencher Iowa Black RQ-Sp. ROX dye (50 nM) was used as reference control for each sample. For quantification of efficiency of DsiRNA knock down effect on mRNA level the comparative quantification method (Pfaffl, 2001) was used. Outlier values were determined by the Mehalanobis distance technique (D^2; Mehalanobis, 1936) and percent of knock down was calculated using the formula (1) where $\Delta Ct\_Control$ correspond to Ct difference between VHSV infected control without treatment of DsiRNA

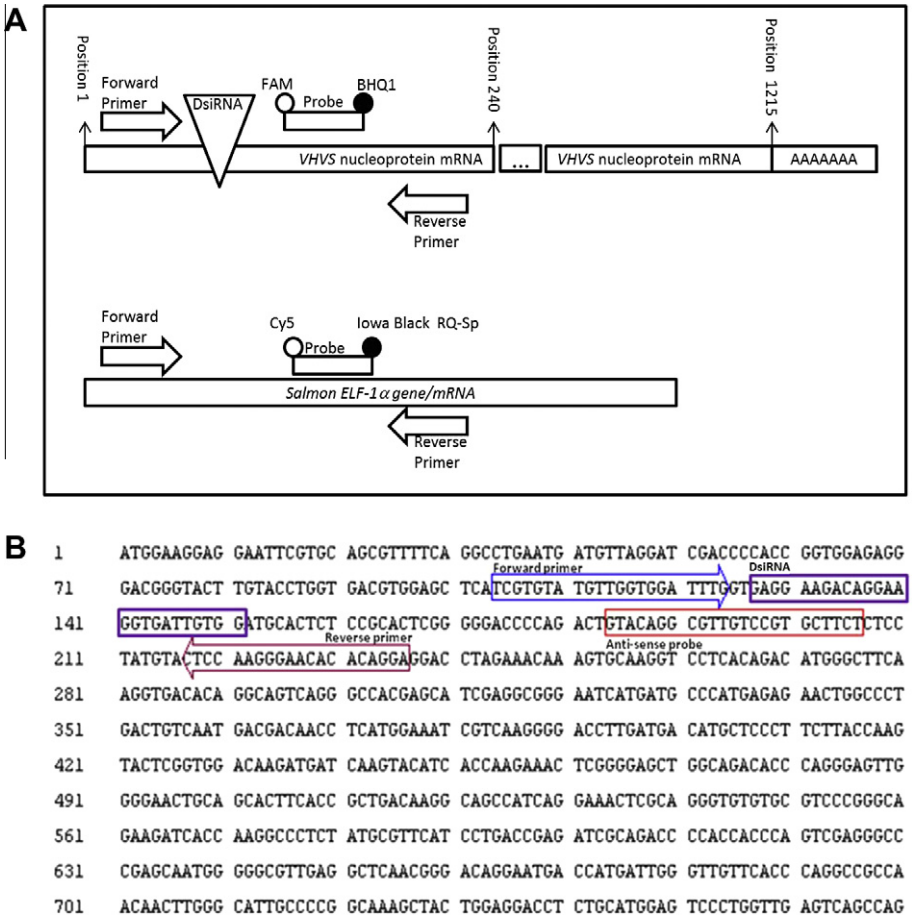

Fig. 1. Strategy for quantification of VHSV nucleocapsid knock down by 5′-nuclease quantitative RT-PCR VHSV. (A) Localization of primer sets probes and the DsiRNA target position in the VHSV nucleoprotein mRNA sequence and the positions of primers and probes in the rainbow trout ELF mRNA sequence which was used for normalization of the VHSV N mRNA level. (B) The nucleotide sequence of VHSV N (Acc. No. AF012093) with positions of primers and probe indicated.

and endogenous control ELF-1α and *ΔCt_siRNA* correspond to Ct difference between VHSV infected sample with DsiRNA treated and endogenous control ELF-1α.

Determination of percent of efficiency of DsiRNA or knockdown of mRNA

$$\% \text{ Efficiency} = 100x\left(1 - 2^{\Delta Ct\_Control - \Delta Ct\_siRNA}\right) \quad (1)$$

2.5. Effect of nucleocapsid targeting DsiRNA at the protein level

Immunofluorescence antibody test (IFAT) for cells transfected with different DsiRNA concentrations and subsequently challenged with virus was carried out. Briefly, cell layers in the 96 well-plates were fixed using 80% cold acetone followed by two washes with PBS-T-BSA and incubation with the VHSV-N specific monoclonal mouse antibody IP5B11 (Lorenzen et al., 1988) diluted 1:100 in PBS-T-BSA for 1 h at 37 °C. Following the three washing steps plates were incubated for 1 h at 37 °C with the polyclonal antibody anti-mouse IgG conjugated with FITC (KPL, USA) diluted 1:100. After three washes the wells were read by fluorescence microscopy (Leica DMIL microscope equipped with a filter for detection of fluorescein).

2.6. Effect on viral replication over time

For tests of DsiRNA effect on viral replication the 15 nM DsiRNA concentration and a dilution of 10^{-3} of the VHSV batch was selected based on the previous experiments. CHSE-214 cells in 96 well plates were infected with VHSV or IHNV as a heterologous viral control (Schyth et al., 2006) and subsequently transfected with DsiRNAs targeting VHSV-N or a DsiRNA targeting the nucleocapsid of flu H1N1-2009 as control. As negative controls cells without virus but transfected with DsiRNAs were used. Cell line supernatants were evaluated at different time points following transfection (0 h, 24 h, 48 h, 72 h and 96 h). Briefly, 50 μl of supernatant from each well was taken, diluted (1:4, 1:16 and 1:64) with PBS-T-BSA 1% Triton X-100 and subjected to a previously developed sandwich ELISA assay for the detection of the viral nucleoprotein (Mortensen et al., 1999). Briefly, ELISA plates (NUNC, Finland) were coated with either polyclonal antibody anti-VHSV F1-255 or anti-IHNV K33 diluted 1:1000 in coating buffer (1.6 μg/ml Na_2CO_3, 3 μg/ml $NaHCO_3$, pH 9.6), incubated at 4 °C for 12 h and washed 2 times with automatic washer Well Wash AC (Thermo Scientific, USA). Cell culture supernatant were added to wells in dilutions and incubated for 1 h at 37 °C. The plates were washed and incubated for 1 h at 37 °C with either anti-VHSV N Mab IP5B11 or anti-IHNV N Mab Hyb136-3 (Lorenzen et al., 1988; Fregeneda-Grandes et al., 2009). After a washing step, 50 μl of polyclonal rabbit anti mouse Ig antibodies conjugated with horseradish peroxidase (Dako; P260) diluted 1:1000 was added per well and incubated for 1 h at 37 °C. Finally, color reactions were developed by the use of H_2O_2 and OPD (KEM-EN-TEC diagnostic, Denmark). Reactions were stopped by H_2SO_4 2 N and each plate was read in an ELISA Multiscan EX reader (LabSystems Genesis, USA) with dual reading using 492 filter subtracted by the background read at 620 nm. ELISA values for the 1:16 dilution of the supernatant were compared as this

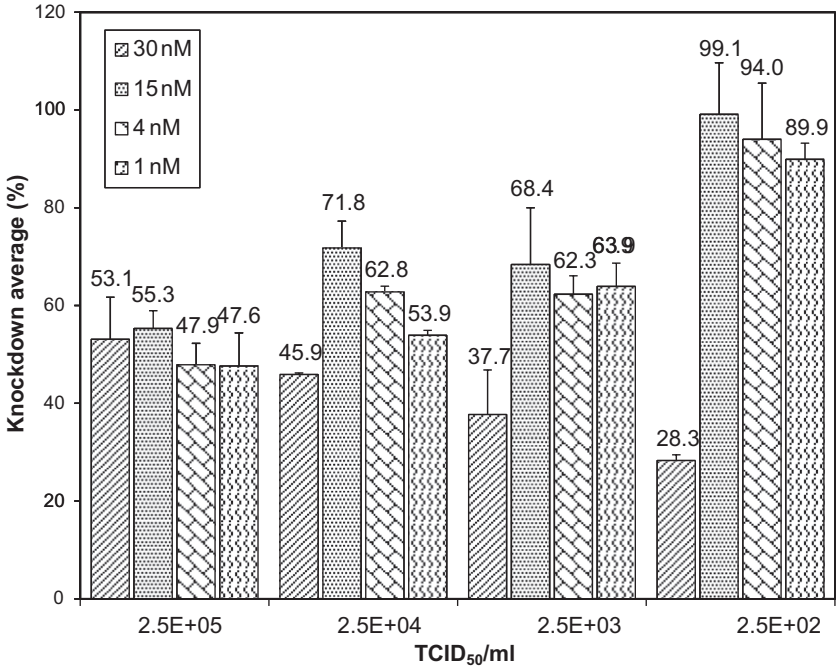

Fig. 2. Effect of nucleocapsid targeting DsiRNA on VHSV-N mRNA level as related to DsiRNA concentration and amount of VHS virus inoculated onto the DsiRNA transfected cells in the assay. A dilution series of VHSV was inoculated onto CHSE-214 cells transfected with antiVHSV-N DsiRNAs at varying concentrations as described in the materials and methods section. The data are shown as mean percent reduction ± standard deviation. $N = 6$ replicates pér combination.

proved to be in the linear range of the assay. For comparing ELISA absorbance values to viral titers standard curves were produced by testing a twofold dilution series (non-diluted to 1:128) of a new batch of the VHSV and IHNV strains in the two ELISAs.

3. Results

3.1. The effect on viral mRNA level is dependent upon DsiRNA concentration and amount of virus used

Transfection efficiency of the fluorescein labeled siRNA formulated in Trifectin reagent was estimated to be around 75.3% in CHSE-214 cells after 24 h of incubation when using 40 nM of the DsiRNA (data not shown). Using the same transfection protocol with variable DsiRNA concentration and adding a VHSV inoculation step following transfection, we found that DsiRNAs targeting the VHSV N gene were able to reduce mRNA levels of VHSV nucleoprotein as evaluated by one-step qPCR. As expected, reduction of VHSV N mRNA was dependent on the concentration of DsiRNA used for the transfection and dependent on dilution of the viral stock used for challenging the cell layer (Fig. 2). In general, lowering the concentration of virus inoculated on the cells (higher dilution) seemed to work better for seeing the RNAi effect, although this was not the case for the highest concentration of DsiRNA (30 nM). The most potent knock down was seen using 15 nM DsiRNA for all the 4 viral dilutions tested although a high knock down efficiency was seen even using only 1 nM of DsiRNA. A maximum of 99% inhibition of replication was accomplished using the 10^{-4} dilution of VHSV and 15 nM of DsiRNA (Fig. 2). The experiment was repeated with 30, 15, 7.5, 3.75 and 1.88 nM DsiRNA ($n = 6$) using a 10^{-4} dilution of VHSV and this experiment showed the same trend (data not shown). Therefore, the 15 nM DsiRNA concentration was used in all the subsequent experiments whereas the viral inoculations were 10^{-3} and 10^{-4} dilutions of the stock virus solution.

3.2. VHSV-N specific DsiRNAs reduce the level of nucleoprotein

CHSE-214 cells transfected with DsiRNA and subsequently infected with VHS virus were stained by immunofluorescence (IF) using specific antibodies against the VHSV-N protein. The number of VHSV-N positive fluorescent cells was clearly lower in wells containing cells transfected with the VHSV-N targeting DsiRNAs than in wells containing mock transfected cells (Fig. 3).

3.3. Effect on replication of VHSV and a control virus demonstrates a high degree of specificity

We tested the effect of the VHSV-N targeting DsiRNA on the nucleocapsid protein levels of cell layers infected with VHSV relative to the infected cells without treatment at 0 h, 24 h, 48 h, 72 h and 96 h post infection and transfection with DsiRNA. We compared these results to results gained with cells transfected with influenza H1N1 specific DsiRNA which served as the control of specificity in this experiment (Fig. 4A). VHSV N level in cell supernatants was indirectly measured as absorbance gained in a sandwich ELISA. From 0 to 96 h absorbance increased in both supernatant from cells transfected with VHSV specific DsiRNA and in supernatants from cells transfected with the control DsiRNA indicating that the virus was replicating and releasing free viral particles to the supernatant. But absorbance was significantly lower in supernatants from wells transfected with the VHSV-N specific DsiRNAs. Absorbance was reduced with 47.5–51.8% from 24 to 72 h post viral inoculation as compared to the level in the control cells treated with DsiRNA targeting influenza. This difference had diminished to 34.1% at 96 h (Fig. 4A). According to the standard curve in Fig. 4A this difference could be related to a reduction of approximately $2 * 10^7$ $TCID_{50}$/ml between the control-DsiRNA treated wells and wells treated with the VHSV specific siRNA. For evaluating the specificity of treatment we inoculated DsiRNA transfected CHSE-214 cell cultures with the VHSV related virus

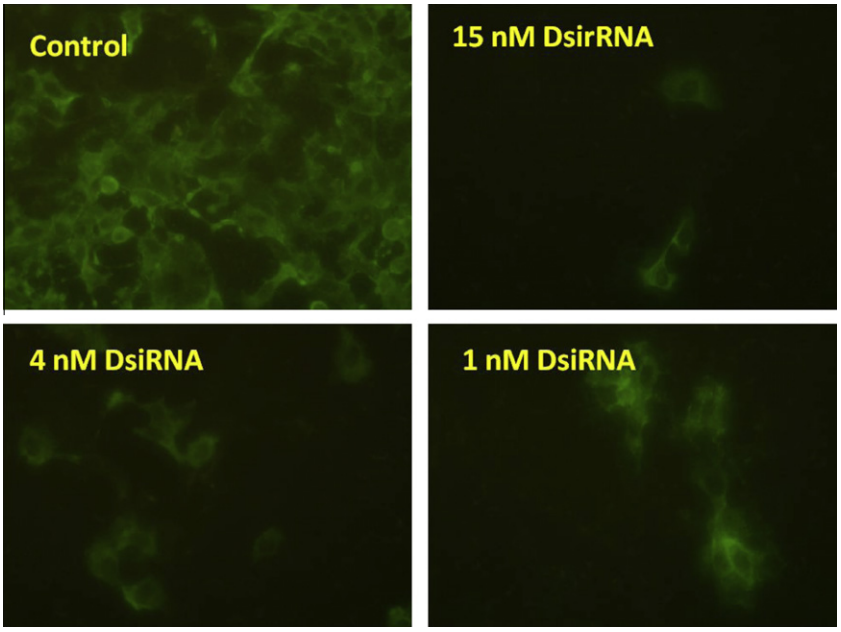

Fig. 3. Effect of treatment with DsiRNA targeting VHSV-N. Immunofluorescence antibody staining for the VHSV nucleocapsid (N). Pictures of mock treated control cells infected with VHSV (10^{-4} dilution of stock – see Section 2) and cells infected with VHSV but treated with 15 nM, 4 nM and 1 nM respectively of DsiRNAs targeting VHSV-N.

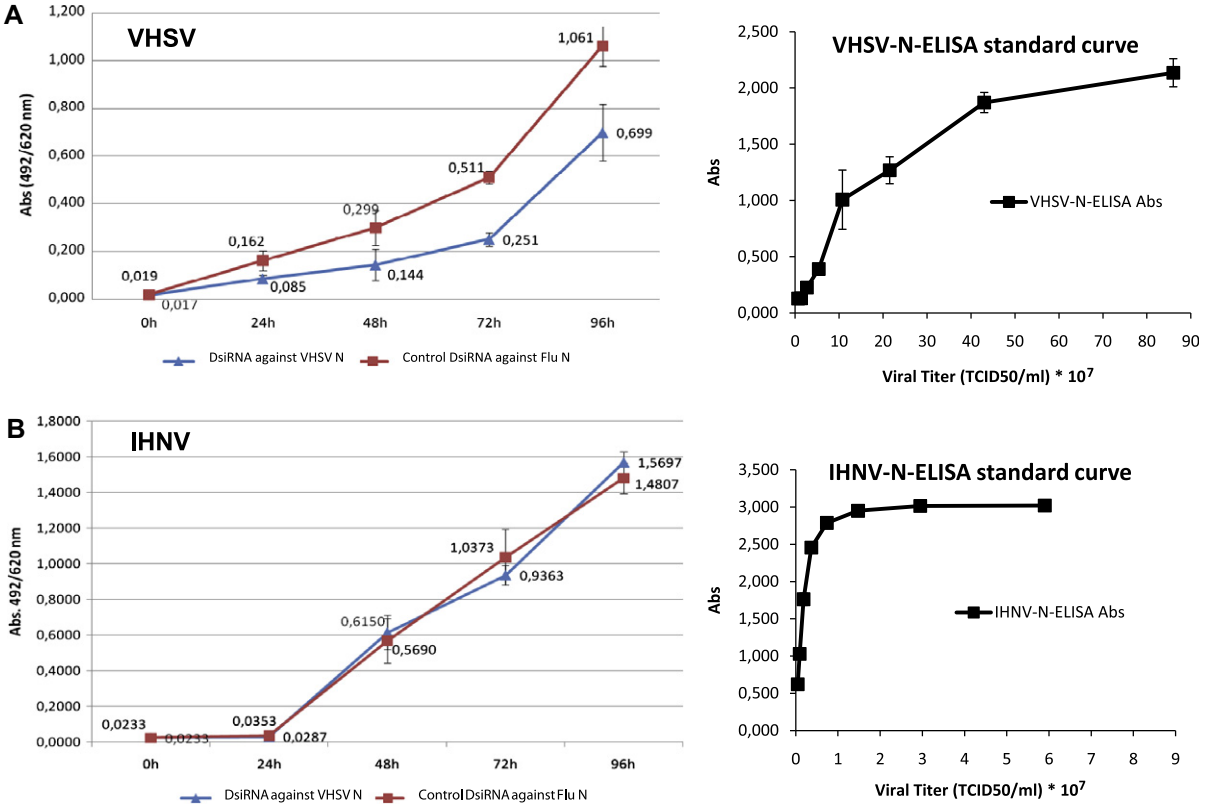

Fig. 4. DsiRNA targeting the VHSV nucleocapsid was able to reduce replication in a species specific manner. Levels of VHSV and IHNV N protein were determined in the cell culture supernatant of DsiRNA transfected cell cultures at various time points following challenge with VHSV (upper panel) and IHNV (lower panel) respectively. (A) Results from VHSV infected cells transfected with DsiRNAs targeting VHSV-N and cells transfected with a control DsiRNA targeting Flu-N H1N respectively. (B) Results gained when the same two DsiRNAs were used against the heterologous virus IHNV. Three replicate wells were used for all treatments. The supernatant was tested in different dilutions. The data presented here corresponds to a 1:16 dilution of the cell culture supernatant as ELISA absorbance values produced using this dilution were all in the linear quantitative range of the assay. The titer values of the VHSV and IHNV stocks are given in the materials and methods section. $N = 4$ replicates pér treatment. A standard curve relating absorbance values with viral titers ($TCID_{50}$/ml) is included for each ELISA assay.

infectious hematopoetic necrosis virus (IHNV) alongside the experiments with VHSV. Differences were not observed between IHNV infected cells transfected with control and VHSV specific DsiRNA respectively (Fig. 4B). This indicated that the effect of the VHSV targeting DsiRNA was highly specific.

4. Discussion

A connection between Dicer, the siRNA processing enzyme, and loading of siRNAs into the RISC complex has been described (Liu et al., 2006, 2007). The strategy of using dicer substrate siRNAs takes advantage of this link. The DsiRNAs act as substrates for dicer which uses them to produce siRNAs which can then be loaded directly from Dicer into RISC (Kim et al., 2005). We show here that this type of siRNA substrate can work both specifically and effectively against viral replication of an economically important virus of cold water aquaculture fish.

By using a DsiRNA designed to target the VHSV nucleocapsid N gene we were able to reduce the mRNA level and the level of N protein in the salmonid fish cell line CHSE-214. But most importantly, we were able to reduce the replication of VHSV in a species specific manner in as much as the VHSV-N targeting DsiRNA did not inhibit replication of the closely related IHN virus, which was used as a heterologous viral control (Schyth et al., 2006). The level of viral particles released to the supernatant from DsiRNA treated cells was measured by the use of a sandwich ELISA. This was based on primary catching polyclonal antibodies directed against total VHSV or IHNV particles combined with secondary monoclonal antibodies specific for the VHSV or IHNV nucleoprotein. During the infection rhabdoviral nucleoprotein has been shown to gather in a soluble cellular pool wherefrom it is recruited to the viral RNA (Hsu et al., 1979) a step which precedes the attachment of the viral L and P proteins necessary to form nucleocapsids which will in the final particle be situated approximately 9 bases apart on the RNA genome (Thomas et al., 1985). Although we have not been able to find any studies on the possibility of formation of viral particles with less nucleocapsids on the viral genome we expect from the above reason the nucleoprotein level measured in the supernatant to be a good indicator of the amount of viral particles. As the nucleoprotein is by far the most abundant protein in the rhabdoviral particle the use of antibodies against N in the ELISA was the strategy resulting in the most sensitive assay.

VHSV and IHNV are both fish pathogenic rhabdoviruses of the genus novirhabdovirus (Hoffmann et al., 2005). Despite sequence similarity we show in Fig. 5 that there are substantial differences between the mRNA sequence of VHSV and IHNV in the sub-sequence targeted by our VHSV-N specific DsiRNA. But the ability of siRNAs to reduce non-target genes in a microRNA like fashion, by allowing mismatches while pairing to mRNAs, has been postulated (Zhao et al., 2008). To rule out this possibility we searched the IHNV US220-90 genome sequence for possible microRNA targeting by our VHSV-N specific DsiRNA (microRNA target scanning program RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid; Krüger and Rehmsmeier, 2006). Although nearly perfect seed matches were seen among the alignments the best hits (mfe values below −30 kcal/mol) produced between 9 and 12 internal mismatched between the VHSV targeting DsiRNA and the IHNV genome sequence meaning that we would not expect the DsiRNA to target IHNV. In addition, the ability of VHSV and IHNV to replicate are both sensitive to interferon stimulation of host cell layers (Schyth et al., 2006; Purcell et al., 2006). Interferon stimulation in siRNA assays represent a problem with respect to proving specificity of effect as has been shown in both fish (Schyth et al., 2007) and in higher vertebrates (Ma et al., 2005). We therefore believe that in the current set-up IHNV was the best type of control to monitor the specificity of the DsiRNA effect.

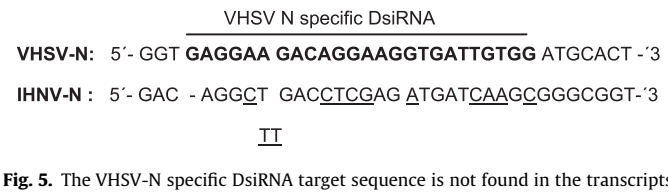

Fig. 5. The VHSV-N specific DsiRNA target sequence is not found in the transcripts of IHNV. Alignment of the nucleoprotein sequence of VHSV with the corresponding sequence in the heterologous control virus IHNV. In the alignment the target-site is presented in bold. Underlined bases in the IHNV-N sequence would produce mismatch with the DsiRNA sequence. Also the target sequence of the influenca specific DsiRNA used as control (see the text) was compared to the VHSV genome producing an alignment with 14 mismatches and 2 gaps (data not shown). An analysis of possible microRNA-like targets of the VHSV-N specific DsiRNA in the IHNV transcriptome revealed no obvious targets (see Section 4).

The efficiency of VHSV-N mRNA knock down was dependent upon siRNA concentration and the titer of the inoculated VHSV dilution. Knock down efficiency showed an optimum at 15 nM and 4 nM concentrations of DsiRNA (Fig. 2) and we speculate that the reason for the lower effect on viral inhibition at 30 nM of DsiRNA maybe due to saturation of the RNAi machinery thereby affecting the ability of the cell to steer physiological processes by microRNAs needed for antiviral defense. Indeed there are reports on effects of siRNAs on the microRNA pathway (Zhao et al., 2008). In any case, our data show that a high degree of viral inhibition could also be achieved with low concentrations of DsiRNA down to 1 nM. Knock down efficiency decreased when a higher viral load was inoculated onto the DsiRNA transfected cells, most probably because the number of siRNA molecules become insufficient to target all nucleoprotein transcripts. These results show, that in order to detect an antiviral effect of siRNAs *in vitro*, it is important to titrate both virus and siRNAs in such assays. We can not explain why it was possible to achieve a 99% reduction of VHSV N when we could only achieve a transfection efficiency of 75% with our fluorescently labeled siRNAs. We can speculate that all the cells are transfected to some degree with our fluorescently labeled control siRNAs, but that only highly transfected cells give a strong signal. This would mean that our transfection estimate could be an underestimate. We do not know what is the cellular threshold of siRNAs needed for an efficient knock down although from our results we did see that even small amounts of DsiRNA used for transfection was able to give a high reduction in the target gene. An alternative explanation could be that in our cells the uptake kinetics of 27/25 mer siRNAs is higher than for the 21/21-mer fluorescently labeled control siRNA.

In the time-course study (Fig. 4) an increase in viral inhibition until 72 h post viral inoculation (compare *VHSV* N ELISA absorbance values for control DsiRNA transfected and VHSV specific DsiRNA transfected cells) seemed to decrease only little at 96 h. We did not pursue a further examination of the dependency of timing, but we expect that at some time point the cytoplasmic pool of siRNAs might be depleted in the cells due to cell division and break down of siRNA. The timely take-over by the virus can presents a problem for the *in vivo* use of DsiRNAs. We anticipate that the use of plasmid vectors encoding small hairpins RNAs (shRNAs) for Dicer cleavage into mature siRNAs by the same pathway as the DsiRNAs might share the same benefits including sustained production of the siRNAs which should make viral take-over harder for the virus. With respect to this, a novel strategy where gene target specific shRNA sequences are cloned into vectors containing a pre-microRNA backbone have recently been employed for combating iridoviral replication in fish cells (Dang et al., 2008a). But despite the potential of this idea, expression from the vector in the study by Dang et al. (2008a) elicited an interferon response resulting in non-specific non-RNAi based antiviral effect. This again

reinforces the use of interferon sensitive target controls in viral siRNA knock down assays.

Although we were not able to fully suppress the replication of VHSV, our study shows that a specific antiviral effect of siRNAs in fish cells is a realistic goal. Lately, siRNAs have also been used successfully to inhibit replication of the warm water red seabream iridovirus through targeting of its major capsid protein (Dang et al., 2008b). There are still a limited number of studies on the inhibition of lower and higher vertebrate rhabdoviruses like the VHSV, rabies and vesicular stomatitis virus (VSV) (Barik, 2004; Schyth et al., 2006, 2007; Brandão et al., 2007; Ruiz et al., 2009; Israsena et al., 2009). Common to most studies on the antiviral effect of siRNAs, is that the use of a heterologous target control virus, which according to our observations is essential for confirming knock down specificity (Schyth et al., 2006), has not been used. The alternative, using a non-targeted viral gene of the target virus as control for specificity during the first rounds of replication has been employed in one of these studies (Barik, 2004) but does not allow detection of general antiviral effects like IFN induction by the siRNAs. In the present study we test our DsiRNA successfully using IHNV as a heterologous viral control for inhibition of VHSV thereby giving evidence for high specificity of the effect. To our knowledge, this is the first study showing specific inhibition of a fish pathogenic rhabdovirus by RNA interference. Whether DsiRNAs would also work specifically in fish *in vivo* remains to be determined. The successful use of DsiRNAs in cold water fish cells gives perspectives for its use in gene functional studies as well as the use of the RNAi mechanism for specific treatment against viral disease in such organisms. This perspective is only strengthened by the existing reports on the *in vivo* activity of DsiRNAs in mammals (Amarzguioui et al., 2006; Kim et al., 2006).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.03.174.

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