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ASSESSMENT OF THE INHIBITORY EFFECT OF RIBAVIRIN ON THE RAINBOW TROUT RHABDOVIRUS VHSV BY REAL-TIME REVERSE-TRANSCRIPTION PCR

Running title: Q-RT-PCR antiviral assay for trout virus

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Abstract

Viral hemorrhagic septicemia virus (VHSV) is one of the most ubiquitous viruses in salmonid aquaculture in Europe. This infectious disease results in significant losses in the farming industry and therefore effective therapeutic agents are needed to control outbreaks caused by this pathogen. Thus, accurate methods to test new antiviral compounds need to be developed. Our goal was to establish a model system for testing novel antivirals with potential applications to aquaculture. In a previous study a TaqMan® real-time RT-PCR assay was designed to detect and quantitate VHSV in rainbow trout tissues (Chico et al., 2006). In this report, we applied the real-time RT-PCR assay to the evaluation of the inhibitory effect of ribavirin, a well-known broad spectrum antiviral drug, in a cell culture system. When added from the beginning of the infection, ribavirin caused a dose-dependent reduction of VHSV RNA accumulation. Real-time RT-PCR measurements showed 99.8% inhibition at 25µg/ml ribavirin, with an IC50 of 0.43µg/ml. Ribavirin maintained its inhibitory activity against VHSV when added at 6 hours post-infection. Quantitation of N protein messenger RNA and plus-stranded RNA showed a substantial decrease of viral transcription in ribavirin-treated cells. Partial reversion of the effect of ribavirin by addition of GTP was observed, confirming that ribavirin targets the synthesis of guanidine nucleotides in the cells. This is the first report of a real-time PCR-based assay for addressing the efficacy and mechanism of action of an antiviral agent for rainbow trout.

Keywords: viral hemorrhagic septicemia virus, real-time PCR, antiviral
1. Introduction

Viral hemorrhagic septicemia virus (VHSV) is the agent of an important viral disease of rainbow trout (*Oncorhynchus mykiss*). Infection with this virus may lead to high mortality rates, occurring usually among juvenile fish. VHSV belongs to the *Novirhabdovirus* genus of the *Rhabdoviridae* family, a group of bullet-shaped enveloped viruses containing one molecule of negative-sense single-stranded RNA, coding for the nucleoprotein (N), the glycoprotein (G) and four other viral genes (3´-N-P-M-G-NV-L-5´) which are expressed as individual transcripts (Schütze et al., 1999).

Search for specific and efficacious antiviral compounds is a matter of concern in the control of fish virus infections. This search should be done in combination with an experimental technique capable of providing rapid and reliable evaluation of the efficacy of candidate molecules. We have recently developed a quantitative real-time RT-PCR (Q-RT-PCR) to measure VHSV loads both *in vitro* and *in vivo* (Chico et al., 2006). Earlier, successful detection of another rainbow trout rhabdovirus, *infectious hematopoietic necrosis virus* (IHNV), in brain and kidney by Q-RT-PCR was described (Overturf et al., 2001). In both studies, Q-RT-PCR was shown to be a fast, sensitive and reliable method to measure viral RNA accumulation in cultured cells as well as in fish tissues. Other authors have provided evidence that Q-RT-PCR can be a suitable technique to test the activity of antiviral compounds by assessing the inhibitory effect of a series of chemicals on herpesviruses (Fryer et al., 2004; Macé et al., 2003; Stránská et al., 2002) and retroviruses (Haffar et al., 2005; Pesce et al., 2005). Efficacy evaluation of antiviral molecules *in vitro* (on cell culture) by Q-RT-PCR has been reported for a number of RNA viruses (Garcia et al., 2001; Günther et al., 2004; Wright et al., 2004).
To test the performance of Q-RT-PCR in our VHSV/carp cell system, the nucleoside analogue ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was chosen for this study for its well-known broad spectrum antiviral activity. Ribavirin was discovered to have an inhibitory effect on the fish pathogen *infectious pancreatic necrosis virus* (*IPNV*) *in vitro* (Hudson et al., 1988; Migus and Dobos., 1980). Later it was shown that the ribavirin analogues EICAR and pyrazofurin were able to inhibit IPNV replication *in vitro* as well as in trout fry (Jashes et al., 1996; Moya et al., 2000). With respect to rhabdoviruses, ribavirin was found to block *vesicular stomatitis virus* (*VSV*) replication in hamster cells (Toltzis and Huang, 1986; Toltzis et al., 1988). For fish rhabdoviruses, *in vitro* inhibition of IHNV by ribavirin has been reported (Hudson et al., 1988). Due to its broad spectrum effectiveness, ribavirin has been used in recent years as a model antibiotic in studies where the inhibition of viral RNA synthesis was assessed by Q-RT-PCR (Castelain et al., 2004; Friedrichs et al., 2004; Garcia et al., 2001; Günther et al., 2004; Takhampunya et al., 2006; Wright et al., 2004).

We report in this paper the employment of a Q-RT-PCR assay with the fluorogenic TaqMan® probe to study the effect of ribavirin on VHSV replication. We characterized the effect of ribavirin on viral total RNA, plus-stranded RNA and messenger RNA accumulation, establishing a model system to test the efficacy of VHSV inhibitors *in vitro*, as well as a valuable tool to investigate the mechanism of action of novel antiviral drugs.

2. Materials and methods
2.1. Cell lines and virus infection assay

The carp *Epithelioma papulosum cyprini* (EPC) cells were purchased from the European Collection of Cell cultures (ECACC#93120820, Salisbury, UK). *Viral hemorrhagic septicemia virus* (VHSV, 07-71 European strain) was grown on EPC cells. EPC cells were seeded on 24-well plates in RPMI 1640 medium (Gibco) containing 10% fetal calf serum (FCS). VHSV was added to the EPC cells at a multiplicity of infection (m.o.i.) of 0.05 and incubated at 14°C in RPMI 1640 plus 2% FCS. For virus titration purposes, VHSV culture supernatants were collected at 3 days p.i.

2.2. Cytotoxicity assay

Ribavirin (Sigma-Aldrich) stocks were prepared in sterile water and stored at –20°C until use. Toxicity of ribavirin on EPC cells was evaluated by means of the colorimetric MTS method (CellTiter 96® AQueous Cell Proliferation Assay, Promega Corporation). Briefly, cells were seeded in 96 well plates and allowed to reach confluence. Ribavirin in concentrations up to 100µg/ml was added and the cells were incubated in the presence of the compound for 14 hours. The experiment was performed in triplicate wells following the protocol provided by the manufacturer. Cell viability was determined by measuring absorbance at 492nm.

2.3. End-point dilution assay

Briefly, 10-fold serial dilutions of supernatants from VHSV-infected cells were added to 96-well plates with EPC cells and incubated at 14°C for 3 days. Wells with cytopathic effect were counted after staining with a 1% crystal violet solution.
Determination of virus titer (expressed as TCID50/ml) was done by the Reed-Münch end-point calculation method.

2.4. Isolation of RNA

Total RNA was extracted from EPC cells cultivated in 24 well plates by using the RNAgent® Total RNA Isolation System extraction kit (Promega Corporation), following the manufacturer’s instructions. Each RNA pellet was resuspended in 25µl RNase-free water and the quantity of RNA was checked by measuring absorbance at 260 nm.

2.5. TaqMan® PCR primer and probe design

Amplicons in the VHSV N and VHSV G genes suitable for real-time PCR were identified with the Primer Express™ 2.0 software (Applied Biosystems). Primers and 5’-FAM-labeled probes were designed and standard curves were performed to check the efficiency of the amplification reactions (Chico et al., 2006).

For β-actin, sequences were selected from a exon region of common carp β-actin sequence (GenBank M24113): primer forward (5’-GCTGACAGGATGCAGAAAGAGA-3’), and reverse (5’-GGGCAATGATCTTGATTTTCATT-3’) generated a 67bp amplicon; the FAM-TAMRA probe was (5´-ACATCCCTGGCCCCCAGCA-3´).

2.6. Real-time RT-PCR assay

Unless otherwise indicated, 1µg total RNA was reverse-transcribed with 90ng random hexamers, 0.5mM deoxynucleoside triphosphates (dNTPs) mix. After denaturing for 5 min. at 65°C, 10 mM DTT, 20 units ribonuclease inhibitor, and 100 units MMLV-RT
enzyme (Invitrogen) were added in a final reaction volume of 20µl. The RT reaction profile was: 10 min at 25°C, 50 min at 37°C and 15 min at 70°C.

Quantitative PCR assays were performed using an ABI PRISM® 7700 Sequence Detector System (Applied Biosystems). Reactions were carried out in a final volume of 25µl, containing 300nM of each primer, 100nM probe, 2µl cDNA (unknown samples) and 1X Absolute™ QPCR Rox Mix (ABGene). Samples were subjected to the following thermal cycler conditions: 2min at 50°C, 15min at 95°C, and 40 cycles (15s 90°C/1min. 60°C). Endogenous control for quantitation was the 18S ribosomal RNA gene. 18S rRNA levels were determined with the TaqMan® Ribosomal RNA Control Reagents kit (Applied Biosystems) following the manufacturer’s guidelines.

For messenger RNA (mRNA) quantitation, the RT reaction was performed as described above, but using 25 pmol oligo dT’s instead of random hexamers.

2.7. High temperature primer-specific RT reaction

For quantitation of positive-sense RNA, reverse transcription was initiated by a reverse primer, N996rev (5’-CGTAGCGCTCTTGGATGGAC-3’; nucleotide position 977-996, N-gene sequence, antisense). Plus-strand specific cDNA synthesis was performed using the high temperature reverse transcription capability of the Tth DNA polymerase. cDNA complementary to plus-stranded VHSV (N gene) RNA was synthesized in a 40µl RT reaction mix containing 5µl RNA (0.5 - 1µg), 25pmol N996rev primer, 200µM dNTPs, 1 mM MnCl2, 10 units RNAGuard™ RNase inhibitor (Amersham Biosciences) and 2.5 units Tth DNApol (Promega Corporation). RT mixes were incubated 2 min at 60°C and 20 min at 70°C. An additional incubation of 30 min at 98°C with a Mn chelating buffer is carried out to inactivate reverse transcriptase.
3. Results

3.1. Determination of VHSV susceptibility to ribavirin by real-time RT-PCR

In the initial evaluation of the toxicity of ribavirin on the EPC carp cell line we determined that it did not exert a cytotoxic effect at concentrations \( \leq 100 \mu g/ml \). Numbers of viable living cells in cultures treated with 100µg/ml ribavirin for 14 hours were 127.4 ± 17.1% compared to the untreated controls (100 ± 9.8%). Past reports had shown a ≈60% suppression of cellular RNA synthesis by 50 µg/ml ribavirin in a hamster cell line (Toltzis and Huang, 1986). Thus, the dose-effect study of ribavirin was carried out at concentrations ranging from 1 to 25 µg/ml, examining cytopathic effect and measuring virus yield. A very strong inhibition of virus production was observed at 5, 10 and 25 µg/ml ribavirin (Fig. 1, table). Virus titers in the infected cells supernatants were 4 to 5 logs lower in those cells where ribavirin was added at the beginning of the infection (0 hours p.i.). No toxic effect was observed up to 25 µg/ml ribavirin.

To investigate whether our Q-RT-PCR protocol could be suitable to measure the inhibitory effect of ribavirin on virus replication, VHSV-infected EPC cells were incubated with concentrations of ribavirin ranging from 0.1 µg/ml to 25 µg/ml and VHSV RNA levels at 9 hours p.i. were determined by Q-RT-PCR (Fig. 1). A dose-dependent inhibition of VHSV RNA accumulation was found in the cells treated with ribavirin: 83.3% inhibition at 1 µg/ml ribavirin, 93.9% inhibition at 10 µg/ml, and 98.5% inhibition at 25 µg/ml (the latter point is not shown in Fig. 1). Ribavirin inhibitory concentration 50% (IC50) was calculated after fitting data points to a curve by using the Origin™ 7.0 software: IC50 = 0.43 µg/ml (1.76 µM). The closest concentration to the IC50 tested in the experiment was 0.5 µg/ml ribavirin, resulting in 54.9% inhibition of VHSV RNA synthesis.
3.2. Kinetics of VHSV RNA accumulation. Effect of ribavirin

To further examine the effect of ribavirin on VHSV, total RNA was extracted from infected EPC cells from 2 to 10 hours p.i. Q-RT-PCR measurements of viral RNA accumulation reflect the progress of infection (Fig. 2), with levels of VHSV RNA increasing with time: ≈3000-fold more viral RNA at 10 h p.i. than at 2 h p.i., indicating an active replication of the virus within the cells from hour 6 post-infection. When 25 µg/ml ribavirin was added at 0 h p.i. a high reduction of viral RNA accumulation occurred: at 10 hours p.i. 99.8% inhibition of VHSV RNA was achieved by treatment with ribavirin.

3.3. Effect of ribavirin on VHSV transcription

According to previous reports on VSV (Toltzis et al., 1988) ribavirin was expected to target viral RNA synthesis: genome replication, mRNA synthesis, or both. Firstly, to assess whether ribavirin was blocking an early and/or late step of the VHSV life cycle we carried out a simple time-of-addition experiment (Fig 3A) where ribavirin was added to the infected cells at 0 or at 6 hours p.i. and total RNA was extracted at 9 h p.i. and analysed by Q-RT-PCR. As expected from our previous findings ribavirin almost completely inhibited VHSV RNA accumulation when added at time 0 of infection (99.5% inhibition). When ribavirin was added at 6 h p.i. it still retained its inhibitory activity (81.9% inhibition). In an independent experiment where ribavirin was added at a later time (9 h p.i.) and VHSV RNA analyzed at 24 h p.i., 81% inhibition of viral RNA accumulation was observed. These results suggested that ribavirin exerts its action also at a late stage of the virus life cycle.

To measure messenger RNA levels and get an insight on the effect of ribavirin on VHSV transcription, RNA extracted from infected cells was converted to cDNA by using
oligo dT’s. Subsequently real-time PCR analysis was carried out with primers for the VHSV G protein and N protein genes, and the relative amount of mRNA in each sample was determined (Fig. 3B). A strong inhibition of 99.7% in VHSV G mRNA and 99.3% in VHSV N mRNA levels was found when ribavirin was present since the beginning of the infection. When added at 6 h p.i. inhibition of 59.9% and 54.1% for VHSV G and VHSV N mRNAs levels were found, respectively. Ribavirin did not have a negative effect on the transcription of the cellular β-actin gene at the concentration used in the experiment (25µg/ml). The 3’- 5’ attenuation of transcription is a well known feature of rhabdovirus gene expression. According to our Q-RT-PCR analysis VHSV N mRNA levels were 3.3-fold higher than VHSV G mRNA, confirming the validity of the Q-RT-PCR assay as a quantitative technique.

3.4. Analysis of VHSV plus-stranded RNA in ribavirin-treated cells.

To further examine the effect of ribavirin on VHSV RNA accumulation minimizing potential problems with secondary structures in RNA templates during cDNA synthesis, a Q-RT-PCR protocol using a sequence-specific primer and high temperature reverse transcription was developed. EPC cells were infected with VHSV and 25µg/ml ribavirin was added at 6 hours post-infection. RNA was extracted at 7, 8 and 9 h p.i. and the N gene sequence specific primer N996rev was used to convert plus-stranded VHSV RNA to cDNA in a RT reaction with Tth DNA polymerase at 70°C prior to TaqMan® real-time PCR analysis. Total cDNA was also synthesized with random hexamers and MMLV reverse transcriptase (at 37°C) for β-actin RNA quantitative analysis (Fig. 4). Positive-sense VHSV N RNA accumulates in the infected cells from 4 hours p.i. with a sharp increase from hour 7 p.i., likely indicating the bulk of viral transcription. When ribavirin is added VHSV RNA
dropped to undetectable levels in two hours. VHSV infection does not seem to affect β-
actin RNA levels in EPC cells. In accordance with a previous result (Fig. 3B) the synthesis
of carp β-actin RNA did not appear to be sensitive to ribavirin treatment at the
concentration used.

3.5. Reversibility of ribavirin inhibitory effect by GTP

Ribavirin has been described as an inhibitor of the inosine monophosphate
dehydrogenase (IMPDH) enzyme, causing a depletion of the guanidine nucleotides pool
within the cell (Graci and Cameron, 2002; Graci and Cameron, 2006; Parker, 2005).
Therefore, some reversion of ribavirin action should be achieved by replenishing GTP
levels, as it has been shown in VSV-infected cells (Toltzis and Huang, 1986). In VHSV-
infected EPC cells viral RNA levels raised from $2.46 \pm 0.24\%$ to $27.79 \pm 3.07\%$ when GTP
was added to the ribavirin-treated cells (Fig. 5). Thus, an approximately 10-fold increase in
VHSV RNA synthesis is induced by the addition of GTP to ribavirin-treated cells. GTP
alone did not cause an augmentation of VHSV RNA levels; rather, they dropped to $69.54 \pm
0.68\%$ compared to control VHSV-infected cells. These results support the notion that one
of the targets of ribavirin is the guanidine nucleotide synthesis pathway.

4. Discussion

Viral diseases pose a significant threat to aquaculture production. With respect to
salmonid species, *viral hemorrhagic septicemia virus* (VHSV) and *infectious pancreatic
necrosis virus* (IPNV) cause the two major diseases in Europe with outbreaks that may lead
to mass mortalities. Research efforts are aimed to develop effective virus-specific agents
against fish pathogens. One of the most promising studies on this topic described the *in vivo*
inhibition of IPNV by ribavirin analogues (Jashes et al., 1996; Moya et al., 2000). This was in accordance with earlier findings on the anti-IPNV activity of ribavirin in vitro (Migus and Dobos, 1980). Ribavirin, a nucleoside-like molecule is a broad spectrum antiviral whose inhibitory activity against rhabdoviruses, both mammalian (vesicular stomatitis virus, VSV) and piscine (infectious haematopoietic necrosis virus, IHNV), has been reported (Hudson et al., 1988; Toltzis and Huang, 1986). Taking all of the above in consideration, we pursued to develop an assay to test the efficacy of nucleoside analogues and other compounds against fish viruses in vitro, as a previous step to the in vivo trials in experimental aquaria. This was achieved in combination with an accurate method for viral RNA quantitation: real-time RT-PCR with TaqMan® probes.

In a previous work by our group a Q-RT-PCR assay was designed to run a reliable diagnostic test for VHSV in experimentally challenged rainbow trout (Chico et al., 2006). During the past years VHSV has been proven to be a good experimental model: it grows well in cell culture, it is not hazardous for humans, and animal tests with large numbers of individuals can be performed in experimental aquaria. Thus, a Q-RT-PCR assay for VHSV was set up on laboratory grown virus and subsequently tested on different tissue samples from VHSV-infected fish with success. The Q-RT-PCR analysis had a sensitivity of 1.6 TCID50, and it was capable of identifying more VHSV-positive fish than the standard cell culture assay (Chico et al., 2006).

Quantitative real-time PCR has been successfully employed to test the efficacy of antiviral compounds in cell culture (Garcia et al., 2001; Günther et al., 2004). In this work, the inhibition of VHSV RNA synthesis in vitro by ribavirin was determined by Q-RT-PCR. At 10 hours post-infection 99.8% inhibition of viral RNA accumulation was determined in VHSV-infected cells treated with 25µg/ml ribavirin. The IC50 was calculated as 0.43µg/ml.
This value is about 2 logs lower than those reported for ribavirin inhibition of VSV, where the IC50 was determined by measuring radioactive-labelled viral RNA (Toltzis and Huang, 1986). For Lassa virus (Arenaviridae) a concentration of 9 µg/ml ribavirin caused 50% inhibition of viral RNA accumulation as determined by Q-RT-PCR (Günther et al., 2004).

It is known that the Q-RT-PCR assay leads to lower IC50 values than standard (i.e. plaque assay) methods for virus quantitation (Macé et al., 2003; Stránská et al., 2002), likely due to the fact that viral RNA species exist which do not correspond to infectious virus particles.

Ribavirin was effective at reducing VHSV total RNA as well as mRNA synthesis. Early studies suggested a direct inhibitory effect on the VSV RNA polymerase (Toltzis et al., 1988). For the IPNV birnavirus, the inhibition of both viral transcription and replication by ribavirin has been speculated (Migus and Dobos, 1980). Our data from the late addition of ribavirin to the infected cells also support this notion. Interference with both replication and transcription may explain the difference between ribavirin effect on VHSV total RNA (81.9% inhibition) and the effect on VHSV mRNAs (54 – 59% inhibition) when the drug is added at 6 h p.i.: by detecting only mRNA molecules the inhibitory effect of ribavirin can be underestimated, in contrast to the analysis of total RNA.

Regarding the Q-RT-PCR assay, it has been pointed out by a number of authors (Purcell et al., 2006) that performing the RT reaction at a relatively low temperature (i.e. 37°C) may lead to inaccurate quantitation of the target RNA due to false priming, self-priming and random priming events, resulting in synthesis of contaminating cDNA. To check this issue we set up a RT protocol using a VHSV N sequence specific primer (instead of random hexamers) and Tth DNApol, a polymerase capable of functioning up to 70°C. This method allowed us to analyze plus-stranded VHSV RNA (N gene mRNAs as well as whole length anti-genomes) and reassess the inhibitory effect of ribavirin. Consistently with
our previous experiments, a high reduction of viral plus-stranded RNA levels (81.6% inhibition after one hour of treatment) was found.

One of the proposed mechanisms of action of ribavirin is the inhibition of the enzyme inosine monophosphate dehydrogenase (IMPDH), causing the depletion of the intracellular guanidine nucleotides pool (Graci and Cameron, 2002; Graci and Cameron, 2006; Parker, 2005). We examined this possibility, finding a 10-fold increase of VHSV RNA levels when GTP was added to the ribavirin-treated infected cells compared to ribavirin alone, strongly supporting the hypothesis of ribavirin targeting the metabolic pathway leading to GTP formation. We observed a ≈ 30% decrease in VHSV RNA accumulation due to GTP treatment. Although we did not pursue the investigation of this phenomenon, we can speculate that the imbalance in the cellular NTPs pool caused by addition of GTP might lead to a lesser efficiency of the viral RNA polymerase.

We are aware that by monitoring viral load by a PCR-based assay other antiviral activities that lead to production of non-infectious virus can be overlooked. At the concentration tested (25 µg/ml = 102 µM) ribavirin has a lethal mutagen activity against a number of RNA viruses (Graci and Cameron, 2002). The combination of two or more mechanisms of action could explain the 10⁵-fold reduction in infectious virus production caused by ribavirin.

In conclusion, Q-RT-PCR can be used, in combination with the measurements of viral titers by classical methods, to study the progression of infection and the kinetics of virus replication. By providing accurate quantitation of viral RNA accumulation, Q-RT-PCR is a particularly suitable technique to assess the inhibitory activity of antiviral molecules. Bearing in mind practical applications we should stress that there is not always a direct correlation between in vitro results and protection of fish, so in vivo testing is
necessary. A previous work where the ribavirin analogue EICAR was administered daily by immersion to IPNV-infected fish during a 20 day period, resulted in a statistically significant reduction of mortality rates (Moya et al., 2000). Further research needs to be conducted to determine the most cost-effective way to deliver antiviral compounds to trout fry.

Finally, we believe that the real-time PCR technique will be increasingly implemented to study the pathogenesis of fish virus infections, assess the efficacy of new antiviral drugs, and address their mechanism of action. The findings obtained in the present study may contribute to the further development of antiviral agents for VHSV and other fish viruses.

Acknowledgements

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References


Figure Legends

Fig.1. Dose-dependent effect of ribavirin on VHSV replication. Left: VHSV-infected EPC cell monolayers (m.o.i. = 0.05) were incubated with 0.1, 0.5, 1, 2.5 and 10 µg/ml ribavirin. Cells were harvested at 9 hours p.i. and total RNA was prepared. VHSV RNA levels were measured by real-time RT-PCR by amplifying a sequence of the VHSV N gene. Each PCR reaction was done in triplicate. The percent values represent the inhibition of VHSV RNA accumulation compared to the non-treated VHSV control. Right: effect of ribavirin on virus yield. Aliquots of VHSV-infected cells supernatants were sampled at 9 h p.i. and virus titers were determined by the end-point dilution method. Ribavirin was added at 0 h p.i.

Fig.2. Time course of VHSV RNA accumulation in EPC cells infected with a m.o.i. of 0.05. Cell monolayers were harvested at the indicated times and samples were subjected to the Q-RT-PCR assay as in figure 1 (filled squares). 25 µg/ml ribavirin was added to the cells (open circles) at the same time as the virus (0 h p.i.). Arbitrary units: Y axis values represent the ratio between the amount of VHSV RNA in a given sample and the amount of VHSV RNA at 2 h p.i. TaqMan® PCR reactions were run in triplicate. Error bars indicate the standard deviations of those replicates.

Fig.3. A: Effect of ribavirin time of addition on VHSV RNA accumulation: 25µg/ml ribavirin was added at 0 h p.i. and at 6 h p.i. EPC cell monolayers were harvested at 9 hp.i. and VHSV (N gene) RNA levels were determined by Q-RT-PCR (random hexamers RT). B: Effect of ribavirin on VHSV transcription. RNA from VHSV-infected cells was converted to cDNA by oligo dT’s RT reaction prior to Q-PCR analysis of VHSV G and VHSV N sequences. A carp β-actin gene sequence was also amplified as a control of...
cellular messenger RNA. The internal control to normalize β-actin and VHSV RNA data was the 18S rRNA (random hexamers RT). Results in panel B are expressed as the ratio between the expression of mRNA in a given sample and the expression of VHSV G mRNA after treatment with ribavirin from time 0 (lowest value of the experiment). Data are mean ± SD for one experiment performed in triplicate.

Fig. 4. Time course of plus-stranded VHSV N gene RNA (squares) and β-actin RNA (triangles) in EPC cells infected with VHSV (m.o.i. = 0.05). Infected cells were treated with 25 µg/ml ribavirin from 6 hours post-infection (arrows; open symbols) and harvested at the indicated times for RNA extraction and real-time RT-PCR analysis. The sample with the lowest target gene expression (VHSV N at 2 h p.i.) is given value = 1 and used as the reference. VHSV N RNA at 8 h p.i. and 9 h p.i. in cells treated with ribavirin were below detection limit (assigned value = 0). Data are mean ± SD for one representative experiment performed in triplicate.

Fig. 5. Reversibility of ribavirin effect by GTP. EPC cells were infected with VHSV and incubated in the presence of 25 µg/ml (102 µM) ribavirin (RIB) and/or 500 µM GTP for 9 hours. Total RNA was prepared and subjected to Q-RT-PCR analysis as in figure 1. Relative percentages of inhibition were calculated in comparison to the untreated VHSV infection control. TaqMan® PCR reactions were run in triplicate. Error bars indicate the standard deviations of those replicates.
Figure 3

(A) VHSV RNA (arbitrary units)

(B) mRNA expression (arbitrary units)

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