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Inhibitory Effect of a Nucleotide Analog on Infectious Salmon Anemia Virus Infection▽

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The infectious salmon anemia virus (ISAV), which belongs to the *Orthomyxoviridae* family, has been responsible for major losses in the salmon industry, with mortalities close to 100% in areas where Atlantic salmon (*Salmo salar*) is grown. This work studied the effect of ribavirin (1-β-D-ribofuranosyl-1,2,3-triazole-3-carbaxaide), a broad-spectrum antiviral compound with proven ability to inhibit the replicative cycle of the DNA and RNA viruses. The results show that ribavirin was able to inhibit the infectivity of ISAV in *in vitro* assays. In these assays, a significant inhibition of the replicative viral cycle was observed with a 50% inhibitory concentration (IC_{50}) of 0.02 μg/ml and an IC_{90} of 0.4 μg/ml of ribavirin. After ribavirin treatment, viral proteins were not detectable and a reduction of viral mRNA association with ribosomes was observed. Ribavirin does not affect the levels of EF1a, nor its association with polysomes, suggesting that the inhibition of RNA synthesis occurs specifically for the virus mRNAs and not for cellular mRNAs. Moreover, ribavirin caused a significant reduction in genomic and viral RNA messenger levels. The study of the inhibitory mechanism showed that it was not reversed by the addition of guanosine. Furthermore, *in vivo* assays showed a reduction in the mortality of *Salmo salar* by more than 90% in fish infected with ISAV and treated with ribavirin without adverse effects. In fact, these results show that ribavirin is an antiviral that could be used to prevent ISAV replication either *in vitro* or *in vivo*.

The infectious salmon anemia virus (ISAV) is a pathogen that principally affects Atlantic salmon (*Salmo salar*), causing multisystemic disorders. Since 1984, it has been associated with high mortality in the aquaculture industry (48), reaching 100% in some cases (5, 17, 27, 39, 48). ISAV is a member of the *Orthomyxoviridae* family and is the only member of the *Isavirus* genus (24, 32). ISAV has a pleomorphic structure, with spike projections composed of hemagglutinin-esterase (HE) protein that interacts with the sialic acid molecule of cell receptors (20). Similar to the influenza A and B viruses, ISAV is an envelope virus that contains eight segments of negative single-stranded RNA as the genome (8). The functions of most proteins encoded by the segments of ISAV have been assigned according to their similarities to the proteins encoded by influenza A virus, suggesting that ISAV uses its own polymerase to copy and transcribe its genome. Thus, ISAV polymerase synthesizes both mRNA and viral RNA (vRNA) and seems to be constituted by three subunits: polymerase basic 2 (PB2), encoded by segment 1 (43); polymerase basic 1 (PB1), encoded by segment 2 (24); and polymerase acid (PA), encoded by segment 4 (3, 38, 43). Moreover, as with influenza virus, RNA replication and transcription occur in the cell nucleus, while mRNA and vRNA are transported to the cytoplasm for translation and packaging, respectively (18).

Ribavirin (1-β-D-ribofuranosyl-1,2,3-triazole-3-carbaxaide) is a broad-spectrum antiviral agent with *in vitro* and *in vivo* inhibitory activity against DNA and RNA viruses. Ribavirin has proven to be an inhibitor of several ortho- and paramyxoviruses (42). Furthermore, ribavirin inhibits *in vitro* and *in vivo* replication of influenza virus (15, 33, 49, 50), the most typical member of the *Orthomyxoviridae* family.

In particular for viral pathogens of fish, ribavirin dramatically affects the infective cycle of some viruses, such as viral hemorrhagic septicemia virus (VHSV) (29), infectious pancreatic necrosis virus (IPNV) (22), infectious hematopoietic necrosis virus (IHNV) (21), and chum salmon reovirus (CSV) (14). There are several ways in which ribavirin inhibits viral replication. Ribavirin is quickly phosphorylated in cells into monophosphate (RMP), diphosphate (RDP), and triphosphate (RTP), the last being one of the major forms in the cell (44). In some cases, ribavirin inhibits the cellular IMP dehydrogenase (IMPDH), the enzyme that converts IMP to XMP (21), reducing the intracellular pools of GMP, GDP, and GTP (26, 33, 44, 45); decreasing the rate of RNA synthesis; and affecting the level of both viral and cellular RNA molecules (22, 26). Moreover, by decreasing the levels of guanosine, ribavirin could inhibit capping of mRNAs (6, 19). Additionally, ribavirin could be incorporated by poliovirus (PV) polymerase 3Dpol as either a GTP or an ATP analog, causing mutagenesis of viral RNA and thus inhibiting the infective ability of the

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poliovirus (10, 11). Ribavirin can be incorporated into nascent genomic RNA, causing an early termination of RNA synthesis or an error catastrophe through the misincorporation of cytidine and uridine instead of guanidine or adenine, which results in lethal mutagenesis (11, 47). Ribavirin also inhibits HIV (35, 40), vesicular stomatitis virus (VSV) (16), and La Crosse virus (7) polymerase; reovirus transcriptase (36); vaccinia virus guanylyltransferase (19); and influenza virus polymerase (15, 50).

Because of the similarities between the ISA and influenza viruses, in this study we evaluated the *in vitro* and *in vivo* antiviral effects of ribavirin on ISAV infection. We demonstrated that ribavirin causes a dramatic decrease in ISAV RNA accumulation, making it a plausible candidate for preventing this disease among farmed *Salmo salar*.

MATERIALS AND METHODS

Virus and cells. Monolayers of salmon head kidney cells (SHK-1) were grown at 15°C in Leibovitz's 15 medium (L-15) supplemented with 10% fetal bovine serum (FBS), L-glutamine (200 mM), β-mercaptoethanol (55 mM), and gentamicin (10 mg/ml).

Monolayers of Chinook salmon embryo cells (CHSE-214) were grown at 15°C in minimum essential medium balanced with Earle's salts (E-MEM) supplemented with 10% FBS, streptomycin-penicillin (50 mg/ml), HEPES (10 mM), and 1× amphotericin B (Fungizone).

The viral inoculum ISAV genotype 7b, previously obtained in our laboratory (9), was propagated in monolayers of SHK-1 cells at 70% confluence with a multiplicity of infection (MOI) of 0.01. Cells were infected 3 days after seeding. After 2 h of absorption at 16°C, the monolayer was washed twice with phosphate-buffered saline (PBS) and supplemented L-15 medium was added. The cells were incubated for 14 days postinfection (dpi) or until the appearance of cytopathic effect (CPE) (12). Subsequently, the supernatants of the culture were harvested and stored at −20°C. The viral titer was determined by real-time quantitative reverse transcription-PCR (real-time qRT-PCR).

CHSE-214 monolayers were infected with the virus of infectious pancreatic necrosis (IPNV) genotype SP with an MOI of 0.05. After 1 h of adsorption at 15°C, the monolayer was washed 2 times with PBS and E-MEM supplemented with 2% fetal bovine serum (FBS) was added. The cells were incubated for 24 h or until the appearance of CPE. The culture supernatants were harvested and stored at −20°C. The viral titer was determined by plaque assay analysis (28).

Cytotoxicity of ribavirin. Monolayers of SHK-1 cells were grown in 6-well plates until 90% confluence. Then, increasing concentrations of ribavirin (W = 244.2) were added to each well and incubated for 7 days at 15°C. Subsequently, the supernatant was removed and cells were washed twice in PBS and detached with 200 μl of a solution of 0.5 mM EDTA and 0.02% trypsin. The cells were centrifuged for 10 min at 1,000 × *g* and resuspended in PBS-2% FBS. Propidium iodide (PI) was added at a final concentration of 0.75 μg/ml, and viability was determined by flow cytometry from 100,000 cells. The result of the 50% cytotoxic concentration (CC_{50}) determination is expressed as a percentage of living cells.

Ribavirin treatment *ex vivo*. ISAV was incubated for 1 h in L-15 without FBS with increasing concentrations of ribavirin (Sigma-Aldrich) and then used to infect the SHK-1 cells with an MOI of 0.01. After 2 h of absorption at 16°C, the cellular monolayer was washed and supplemented L-15 medium was added with ribavirin at the same concentration as that in the preincubation. At 7 dpi, the supernatant or cells were analyzed by real-time qRT-PCR to determine viral titer.

Real-time qRT-PCR. The viral RNA present in the supernatant from infected SHK-1 cells was extracted with the EZNA Total RNA Kit I (Omega Bio-tek). The viral titer (copies/ml) was determined by quantitative real-time reverse transcription-PCR (qRT-PCR) with the F5/R5 primers initially described in reference 13 using the Brilliant II SYBR green QRT-PCR Master Mix kit, 1-Step (Agilent Technologies), in an Agilent Technologies MxPro3000P instrument. The thermal profile of real-time qRT-PCR was as follows: reverse transcription (RT), 1 cycle of 20 min at 42°C; predenaturation, 1 cycle of 10 min at 95°C; PCR, 40 cycles of 15 s at 95°C, 15 s at 60°C, and 15 s at 72°C; and disassociation curve, 60 to 95°C with an increase of 0.1°C/s. For performing the real-time qRT-PCR of EF1a, the extracted RNAs were treated with DNase and then subjected to RT carried out with Moloney murine leukemia virus (M-MLV) and random primers for 1 h at 42°C. For the real-time qPCR, the EF1aF (5′ ATGGGGACAACAT GCTGGAR 3′) and EF1aR (5′ CGGGAKGGGGGCAGGAT 3′) primers were used. The thermal profile of real-time qPCR corresponds to the following: predenaturation, 1 cycle of 10 min at 95°C; PCR, 40 cycles of 15 s at 95°C, 15 s at 58°C, and 15 s at 72°C; and disassociation curve, 60 to 95°C with an increment of 0.1°C/s.

Guanosine treatment. The inoculum of ISAV was preincubated with ribavirin, guanosine, or both and used to infect SHK-1 monolayers. After the absorption period, medium was replaced with supplemented L-15 plus ribavirin, guanosine, or both at the same concentrations used in the period of preincubation. After 4 dpi, RNA was extracted from the supernatant to determine the virus titer by real-time qRT-PCR.

IPNV inoculum was preincubated for 1 h with ribavirin, guanosine, or both in medium and then used to infect CHSE-214 cells. After 1 h of adsorption, the infection medium was replaced by E-MEM–2% FBS plus ribavirin, guanosine, or both at the same concentrations previously used in the period of preincubation. At 16 h postinfection (hpi), the supernatant was stored at −20°C and cell lysate was subjected to extraction of genomic RNA and visualized by electrophoresis on a 7% polyacrylamide gel for 18 h at 180 V; the gel was stained with silver nitrate (30).

Detection of genomic RNA and mRNA. RNA was extracted from infected SHK-1 cells with an MOI of 0.01 of ISAV, with or without ribavirin. The RNA from each well was divided, and two RT reactions were conducted with M-MLV individually using primer F5 or R5 for 1 h at 42°C, according to the supplier's instructions. The cDNA obtained was quantified by real-time qRT-PCR.

Polysomal profiles. ISAV-infected and uninfected SHK-1 cells, with or without 0.5 μg/ml or 5 μg/ml of ribavirin, were incubated for 10 min with cycloheximide (100 μg/ml). Total extract of cells was obtained by lysis with buffer (Tris-HCl, 20 mM, pH 7.4; $MgCl_2$, 3 mM; KCl, 150 mM; NP-40, 0.5%; cycloheximide, 100 μg/ml; dithiothreitol [DTT], 1 mM). To analyze the polysomal profiles, the extracts were diluted in 2 volumes of gradient buffer (Tris-HCl, 20 mM, pH 7.4; $MgCl_2$, 3 mM; KCl, 150 mM; cycloheximide, 100 μg/ml; DTT, 1 mM) and placed on a sucrose gradient (15 to 45% [wt/wt] sucrose, prepared in the same buffer). Centrifugation was carried out with a SW41 Ti rotor in a Beckman ultracentrifuge at 39,000 rpm for 3 h. The RNA of each fraction of 200 μl was extracted using the EZNA extraction kit. The RNAs of ISAV and of EF1a associated with each fraction were quantified by real-time qRT-PCR.

IFAT. For the immunofluorescence antibody test (IFAT), SHK-1 monolayers grown on coverslips were infected as described above. At the indicated times, the monolayers were washed twice with PBS and fixed in 4% formaldehyde in PBS for 30 min. Fixed cells were washed with PBS and permeabilized with 0.3% Triton X-100 in PBS for 10 min. The coverslips were incubated in PBS for 1 h in blocking buffer (3% [wt/vol] bovine serum albumin [BSA] and 0.1% Triton X-100 in PBS) and then incubated for 1 h with anti-ISAV nucleoprotein (clone 2C2/H4; Grupo Bios Chile) as the primary antibody diluted in blocking buffer, washed 3 times with PBS, and finally incubated for 1 h with anti-mouse Alexa Fluor 488 (Invitrogen) as the secondary antibody. The coverslips were then washed once with 1 μg/ml PI in PBS and 6 times with PBS alone and then mounted on glass slides with DABCO [1,4-diazabicyclo(2,2,2)octane]. Images were obtained with a Zeiss LSM 510 confocal microscope. Alexa Fluor 488 was detected by using the 488-nm excitation line and analyzing emissions between 500 nm and 535 nm. PI was detected by using the 543-nm excitation line and analyzing emissions between 575 nm and 650 nm.

Ribavirin treatment *in vivo*. Healthy postsmolt Atlantic salmon (*Salmo salar*) with an average weight of 75 g were kept in 200-liter tanks with 25 ppt of salinity at a density of 22 kg/m^3, a temperature of 14 to 18°C, and an oxygen rate of 5.8 to 7.1 mg/liter. After 9 days of acclimatization, 6 groups of 30 fish were infected by cohabitation with fish infected by intraperitoneal injection of 0.1 ml from 1×10^8 copies/ml of virus. At 25 days after infection (37), 3 groups were treated with ribavirin, 6.5 μmol/kg of body weight, for 10 days in feed. The other 3 groups were administered only the vehicle. The fish were fed at 0.5% of body weight to ensure complete intake of the antiviral doses. In addition, 2 groups of the other 30 fish remained as untreated controls. Daily physical-chemical parameters were monitored: nitrite, nitrate, ammonium, pH, temperature, and dissolved oxygen. Also, hematocrit and hemoglobin were analyzed. Daily mortality, activity, and appetite of the fishes were registered. After treatment, the fish were kept for 20 days to monitor performance.

RESULTS

Effect of ribavirin on the *in vitro* replication of ISAV. To determine if ribavirin affects the infection of ISAV, the virus was incubated with increasing concentrations of ribavirin for 1 h before the SHK-1 cell infection and the drug was main-

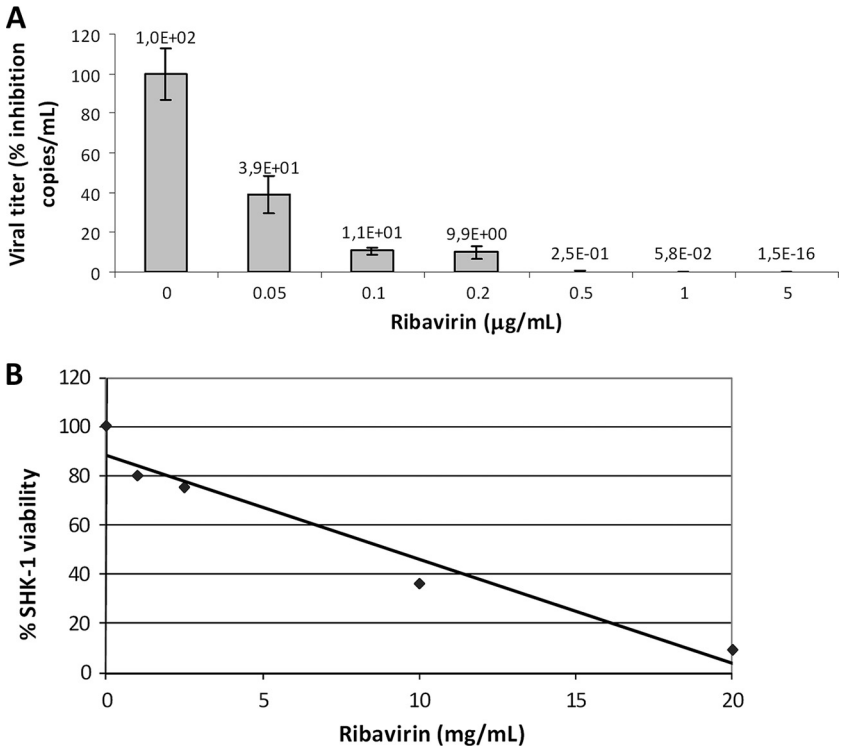

FIG. 1. Evaluation of the antiviral activity of ribavirin on ISAV infection in SHK-1 cells. Cellular monolayers of SHK-1 were infected with an MOI of 0.01 of ISAV in the absence or presence of increasing concentrations of ribavirin. (A) The ISAV titer (copies/ml) was determined by real-time qRT-PCR, normalized to 100, graphed as the average ($n = 6 \pm$ the standard deviation), and shown as the percentage of inhibition. (B) Viability of SHK-1 cells treated with increasing concentrations of ribavirin (graphic representation of 3 measurements).

tained throughout the incubation period. Seven days postinfection, RNA was extracted from the culture supernatant and used to quantify the number of copies of genomic segment 8 RNA of ISAV by real-time qRT-PCR, indicating the viral titer (48). Figure 1A shows a progressive decrease in ISAV titer as ribavirin concentrations increase. The 50% inhibitory concentration (IC_{50}) determined for ribavirin was 0.02 μg/ml, and the IC_{90} was 0.4 μg/ml. An 0.5-μg/ml concentration of ribavirin was considered the IC_{100}, since at this concentration viral RNA is hardly detectable by real-time qRT-PCR (more than 10^{15}-fold inhibition). Ribavirin inhibition occurred at the same magnitude when ISAV was not incubated with ribavirin before the infection (data not shown), suggesting that ribavirin does not interfere with the entry of the virus into the cell.

To determine if ribavirin exerts a cytotoxic effect on SHK-1 cells, the CC_{50} was estimated. Figure 1B shows that the CC_{50} of ribavirin on SHK-1 cells was 9 mg/ml. The major difference (5×10^5 times) between the IC_{50} (0.02 μg/ml) and CC_{50} of ribavirin means that this antiviral would be a safety indicator for *in vivo* studies.

Effect of ribavirin on viral NP protein synthesis. Viral titer is determined as viral RNA on supernatant, assuming that this corresponds to genomic RNA contained within viral particles. To confirm that the action of ribavirin on the viral titer actually affects the synthesis of viral proteins, we analyzed infected cells at 7 dpi by IFAT using an antibody against nucleoprotein (αNP) to detect viral protein synthesis. Figure 2 shows that 40% of SHK-1 cells were infected by ISAV with an MOI of

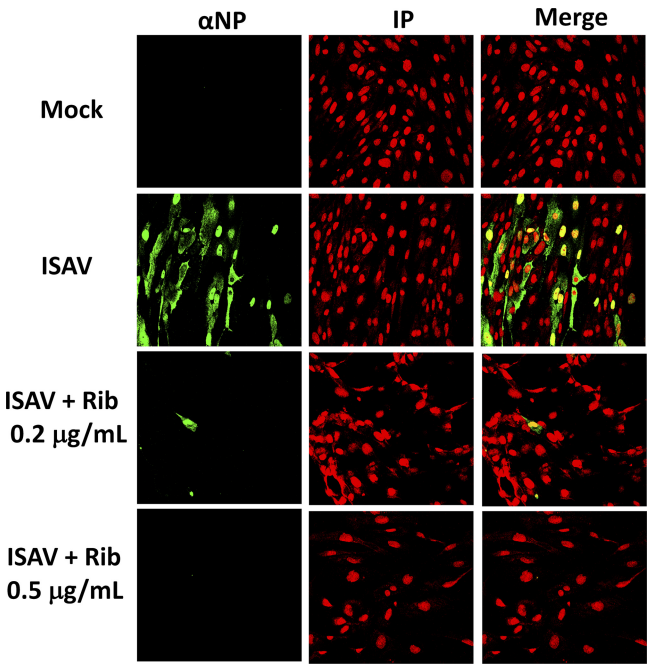

FIG. 2. Effect of ribavirin on viral nucleoprotein expression in cells infected with ISAV. SHK-1 cells were infected with ISAV and treated with increasing concentrations of ribavirin. At 7 dpi the cells were analyzed by IFAT. Nuclei were stained with PI in red, and NP was detected in green (αNP).

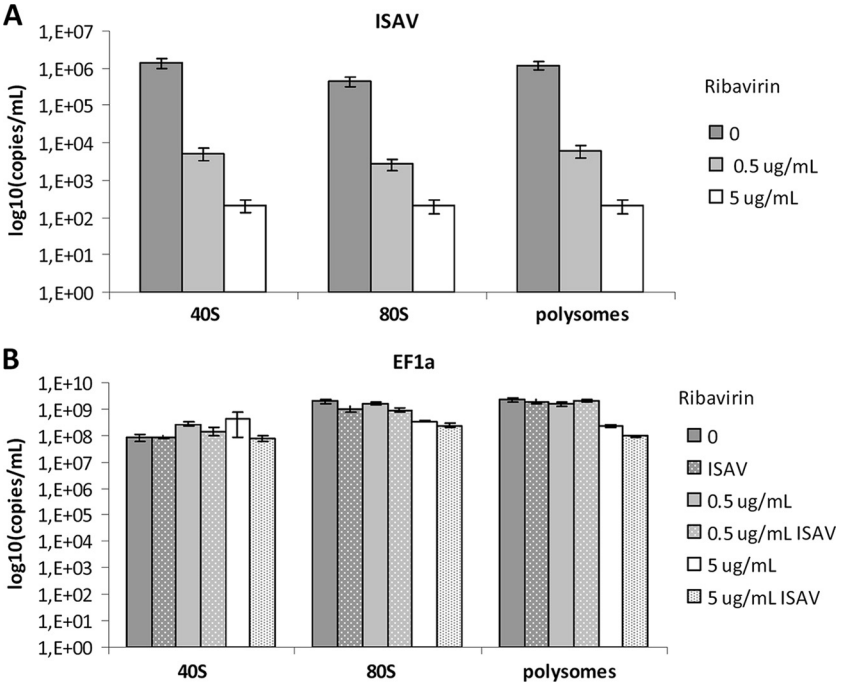

FIG. 3. Effect of ribavirin on the association of ISAV and EF1a mRNA with ribosomal subunits. Total extract of SHK-1 cells infected or not with ISAV and treated with 0, 0.5, and 5 μg/ml of ribavirin for 4 days was fractionated on a sucrose gradient of 15 to 45% (wt/wt). The RNAs of ISAV (A) or EF1a (B) associated with 40S ribosomal subunit, 80S ribosome, and polysomes were determined by real-time qRT-PCR.

0.01 (positive control) according to this technique. However, only 0.001% of infected cells were detected when 0.2 μg/ml of ribavirin was added. Furthermore, no viral protein was detected in the negative control or in cells treated with 0.5 μg/ml of ribavirin. The infected cells treated with ribavirin showed only a low number of cells; this loss of cells by displacement is caused by immunofluorescence procedures but is not due to cytotoxic activity. As no viral protein is detected in the presence of ribavirin, we investigated whether ribavirin affects protein synthesis.

Effect of ribavirin on the association of viral mRNA with polysomes. To determine if the decrease in viral proteins observed is related to the reduction of viral mRNA association with polysomes, a polysomal profile was developed. In this, ribosomal subunit 40S could be distinguished from competent 80S ribosome and from polysomes, through separation on a sucrose gradient, and the association of viral mRNA with these ribosomal subunits could be determined by real-time RT-PCR. Thus, changes in the association of segment 8 mRNA with ribosomes were determined on infected cells treated or not with 0.5 μg/ml or 5 μg/ml of ribavirin. Figure 3A shows that in the polysomal profile obtained, treatment with 0.5 μg/ml of ribavirin decreased mRNA association with the 40S subunits, 80S ribosomes, and polysomes by at least 2 logs, while 5 μg/ml of ribavirin decreased it by an additional 2 logs (reaching the limit of detection by the instrument). Then, decrease in viral proteins after ribavirin treatment could be due to an inhibition of loading of viral mRNA on ribosomes or to an inhibition of RNA synthesis.

Remarkably, none of these treatments affected the association of ribosomes with the elongation transcription factor 1a (EF1a) mRNA in both infected and uninfected cells (Fig. 3B), indicating that inhibition of ribavirin is specific for ISAV. Besides, ISAV did not affect the association of cellular mRNAs with polysomes.

Effect of ribavirin on viral vRNA and mRNA synthesis. The inhibition of viral RNA association with polysomes and protein synthesis could be due to a decrease in the levels of viral mRNA. Thus, we determined the effect of ribavirin on the synthesis of both viral mRNA and vRNA. To do this, total RNA was extracted from cells treated or not with 0.5 and 5 μg/ml of ribavirin and the supernatant was discarded. Quantification of RNAs by real-time qRT-PCR showed that the synthesis of both vRNA and mRNA decreased with the addition of ribavirin (Fig. 4). At 0.5 μg/ml, ribavirin inhibited by the same magnitude both vRNA and mRNA (close to 100 times), but at 5 μg/ml, inhibition was slightly higher for mRNA than for vRNA (around 4 orders of magnitude). It was observed with 5 μg/ml of ribavirin that at 7 dpi both RNAs were still detected on cells but at 9 dpi the inhibition of both RNAs was almost complete (at the sensitivity limit of the method used). This suggests that the inhibition of two types of viral RNA could occur through the same mechanism.

Reversibility of the ribavirin inhibition. Some nucleotide derivatives such as oltipraz or lamivudine (3TC) can irreversibly inhibit viral enzymes such as the HIV polymerase (35, 40). Related to this, one of the inhibitory mechanisms of ribavirin is its irreversible incorporation into the nascent RNA instead of GMP or AMP, causing the misincorporation of cytidine and uridine instead of guanine or adenine, resulting in the production of mutated RNA which could generate noninfective particles, a process called "lethal mutagenesis." Also, previous

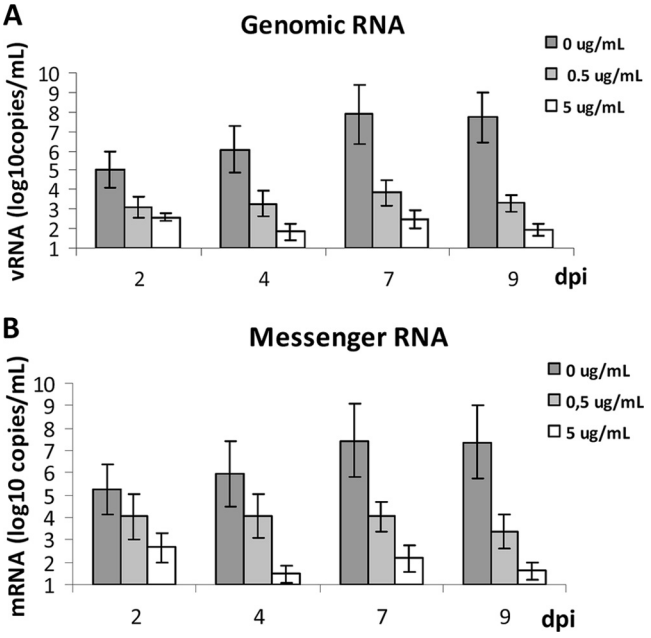

FIG. 4. Effect of ribavirin on genomic and mRNA synthesis of ISAV. Monolayers of SHK-1 cells were infected with ISAV, with or without ribavirin. vRNA (A) and mRNA (B) were quantified with real-time qRT-PCR at 2, 4, 7, and 9 dpi (graphic representations of three independent experiments ± standard errors of the means).

reports had shown that the inhibitory effect of ribavirin on viral or cellular nucleic acid by glutamine depletion could be reversed by replacing the drug-containing medium with fresh medium, demonstrating that this mechanism is reversible (25, 31). To determine whether the mechanism of ribavirin inhibition of ISAV is reversible or not, which could give an insight into the antiviral mechanism, treatment and infection were conducted as in the previous experiment with ribavirin concentrations close to the IC_{50} and the IC_{100} (0.5 and 5 μg/ml, respectively). Then, at 4 dpi the medium with ribavirin was replaced with medium without ribavirin and viral replication was monitored by real-time qRT-PCR at 7 and 14 dpi. As shown in Fig. 5A, at day 4 the inhibition by ribavirin at both concentrations was almost complete. Furthermore, when both concentrations of ribavirin were used, the magnitudes of the inhibition at 7 dpi did not differ. However, at 14 dpi, after 0.5 μg/ml of ribavirin had been removed at 4 dpi, there was almost complete reversal of inhibition, reaching the level of the positive control and increasing at least 10^6-fold. This result shows that, at this concentration, reversal would be complete 10 days after removal of ribavirin, which could be due to residual virus replication not inhibited by ribavirin. However, at 5 μg/ml of ribavirin, reversal of inhibition did not occur at least for 10 days, suggesting that the effect of ribavirin at this concentration is irreversible.

To analyze the effect of ribavirin on the synthesis of viral proteins, the infected cultures were analyzed by immunofluorescence (IFAT) using an antibody which recognizes NP. As shown in Fig. 5B, no NP proteins were detected using 0.5 μg/ml of ribavirin at 4 and 7 dpi. However, when ribavirin was removed at 4 dpi, the viral proteins were detected at 14 dpi, suggesting that virus that escaped the antiviral effect present in the cells at 7 dpi allowed synthesis of new viral proteins after ribavirin removal.

If ribavirin affects the GTP pool, the level of cellular mRNAs must decrease when ribavirin is added. To confirm this, we studied the effect of ribavirin at 7 and 14 days post-treatment on the synthesis of the cellular EF1a mRNA, a common cellular housekeeping mRNA, by treating cells with 0.2 μg/ml or 0.5 μg/ml of ribavirin and replacing or not replacing the medium with fresh medium at day 4. As shown in Fig. 5D, the presence of ribavirin had no effect on EF1a message level and its removal also did not affect EF1a message accumulation, suggesting that the antiviral at this concentration had no effect on the GTP pool and on cellular transcription. In addition, EF1a expression was not affected by ISAV infection either with or without ribavirin. This indicated that ribavirin inhibition of RNA synthesis is specific to ISAV, being innocuous to the cell at doses used in this investigation.

On the other hand, to determine if lethal mutagenesis is the mechanism that explains the irreversible inhibition of ISAV replication by ribavirin, supernatant containing virus from cells treated with the IC_{50} of ribavirin (0.02 μg/ml) was used to infect a new monolayer of SHK-1 cells in the presence or absence of the IC_{50} of ribavirin. To determine the growth ability of this double-inhibited ISAV, a new monolayer was infected and the titer on the supernatant was determined. Whether lethal mutagenesis occurred or not, the virus that results from double ribavirin inhibition must have contained a mutated genome, so that it could not achieve the same viral titer that was obtained in the absence of ribavirin. Figure 5C shows that viral infectiousness remained similar to that of the first infection (Fig. 5A), reaching a similar titer at 7 dpi (10^6 copies/ml), suggesting that particles generated in the presence of ribavirin did not lose infective capacity, leaving the possibility of the occurrence of lethal mutagenesis.

Effect of guanosine on ribavirin inhibition. In order to confirm that the mechanism by which ribavirin inhibits the viral RNA synthesis is not by the inhibition of IMPDH, the effect of guanosine addition on reversal of ribavirin inhibition was determined. To do this, 75 μg/ml of guanosine was used, because this concentration produces an excess of physiological GTP in cell lines (26, 45, 50). Thus, guanosine and 0.2 or 0.5 μg/ml of ribavirin were added to ISAV-infected SHK-1 monolayers. If ribavirin exerts its inhibitory effect by guanosine depletion, addition of guanosine would prevent this inhibition (22). The results (Fig. 6A) show that addition of ribavirin at 0.2 or 0.5 μg/ml inhibits ISAV titer almost 100%. After 75 μg/ml guanosine is added, there is no reversal of the inhibitory effect of ribavirin at both concentrations at 4 dpi, which is the minimum time at which ISAV RNA can be detected in supernatant of cell culture. This result indicated that the mechanism by which ribavirin inhibits the growth of ISAV *in vitro* is not by guanosine depletion. When 50 and 100 μg/ml of guanosine were used, similar results were obtained (data not shown). Detection of the IPNV genome was used as a control in this experiment. IPNV is inhibited by ribavirin and EICAR, another analog of guanosine, at similar concentrations (IC_{50} of 0.5 μg/ml) (22). Moreover, it was demonstrated that the inhibitory effect of EICAR was reversed by guanosine addition (23). Thus, we determined if the inhibitory effect of 5 μg/ml of ribavirin on the

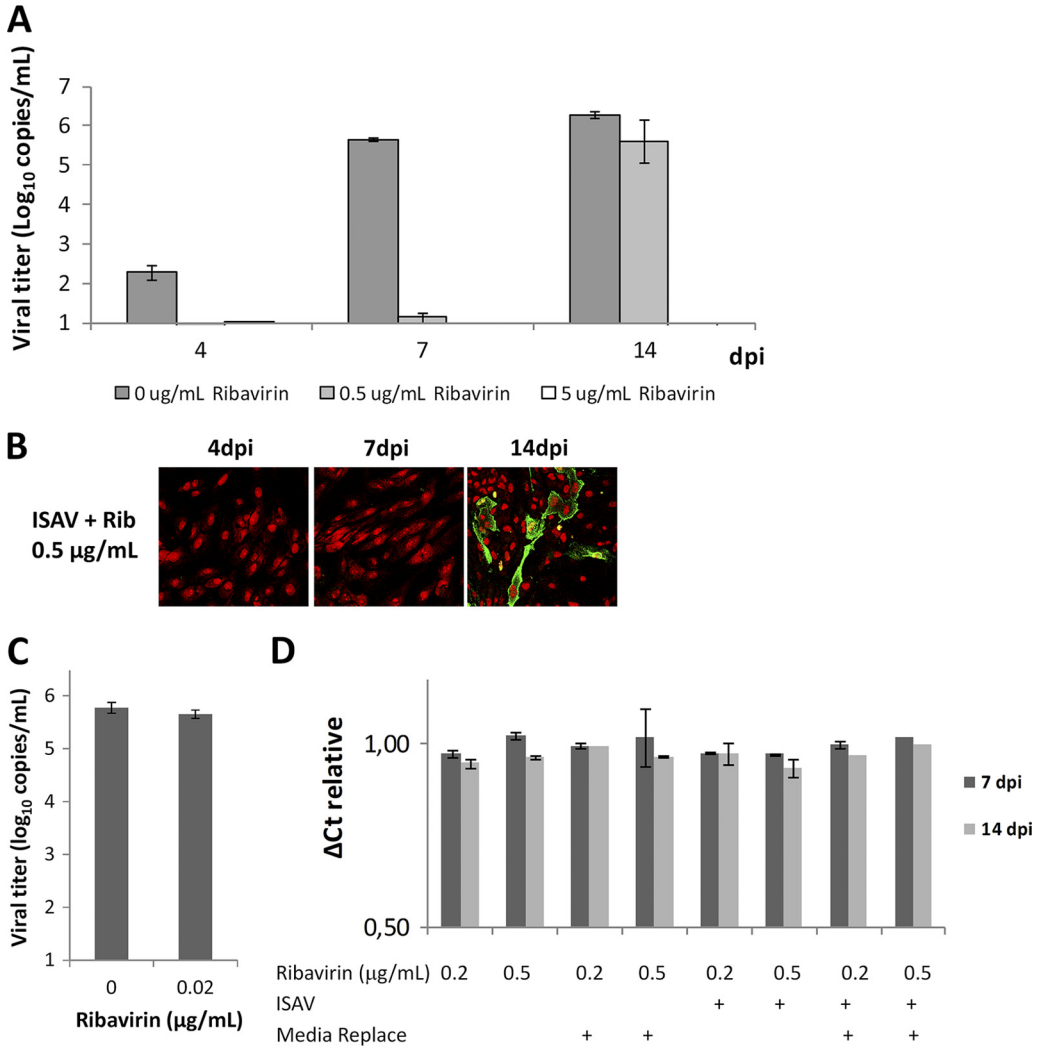

FIG. 5. Study of the reversibility of the inhibition of ribavirin. (A) SHK-1 cells were infected with ISAV, with or without increasing concentrations of ribavirin. At 4 dpi, the medium was changed and the infection was allowed to proceed until 7 or 14 days (graphic representation of 3 independent experiments ± standard error of the mean). (B) Detection of viral nucleoprotein by IFAT of cells infected and treated with 0.5 μg/ml of ribavirin in this experiment with removal of antiviral at 4 dpi. The nuclei were stained with PI in red; NP was detected in green. (C) Supernatant from cells infected and treated with ribavirin at the IC_{50} or not treated was used to infect a new SHK-1 monolayer in the presence or absence of ribavirin inhibition. The titer of the final viral supernatant was determined by real-time qRT-PCR. (D) Effect of ribavirin and ISAV infection on EF1a mRNA expression. SHK-1 cells were or were not infected with ISAV for 7 or 14 days and treated with increasing concentrations of ribavirin. In some cases, medium was replaced at 4 dpi with medium without ribavirin. EF1a expression was determined by real-time qPCR as threshold cycle (C_T) and normalized to the nontreated control (ΔC_T).

IPNV cycle can be reversed by addition of 75 μg/ml of guanosine (Fig. 6B). As is observed, although detection of the IPNV genome is slightly affected by 0.5 μg/ml of ribavirin, the genome is almost undetectable at 5 μg/ml. Noticeably, addition of 50 and 75 μg/ml of guanosine significantly reverses the inhibition by 5 μg/ml of ribavirin. These results indicate that IPNV inhibition by ribavirin can be associated with a decreasing concentration of GTP pool, at a difference of ISAV inhibition levels that is not mediated by a reduction in the GTP pool.

Effect of ribavirin on ISAV infection in Atlantic salmon. Given the antiviral effect observed *in vitro*, we determined if ribavirin can prevent ISAV infection in Atlantic salmon. For this purpose, six groups of 30 postsmolt salmon were infected with ISAV through cohabitation. At 25 dpi, 3 groups (named ribavirin groups 1, 2, and 3) were treated with a dose of 6.5 μmol/kg in feed for 10 days, and the other 3 groups were used as control for ISAV infection (control groups 1, 2, and 3). In addition, in 2 control groups of 30 fish, not infected and not treated, no mortalities were observed throughout the experiment (data not shown). Throughout the trial, mortality was registered daily. Cumulative mortalities are shown in Fig. 7. During the acclimation period, only one fish of control group 2 died. After ISAV infection and before the ribavirin treatment, 4 fish in control group 1 and 2 fish in control group 2 died and the remaining groups lost 1 fish. All dead fish showed symptoms of ISAV, and virus was detected by real-time RT-PCR, showing that they had developed the disease, as was confirmed by necropsy (data not shown). It is important to

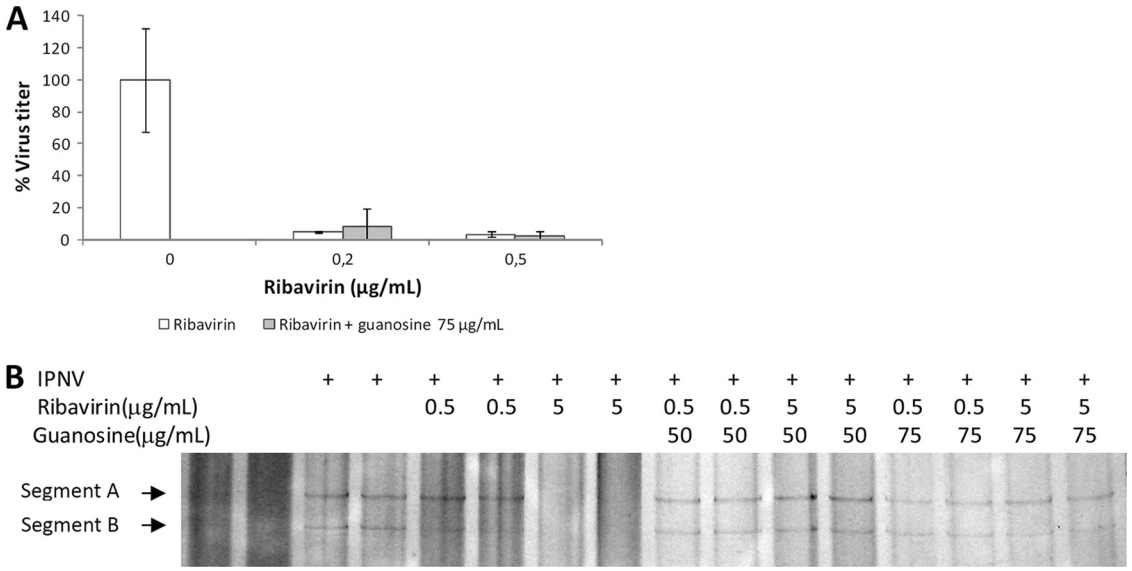

FIG. 6. Effect of guanosine addition on the reversal of ribavirin inhibition on ISAV infection. (A) ISAV inoculum was incubated for 1 h alone or with ribavirin alone or ribavirin plus guanosine and then used to infect SHK-1 monolayers. On the fourth day, the supernatant was assayed for viral titration. (B) IPNV inoculum was incubated for 1 h alone or with ribavirin alone or ribavirin plus guanosine and then used to infect CHSE-214 monolayers. At 16 hpi, RNAs from cells were extracted and visualized by electrophoresis on a 7% polyacrylamide gel for 18 h at 180 V; the gel was stained with silver stain.

note that toxicity testing was performed on *Salmo salar*. Ribavirin was administered for 15 days in feed at concentrations equal to the effective dose, one-half the effective dose, and twice the effective dose. Daily mortality, activity, and appetite of the fish were observed. Furthermore, to determine if the treatment causes anemia as a side effect, necropsies and hematocrit and hemoglobin analysis were performed. Indeed, hematocrit was 30% for nontreated fish and 30, 32, and 30% with treatment with the three doses of ribavirin. In the same way, hemoglobin values were 10% for nontreated control fish and 12, 12, and 10% for ribavirin treatments. These results show that treatment with ribavirin does not change any of the parameters analyzed, showing that it is not toxic and does not produce anemia. It is noteworthy that in three separate experiments, ribavirin protected the fish, with an important reduction in mortality: mortalities of infected tanks without treatment ranged from 43 to 63%, while the use of ribavirin in infected tanks resulted in mortalities between 0 and 3%. Therefore, this experiment showed that ribavirin could be used successfully as an antiviral against ISAV in *Salmo salar*.

DISCUSSION

ISAV has caused millions of dollars of losses to the worldwide salmon industry. In fact, the ISA outbreak in the Faroe

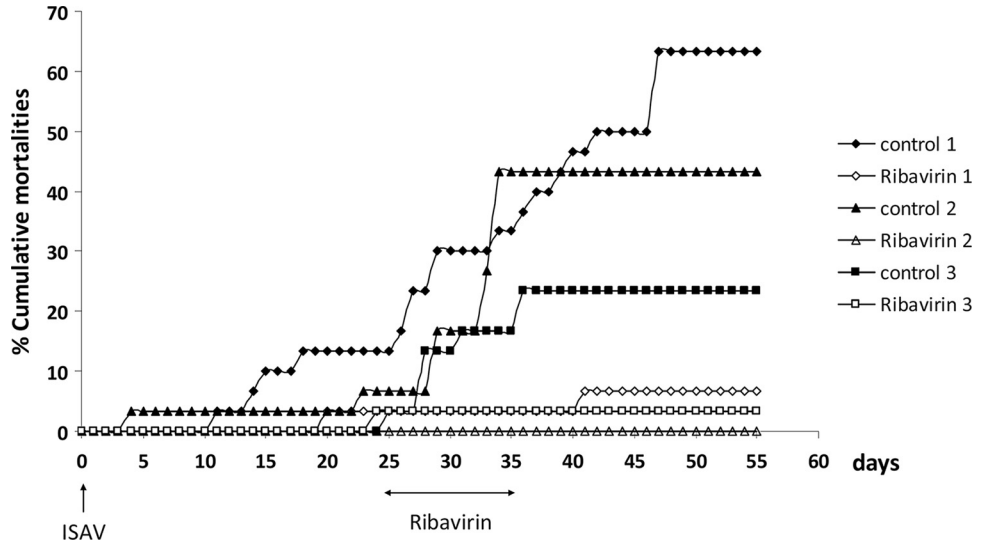

FIG. 7. Effect of ribavirin treatment on Atlantic salmon infected with ISAV. Postsmolt Atlantic salmon were infected with ISAV at day 0 and treated orally in their feed with ribavirin at day 25 for 10 days. Mortalities were monitored daily.

Islands caused a reduction in salmon production from 47,000 tons in 2004 to 12,000 tons in 2006. Furthermore, in less than 1 year, Chile lost its second place after Norway as the world's top producer of Atlantic salmon. Production in 2010 was only 1/10 of that in 2006. Furthermore, ISA is still a serious disease in Norway and Scotland (2).

This work evaluated the effect of the broad-spectrum antiviral ribavirin on the replicative cycle of the ISA virus. It was shown that ribavirin could dramatically inhibit ISAV infection in SHK-1 cells. Moreover, experiments carried out with Atlantic salmon showed that ribavirin inhibits ISAV not only *in vitro* but also *in vivo*. Ribavirin treatment of infected salmon sharply reduced mortality caused by this virus, compared with that in untreated infected salmon. Additionally, the cytotoxicity assays on SHK-1 cells showed that the CC_{50} was 5 orders of magnitude greater than the IC_{50}, which allows us to propose ribavirin as an effective antiviral with certainty of good results in treatment of cultivated salmon that show symptoms of the disease. The inhibitory concentration of ribavirin was a much lower concentration than that reported for other viruses (11, 26, 46).

The selective inhibition of viral enzymes without affecting normal cellular functions is the desired characteristic of an antiviral. Therefore, given the high degree of effectiveness shown by ribavirin, the possible mechanism of this antiviral was examined.

The experiment conducted in order to determine the mechanism by which ribavirin inhibit the replicative cycle of ISAV, showed the following. (i) Guanosine did not reverse the inhibition of ribavirin, and ribavirin exerts a selective inhibition of viral mRNA but not of cellular mRNA (EF1a) synthesis. Guanosine was added at a higher concentration than necessary to produce an excess of physiological GTP in cellular lines (26, 45, 50) and in sufficient quantity to reverse the ribavirin effect on IPNV infection (22). Also, the inhibitory effect of ribavirin on ISAV infection was not reversed by removing it from the medium as occurred in the IPNV experiment (22, 23, 31). It has been reported that ribavirin does not inhibit RNA polymerase II (4), but others show that higher ribavirin doses inhibit cellular mRNA synthesis (31, 51). In this work, that decrease was not detected. This indicates that ribavirin in SHK-1 cells has a high level of effectiveness as an antiviral and that at the concentrations used here it does not affect processes of cellular expression. (ii) The lack of effect on the synthesis of cellular RNA also suggests that ribavirin does not interfere with the incorporation of the cap in the mRNAs, under these conditions. (iii) The titer of virus that is recovered after removal of ribavirin reaches the same titer that was obtained before ribavirin treatment. This suggests that ribavirin is not incorporated in the nascent ribonucleotide chain. Although the rate of incorporation of RTP in other viruses is low (1, 11), causing mutations of 15 nucleotides per genome in poliovirus (PV) and 9 nucleotides in foot-and-mouth disease virus (FMDV), it was demonstrated that even a slight increase in the frequency of mutation dramatically reduces the percentage of infectious vRNAs, being sufficient to cause lethal mutagenesis in PV (10) and supporting the idea that this is probably not the mechanism occurring in ISAV.

A plausible mechanism that could explain the ISAV inhibition by ribavirin could be a direct inhibition of RNA polymerase, as occurs with the La Crosse virus (7), VSV (16), and HIV (35, 40). In fact, it has been shown that ribavirin inhibits influenza virus infection *ex vivo* and *in vivo* by inhibition of the viral polymerase (15, 50). Through *in vitro* assays, the influenza A virus infection is inhibited by RTP but is not inhibited by RMP or by nonphosphorylated ribavirin (15). In cells ribavirin is rapidly phosphorylated to other forms (44). As well, through *ex vivo* assays, ribavirin inhibits various isolates of influenza A, B, and C viruses in MDCK cells at an average concentration of micrograms per milliliter (41, 51). At concentrations higher than 1 mg/ml, ribavirin inhibits the generation of primer and the elongation of the nascent RNA strain in *in vitro* assays using the influenza virus core (49).

The mechanism of direct inhibition of ISAV RNA polymerase by ribavirin that was suggested could be supported by the fact that at 10 days after ribavirin removal, no reversal of the inhibition effect was observed at 5 μg/ml in the assayed time periods. Other studies show that the reversal of inhibition by guanosine depletion occurs within hours to 5 days after removal from the medium (25, 31); if any reversal of inhibitory effect were possible, it would be detectable within 10 days of antiviral removal. This result could indicate that the binding of ribavirin to the polymerase has high affinity. Then, at 0.5 μg/ml of ribavirin and at an MOI of ISAV of 0.01, a percentage of polymerase complexes remains active. Thus, once the ribavirin is metabolized and/or eliminated from the cell (within 24 h of removal from the medium [31, 34]), the transcription and subsequent replication of the viral genome could begin, completing the infective cycle and allowing detection of RNA in the supernatant of the culture.

It is still possible that there is a dual mechanism of inhibition, since the inhibition of the association of viral mRNA with ribosomes was higher than the inhibition of synthesis of viral mRNA by nearly 2 orders of magnitude, suggesting two mechanisms of inhibition affecting both mRNA and protein synthesis in ISAV replication.

The results obtained clearly show that ribavirin efficiently inhibits the replicative cycle of ISAV without affecting cellular mRNA expression. Consequently, ribavirin presents itself as the perfect candidate to be used for palliative treatment against infectious anemia in salmon caused by the ISA virus.

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REFERENCES

1. **Airaksinen, A., N. Pariente, L. Menéndez-Arias, and E. Domingo.** 2003. Curing of foot-and-mouth disease virus from persistently infected cells by ribavirin involves enhanced mutagenesis. Virology **311:**339–349.
2. **Asche, F., H. Hansen, R. Tveteras, and S. Tveteras.** 2009. The salmon disease crisis in Chile. Mar. Resour. Econ. **24:**405–411.
3. **Aspehaug, V., et al.** 2004. Infectious salmon anemia virus (ISAV) genomic segment 3 encodes the viral nucleoprotein (NP), an RNA-binding protein with two monopartite nuclear localization signals (NLS). Virus Res. **106:**51–60.
4. **Barnes, E., et al.** 2004. Impact of alpha interferon and ribavirin on the function of maturing dendritic cells. Antimicrob. Agents Chemother. **48:** 3382–3389.
5. **Bouchard, D., et al.** 1999. Isolation of infectious salmon anemia virus (ISAV) from Atlantic salmon in New Brunswick, Canada. Dis. Aquat. Organ. **35:** 131–137.
6. **Bougie, I., and M. Bisaillon.** 2004. The broad spectrum antiviral nucleoside ribavirin as a substrate for a viral RNA capping enzyme. J. Biol. Chem. **279:**22124–22130.

7. **Cassidy, L. F., and J. L. Patterson.** 1989. Mechanism of La Crosse virus inhibition by ribavirin. Antimicrob. Agents Chemother. **33:**2009–2011.
8. **Clouthier, S. C., T. Rector, N. E. Brown, and E. D. Anderson.** 2002. Genomic organization of infectious salmon anaemia virus. J. Gen. Virol. **83:**421–428.
9. **Cottet, L., et al.** 2010. Bioinformatic analysis of the genome of infectious salmon anemia viruses associated with outbreaks of high mortality in Chile. J. Virol. **84:**11916–11928.
10. **Crotty, S., C. E. Cameron, and R. Andino.** 2001. RNA virus error catastrophe: direct molecular test by using ribavirin. Proc. Natl. Acad. Sci. U. S. A. **98:**6895–6900.
11. **Crotty, S., et al.** 2000. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nat. Med. **6:**1375–1379.
12. **Dannevig, B. H., K. Falk, and E. Namork.** 1995. Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. J. Gen. Virol. **76:**1353–1359.
13. **Devold, M., B. Krossoy, V. Aspehaug, and A. Nylund.** 2000. Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout Salmo trutta after experimental infection. Dis. Aquat. Organ. **40:**9–18.
14. **DeWitte-Orr, S. J., and N. C. Bols.** 2007. Cytopathic effects of chum salmon reovirus to salmonid epithelial, fibroblast and macrophage cell lines. Virus Res. **126:**159–171.
15. **Eriksson, B., et al.** 1977. Inhibition of influenza virus RNA polymerase by ribavirin triphosphate. Antimicrob. Agents Chemother. **11:**946–951.
16. **Fernandez-Larsson, R., K. O'Connell, E. Koumans, and J. L. Patterson.** 1989. Molecular analysis of the inhibitory effect of phosphorylated ribavirin on the vesicular stomatitis virus in vitro polymerase reaction. Antimicrob. Agents Chemother. **33:**1668–1673.
17. **Godoy, M. G., et al.** 2008. First detection, isolation and molecular characterization of infectious salmon anaemia virus associated with clinical disease in farmed Atlantic salmon (Salmo salar) in Chile. BMC Vet. Res. **4:**28.
18. **Goic, B., et al.** 2008. The nucleoprotein and the viral RNA of infectious salmon anemia virus (ISAV) are localized in the nucleolus of infected cells. Virology **379:**55–63.
19. **Goswami, B. B., E. Borek, O. K. Sharma, J. Fujitaki, and R. A. Smith.** 1979. The broad spectrum antiviral agent ribavirin inhibits capping of mRNA. Biochem. Biophys. Res. Commun. **89:**830–836.
20. **Hellebo, A., U. Vilas, K. Falk, and R. Vlasak.** 2004. Infectious salmon anemia virus specifically binds to and hydrolyzes 4-O-acetylated sialic acids. J. Virol. **78:**3055–3062.
21. **Hudson, J. B., E. A. Graham, and M. F. Simpson.** 1988. The efficacy of amantadine and other antiviral compounds against two salmonid viruses in vitro. Antiviral Res. **9:**379–385.
22. **Jashés, M., G. M. M. López-Lastra, E. De Clercq, and A. Sandino.** 1996. Inhibitors of infectious pancreatic necrosis virus (IPNV) replication. Antiviral Res. **29:**309–312.
23. **Jashes, M., G. Mlynarz, E. De Clercq, and A. M. Sandino.** 2000. Inhibitory effects of EICAR on infectious pancreatic necrosis virus replication. Antiviral Res. **45:**9–17.
24. **Krossoy, B., I. Hordvik, F. Nilsen, A. Nylund, and C. Endresen.** 1999. The putative polymerase sequence of infectious salmon anemia virus suggests a new genus within the Orthomyxoviridae. J. Virol. **73:**2136–2142.
25. **Larsson, A., K. Stenberg, and B. Oberg.** 1978. Reversible inhibition of cellular metabolism by ribavirin. Antimicrob. Agents Chemother. **13:**154–158.
26. **Leyssen, P., J. Balzarini, E. De Clercq, and J. Neyts.** 2005. The predominant mechanism by which ribavirin exerts its antiviral activity in vitro against flaviviruses and paramyxoviruses is mediated by inhibition of IMP dehydrogenase. J. Virol. **79:**1943–1947.
27. **Lovely, J. E., et al.** 1999. First identification of infectious salmon anaemia virus in North America with haemorrhagic kidney syndrome. Dis. Aquat. Organ. **35:**145–148.
28. **Luria, S., J. Darnell, D. Baltimore, and A. Campbell.** 1978. General virology, 3rd ed. John Wiley & Sons, Inc., New York, NY.
29. **Marroquí, L., A. Estepa, and L. Perez.** 2007. Assessment of the inhibitory effect of ribavirin on the rainbow trout rhabdovirus VHSV by real-time reverse-transcription PCR. Vet. Microbiol. **122:**52–60.
30. **Merril, C. R., M. L. Dunau, and D. Goldman.** 1981. A rapid sensitive silver stain for polypeptides in polyacrylamide gels. Anal. Biochem. **110:**201–207.
31. **Migus, D. O., and P. Dobos.** 1980. Effect of ribavirin on the replication of infectious pancreatic necrosis virus in fish cell cultures. J. Gen. Virol. **47:**47–57.
32. **Mjaaland, S., E. Rimstad, K. Falk, and B. H. Dannevig.** 1997. Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (Salmo salar L.): an orthomyxo-like virus in a teleost. J. Virol. **71:**7681–7686.
33. **Oxford, J. S.** 1975. Inhibition of the replication of influenza A and B viruses by a nucleoside analogue (ribavirin). J. Gen. Virol. **28:**409–414.
34. **Page, T., and J. D. Connor.** 1990. The metabolism of ribavirin in erythrocytes and nucleated cells. Int. J. Biochem. **22:**379–383.
35. **Prochaska, H. J., Y. Yeh, P. Baron, and B. Polsky.** 1993. Oltipraz, an inhibitor of human immunodeficiency virus type 1 replication. Proc. Natl. Acad. Sci. U. S. A. **90:**3953–3957.
36. **Rankin, J. T., Jr., S. B. Eppes, J. B. Antczak, and W. K. Joklik.** 1989. Studies on the mechanism of the antiviral activity of ribavirin against reovirus. Virology **168:**147–158.
37. **Raynard, R. S., M. Snow, and D. W. Bruno.** 2001. Experimental infection models and susceptibility of Atlantic salmon Salmo salar to a Scottish isolate of infectious salmon anaemia virus. Dis. Aquat. Organ. **47:**169–174.
38. **Ritchie, R. J., J. Heppell, M. B. Cook, S. Jones, and S. G. Griffiths.** 2001. Identification and characterization of segments 3 and 4 of the ISAV genome. Virus Genes **22:**289–297.
39. **Rodger, H. D., T. Turnbull, F. Muir, S. Millar, and R. H. Richards.** 1998. Infectious salmon anaemia (ISA) in the United Kingdom. Bull. Eur. Assoc. Fish Pathol. **18:**115–116.
40. **Severini, A., X. Y. Liu, J. S. Wilson, and D. L. Tyrrell.** 1995. Mechanism of inhibition of duck hepatitis B virus polymerase by (−)-beta-L-2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. **39:**1430–1435.
41. **Shigeta, S., K. K. T. Yokota, K. Nakamura, and E. De Clercq.** 1988. Comparative activities of several nucleoside analogs against influenza A, B, and C viruses in vitro. Antimicrob. Agents Chemother. **32:**906–911.
42. **Shigeta, S., et al.** 1992. Antiviral activities of ribavirin, 5-ethynyl-1-beta-D-ribofuranosylimidazole-4-carboxamide, and 6′-(R)-6′-C-methylneplanocin A against several ortho- and paramyxoviruses. Antimicrob. Agents Chemother. **36:**435–439.
43. **Snow, M., et al.** 2003. Isolation and characterisation of segment 1 of the infectious salmon anaemia virus genome. Virus Res. **92:**99–105.
44. **Streeter, D. G., et al.** 1973. Mechanism of action of 1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a new broad-spectrum antiviral agent. Proc. Natl. Acad. Sci. U. S. A. **70:**1174–1178.
45. **Sun, Y., D.-H. Chung, Y.-K. Chu, C. B. Jonsson, and W. B. Parker.** 2007. Activity of ribavirin against Hantaan virus correlates with production of ribavirin-5′-triphosphate, not with inhibition of IMP dehydrogenase. Antimicrob. Agents Chemother. **51:**84–88.
46. **Toltzis, P., and A. S. Huang.** 1986. Effect of ribavirin on macromolecular synthesis in vesicular stomatitis virus-infected cells. Antimicrob. Agents Chemother. **29:**1010–1016.
47. **Vignuzzi, M., J. K. Stone, and R. Andino.** 2005. Ribavirin and lethal mutagenesis of poliovirus: molecular mechanisms, resistance and biological implications. Virus Res. **107:**173–181.
48. **Workenhe, S. T., M. J. Kibenge, T. Iwamoto, and F. S. Kibenge.** 2008. Absolute quantitation of infectious salmon anaemia virus using different real-time reverse transcription PCR chemistries. J. Virol. Methods **154:**128–134.
49. **Wray, S. K., B. E. Gilbert, and V. Knight.** 1985. Effect of ribavirin triphosphate on primer generation and elongation during influenza virus transcription in vitro. Antiviral Res. **5:**39–48.
50. **Wray, S. K., B. E. Gilbert, M. W. Noall, and V. Knight.** 1985. Mode of action of ribavirin: effect of nucleotide pool alterations on influenza virus ribonucleoprotein synthesis. Antiviral Res. **5:**29–37.
51. **Wray, S. K., R. H. Smith, B. E. Gilbert, and V. Knight.** 1986. Effects of selenazofurin and ribavirin and their 5′-triphosphates on replicative functions of influenza A and B viruses. Antimicrob. Agents Chemother. **29:**67–72.