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Inhibitory effect of mycophenolic acid on the replication of infectious pancreatic necrosis virus and viral hemorrhagic septicemia virus

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ABSTRACT

Infectious pancreatic necrosis virus (IPNV) and viral hemorrhagic septicemia virus (VHSV) remain two of the most important pathogens of farmed trout worldwide. Mycophenolic acid (MPA) is an inhibitor of cellular inosine monophosphate dehydrogenase (IMPDH), an enzyme that catalyzes an essential step in the biosynthesis of GTP. In this report, the antiviral activity of MPA against IPNV and VHSV in cell culture was assessed. Cell viability, virus yield, protein and RNA synthesis determinations were used to evaluate the inhibitory effect of MPA. MPA caused a dose-dependent inhibition of IPNV and VHSV replication. It was found that MPA had a particularly potent effect against IPNV, inhibiting the production of infectious virus more than 10^5-fold. MPA was also highly effective in preventing viral protein synthesis. Quantitative real-time RT-PCR was used to measure viral RNA in cells infected by IPNV or VHSV to evaluate the inhibitory capacity of MPA as well as to compare MPA to the established antiviral drug ribavirin. MPA showed a good efficacy in decreasing accumulation of viral RNA at low concentrations. Finally, time of addition and wash out experiments suggested that MPA may have a dual mechanism of action, targeting both a cell and a viral function. This study provides evidence that MPA can function as a broad-spectrum antiviral drug for use in therapy of rainbow trout diseases.

1. Introduction

Infectious pancreatic necrosis virus (IPNV) and viral hemorrhagic septicemia virus (VHSV) represent important sources of disease in farmed salmonid species such us rainbow trout (*Oncorhynchus mykiss*). IPNV is a member of the family Birnaviridae, a group of non-enveloped double-stranded RNA viruses with a bipartite segmented genome which consists of segments A and B. VHSV is a rhabdovirus, an enveloped negative-stranded RNA virus.

At present, there are no specific antiviral drugs approved for the treatment of those two infectious diseases of trout. A few number of compounds have been tested in vitro as well as in vivo (Hudson et al., 1988; Jashés et al., 2000; Kamei and Aoki, 2007; LaPatra et al., 1998; Mas et al., 2006; Micol et al., 2005; Moya et al., 2000) showing various degrees of efficacy. Here we describe the in vitro activity of mycophenolic acid (MPA) against IPNV and VHSV infection of fish cells. MPA is a non-nucleoside inhibitor of the cellular inosine monophosphate dehydrogenase (IMPDH), an enzyme involved in the synthesis of GMP (Sintchak et al., 1996). Treatment of cells with MPA leads to a decreasing guanine nucleotide pool required for adequate RNA and DNA synthesis. This has been a widely accepted mechanism of action to explain the capacity of MPA to inhibit the replication of several viruses in vitro (Diamond et al., 2002; Robertson et al., 2004; Smee et al., 2002; Sun et al., 2007; Takhampunya et al., 2006). With respect to trout viruses, other compounds with inhibitory activity against IMPDH such as ribavirin (RIB) and EICAR have been proven to protect cultured cells from VHSV and IPNV infection (Marroquí et al., 2007; Migus and Dobos, 1980) as well as rainbow trout from IPNV infection (Moya et al., 2000). Ribavirin, a broad-spectrum antiviral agent with activity against a wide range of DNA and RNA viruses, is a nucleoside analog which after intracellular phosphorylation becomes a competitive inhibitor of IMPDH (Graci and Cameron, 2006; Parker, 2005). In addition to the inhibition of IMPDH, three other mechanisms to explain ribavirin antiviral activity have been proposed: direct inhibition of the viral RNA polymerase (Toltzis et al., 1988), inhibition of the "capping" of the 5′ end of viral mRNAs (Goswami et al., 1979), and induction of error catastrophe as a result of accumulation of mutations (Graci and Cameron, 2002).

We have previously developed a real-time RT-PCR assay to evaluate the antiviral potency of candidate compounds on fish cell cultures. The qRT-PCR assay was proven useful in determining the anti-VHSV activity of ribavirin in vitro (Marroquí et al., 2007). In an effort to find a compound with enhanced antiviral activity against trout viruses, MPA was selected as it has been reported to have

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a more potent antiviral activity than ribavirin on the replication of other RNA viruses (Leyssen et al., 2005). We have examined in detail the antiviral effect of MPA on the production of infectious virus, synthesis of viral proteins and on the accumulation of viral RNA in the infected cells. Our data provide evidence that MPA has a potent inhibitory effect on the replication of both IPNV and VHSV in vitro by preventing the synthesis of viral RNA.

2. Materials and methods

2.1. Cells, viruses and reagents

The fish cell line epithelioma papullosum cyprini (EPC) was purchased from the European collection of cell cultures (ECACC no. 93120820). CHSE-214 (Chinook salmon embryo) and RTG-2 (rainbow trout gonad) cells purchased from the American Type Culture Collection (ATCC CCL 55 and CRL 1681) were used.

The EPC and CHSE-214 were maintained at 28 °C and 20 °C, respectively in a 5% CO_2 atmosphere in RPMI-1640 Dutch-modified medium containing 10% fetal bovine serum, 1 mM pyruvate, 2 mM glutamine and antibiotics (50 μg/ml gentamicin and 2 μg/ml amphotericin B). The RTG-2 cell line was maintained at 20 °C in MEM (with Earle's salts) cell culture medium also supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics (50 μg/ml neomycin sulfonate).

VHSV (07-71 strain) isolated in France (LeBerre et al., 1977) was grown in EPC cells at 14 °C. IPNV (Sp strain) was cultured in CHSE cells at 14 °C. In both cases, when cytopathic effect (CPE) was complete, supernatants from infected cell cultures were clarified by centrifugation and stored in aliquots at −70 °C. Virus yield in cell culture supernatants (TCID 50/ml) was determined in 96-well plates by the end-point dilution method.

Ribavirin and MPA were purchased from Sigma and dissolved in distilled water or methanol, respectively.

2.2. Cytotoxicity determination and CPE reduction assay

Toxicity of MPA on EPC, CHSE and RTG-2 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. Increasing concentrations of MPA from 0.1 μg/ml to 100 μg/ml were added and the cells were incubated in the presence of the compound for 72 h. The experiment was performed in triplicate wells following the protocol provided by the manufacturer. Cell viability was determined by measuring absorbance at 492 nm.

The CPE reduction assay measures the protection of MPA-treated cells against virus-induced lysis by calculating the percentage of viable EPC cells at day 5 post-infection with VHSV. 100% inhibition of CPE represents the average absorbance value of non-infected cells. The IC_{50} value was calculated based on fitting of the dose–response curve using the program Origin.

2.3. Virus yield and immunostained focus assays

CHSE cells (for IPNV infection) and EPC cells (for VHSV infection) were grown on 24-well plates. MPA was added to the cells either 20 h before infection (−20 h) or at the same time as the virus (0 h). Supernatant samples were collected from the infected cells at 24 h, 48 h and 72 h post-infection with IPNV or VHSV (m.o.i. 0.05). The virus yield assay was performed by the end-point dilution method ($TCID_{50}$) with aliquots of cell culture media from MPA-treated infected cells. The aliquots were serially diluted in FCS 2% medium prior to addition to 96-well plates containing confluent EPC cells (for VHSV titration) or CHSE cells (for IPNV). Following a 7-day incubation time at 14 °C the cells were stained with crystal violet and the $TCID_{50}$ was calculated. To determine virus infectivity, a previously developed immunostaining assay (focus forming unit, f.f.u.) was used (Lorenzo et al., 1996; Perez et al., 2002). Confluent EPC cells were incubated with 10 μg/ml MPA for 20 h and then washed before infection with VHSV (m.o.i. = 2×10^{-4} pfu/cell). To other cells 10 μg/ml MPA was added at time 0 and maintained during the infection. At 24 h p.i. the cells were fixed with 100% methanol and stained with a monoclonal antibody to VHSV N protein (2C9). The binding of antibodies was detected by peroxidase-conjugated rabbit antibodies to mouse IgG (Sigma), following incubation with 3,3′-diamino benzidine (DAB) to yield a brownish reaction product. Cell monolayers were washed, dried and clusters of brown-stained cells (foci) were counted under the microscope.

2.4. Western blot analysis

CHSE cells were grown on 24-well plates and infected with VHSV or IPNV at a m.o.i. of 0.05 f.f.u/cell. MPA (10 μg/ml) was added to the cell monolayers at the infection time (t = 0 h p.i.) and cultured at 14 °C. At 24 h, 48 h or 72 h p.i. the media were removed and the cells were washed with PBS ($-Ca^{+2}$, $-Mg^{+2}$) and collected in 200 μl PBS ($-Ca^{+2}$, $-Mg^{+2}$). Cell pellets were obtained by centrifugation at $1000 \times g \times 10$ min and resuspended in electrophoresis buffer sample. Proteins from the virus-infected cell extract were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (12% gels). Prestained SDS-PAGE standards (BioRad Laboratories) were included for molecular mass estimation. Transfer to nitrocellulose membranes was performed in 2.5 mM Tris base, 9 mM glycine, 20% methanol buffer, at 125 mA for 90 min. Following blocking with 2% nonfat dry milk the membranes were incubated with anti-VHSV or anti-IPNV polyclonal rabbit antibodies (1:1500 dilution) before incubation with a peroxidase-conjugated goat anti-rabbit antibody (Sigma). Finally, protein bands were revealed by using the ECL Chemiluminiscence Reagents kit (Amersham Biosciences).

2.5. Determination of time of addition and drug removal effect on the production of viral RNA

For time of addition experiments, confluent CHSE cells were grown on 24-well plates and infected with IPNV or VHSV at a m.o.i. of 0.05 $TCID_{50}$/cell in RPMI-1640 cell culture medium supplemented with 2% FCS. At 0 h, 4 h or 8 h post-infection the culture medium was replaced by medium containing 10 μg/ml MPA. At 24 h and 48 h p.i. RNA was isolated and analyzed by real-time RT-PCR as described below.

To measure the effect of drug removal on viral RNA levels, CHSE cells on 24-well plates were treated with 10 μg/ml MPA for 20 h prior to infection with IPNV (pre-treatment). At 8 h or 4 h before infection the drug was removed by washing the cells with fresh medium. At time 0, IPNV was added to the cells and the infection was allowed to proceed for 24 h. In post-infection treatment experiments, CHSE cells were infected with IPNV (m.o.i. = 0.05) in medium containing 10 μg/ml MPA. At 4 h or 8 h p.i. the cells were washed and replenished with fresh culture media. At 24 h p.i. cell samples were harvested for quantitative RT-PCR analysis.

2.6. Quantitation of viral RNA (real-time RT-PCR assay)

The quantity of VHSV RNA was measured by real-time reverse transcription PCR (qRT-PCR) following an established two-step protocol consisting of reverse transcription with random hexamers followed by quantitative PCR with VHSV N

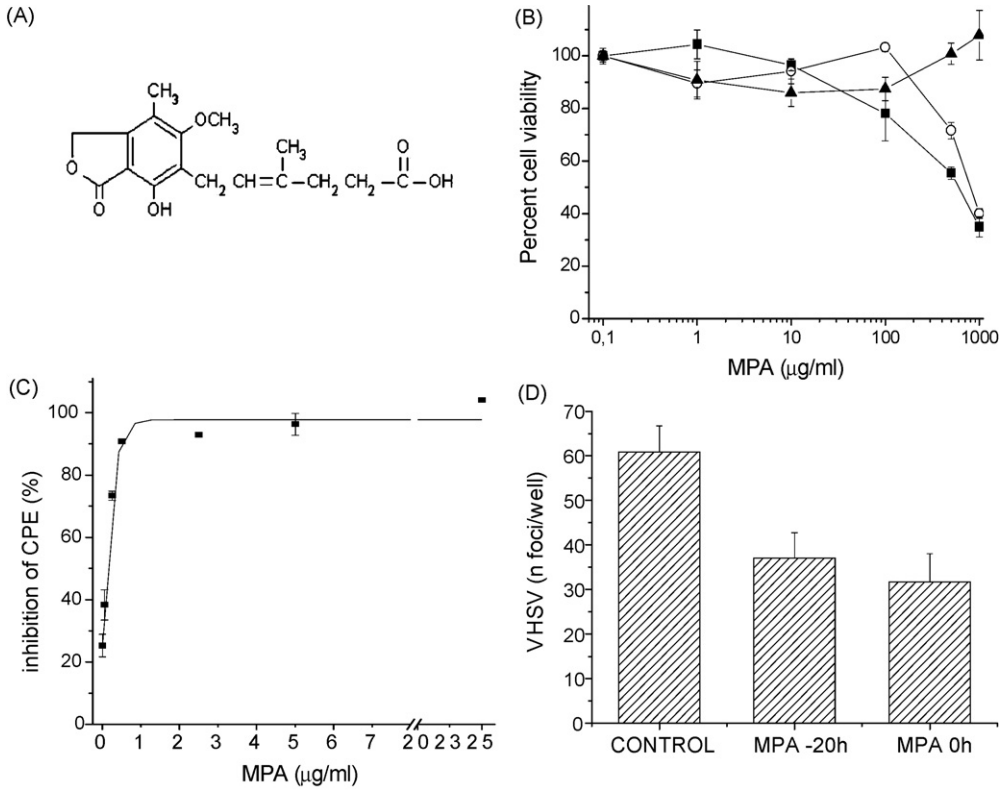

Fig. 1. (A) Chemical structure of mycophenolic acid (MPA). (B) Toxic effect of MPA on EPC (▲), CHSE (○) and RTG-2 (■) cells. Cells were treated with increasing MPA concentrations for 72 h and then tested for viability by using a quantitative colorimetric (MTS) cell viability assay. Mean data and standard deviations (S.D.) from three wells per drug concentrations are expressed. (C) Inhibition of VHSV-induced cytopathic effect on EPC cells by MPA. Concentration–effect curve showing the antiviral activity of MPA against VHSV assessed by the MTS assay, on EPC cells at day 5 post-infection with VHSV. Results are presented as the percentage of CPE reduction referred to non-infected cells (100% inhibition of CPE). Data are expressed as mean ± S.D. from three wells. (D) Reduced infectivity of VHSV in MPA-treated cells. MPA −20 h: incubation of EPC cells for 20 h before infection. MPA 0 h: MPA addition at the time of virus infection. Clusters of positive staining cells (foci) were counted in six wells per treatment. Control: non-treated EPC cells infected with VHSV. Mean values and standard deviations are represented.

gene specific primers and FAM-labeled probes (Chico et al., 2006; Falco et al., 2007). For IPNV RNA, the following A segment specific primers and probe were used (GenBank accession no. AJ622822): IPNV-A-for, 5′-TCTCCCGGGCAGTTCAAGT-3′; IPNV-A-rev, 5′-CGGTTTCACGATGGGTTGTT-3′; IPNV-A-probe, 5′-CCAGAACCAGGTGACGAGTATGAGGACTACAT-3′. For qRT-PCR experiments, total RNA from cells in 24-well plates was extracted with the RNAgents® Total RNA Isolation kit (Promega). For quantity control of cellular RNA, qRT-PCR for 18S rRNA was performed with the 18S rRNA Pre-developed TaqMan® Assay Reagents kit (Applied Biosystems). Samples were analyzed on a 7300 Real-time PCR System (Applied Biosystems). For relative quantitation of viral RNA expression, the comparative method using the $2^{-\Delta\Delta Ct}$ formula was used. The levels of target amplicons (VHSV N gene or IPNV A segment) are compared to a calibrator (i.e. the sample with the lowest level of virus RNA in the experiment) and fold-differences are calculated.

3. Results

3.1. Protection against VHSV-induced CPE in MPA-treated cells

MPA (Fig. 1A) was examined for possible cytotoxic effect in EPC, CHSE and RTG-2 cells, which are the cell lines most commonly used for IPNV and VHSV in vitro studies. This was done both by microscopic examination and by measuring the viability of cell monolayers over a period of 3 days (Fig. 1B). MPA had little effect on cultured cells at concentrations ≤ 100 μg/ml; 500 μg/ml was toxic to RTG-2 and CHSE cells, and 1000 μg/ml MPA had a severe toxic effect in RTG-2 and CHSE cells. Remarkably, EPC cells tolerated up to 1000 μg/ml MPA.

The antiviral activity of MPA was firstly evaluated by determining the viability of EPC cells treated with increasing concentrations of MPA at 5 days post-infection with VHSV, as a measure of the protection of the cells against the VHSV-induced CPE conferred by MPA (Fig. 1C). At 0.5 μg/ml MPA, the viability of VHSV-infected cells were above 90% of mock-infected controls. Based on the CPE inhibition assay the IC_{50} of MPA against VHSV is 0.14 μg/ml (0.43 μM). For further experiments, a drug concentration of 10 μg/ml was chosen since it causes maximal inhibition without reducing cell viability.

We also examined the inhibitory activity of MPA on VHSV growth by an infectious focus formation assay which basically determines the spread of viral infection on a cell monolayer by antibody detection of the VHSV nucleoprotein (Mas et al., 2004). Pretreatment of EPC cells with MPA reduced VHSV foci by (39 ± 9)% while MPA addition at time 0 achieved (48 ± 10)% inhibition of the number of infectious foci on the cell monolayer (Fig. 1D). We have observed that the antiviral effect of MPA is independent of the cell line used in the assay (not shown).

3.2. IPNV and VHSV growth is inhibited by MPA

Given the protective effect of MPA on virus-induced CPE, we next examined the antiviral activity of MPA against IPNV and VHSV by a virus yield reduction assay (Table 1). MPA was tested either by pre-incubation of the cells with the compound (−20 h) or by adding the drug simultaneously with the virus (0 h). In both cases, it was

Table 1
Effect of MPA on the production of infectious IPNV and VHSV progeny

	VHSV ($TCID_{50}$/ml)			IPNV ($TCID_{50}$/ml)		
Hours p.i.	24	48	72	24	48	72
Untreated	3.2×10^4	$2{,}1 \times 10^5$	$2{,}1 \times 10^6$	1×10^5	2.4×10^6	4.7×10^8
MPA, 20 h	3.2×10^3	3.2×10^3	2.1×10^3	<10	<10	<10
MPA, 0 h	<10	3.7×10^3	1.1×10^4	<10	<10	<10

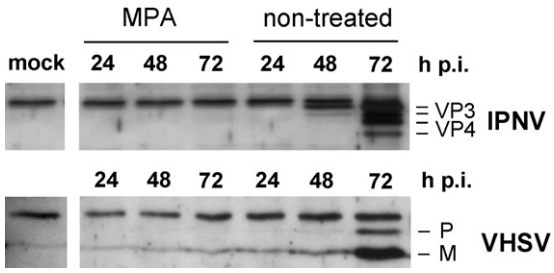

Fig. 2. Inhibition of IPNV and VHSV protein synthesis by MPA. IPNV-infected CHSE cells and VHSV-infected EPC cells were treated with 10 μg/ml MPA (added at 0 h p.i.). Cell monolayers were harvested at the indicated times and analyzed by SDS-PAGE followed by Western blotting. Samples were probed with rabbit anti-IPNV (upper panel) and anti-VHSV (lower panel) polyclonal antibodies. Positions of the viral proteins are indicated.

found that 10 μg/ml MPA had an inhibitory effect on VHSV growth, causing the production of infectious virus at 1–3 days after infection to drop 10 to 1000-fold. Remarkably, a complete inhibition of IPNV replication was achieved: either pre-treatment of the cells with MPA or time 0 addition of the compound resulted in undetectable levels of infectious virus in cell culture supernatants.

3.3. Effect of MPA on viral protein synthesis

The inhibition of IPNV and VHSV replication by MPA was further confirmed by analysis of viral proteins in the infected cells. Thus, we infected CHSE cells with IPNV and EPC cells with VHSV to test if the accumulation of viral proteins was inhibited by addition of MPA. Western blot analysis revealed the presence of viral proteins in the infected cells, beginning at 48 h p.i. and very intense at 72 h p.i. (Fig. 2). In contrast, exposure of the infected cells to MPA resulted in almost complete absence of viral proteins. Similar results were obtained when the presence of viral proteins in cell culture supernatants was analyzed (not shown), in direct correlation with the reduction of virus titers found in MPA-treated cultures.

3.4. Inhibition of viral RNA synthesis by MPA

To investigate whether the absence of viral proteins in MPA-treated cells was a consequence of a reduction of viral RNA templates, the influence of MPA on IPNV and VHSV RNA synthesis was determined. For better comparison, both viruses were tested on the same cell line (CHSE), to exclude the possibility of different activity of MPA on EPC and CHSE cells. Quantitative real-time RT-PCR (qRT-PCR) was performed on total RNA extracted from virus-infected CHSE cells at 24 h, 48 h and 72 h after infection (Fig. 3). qRT-PCR data demonstrated a substantial decrease in VHSV RNA expression in cells exposed to 10 μg/ml MPA (Fig. 3, left): there was approximately a 10-fold reduction at 24 h p.i. and over 1000-fold decrease in viral RNA levels at 72 h p.i. The effect of MPA on IPNV RNA accumulation (Fig. 3, right) was even more severe: a 3-log reduction at 24 h p.i. and over 5 logs reduction at 72 h p.i. Overall, data from the qRT-PCR analysis indicated that MPA was an effective inhibitor of IPNV and VHSV RNA synthesis. This was consistent with the previous results on VHSV and IPNV virus yield and protein synthesis.

3.5. Comparative effect of MPA and ribavirin on IPNV and VHSV RNA synthesis

We had recently reported that ribavirin was effective in inhibiting VHSV infection (Marroquí et al., 2007). To compare MPA to ribavirin the antiviral effect of both compounds against IPNV and VHSV was assessed at 0.1 μg/ml, 1 μg/ml and 10 μg/ml concentrations. Treatment of CHSE cells with MPA and ribavirin at time 0 of infection resulted in a dose-dependent inhibition of viral RNA production as measured by qRT-PCR (Fig. 4). MPA was found to be more potent than ribavirin at the lowest concentration tested: at 0.1 μg/ml MPA achieved over 70% inhibition of the synthesis of IPNV and VHSV RNA, whereas 0.1 μg/ml ribavirin only reduced viral RNA production by 30–40% (Fig. 4, insert). At 1 μg/ml and

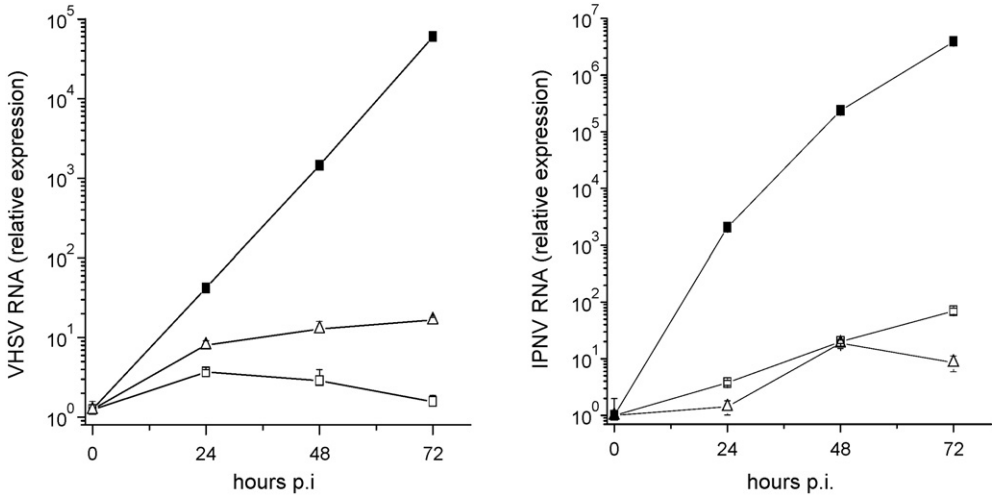

Fig. 3. Antiviral effect of MPA against IPNV and VHSV RNA synthesis as assessed by real-time RT-PCR. CHSE cell monolayers were infected with VHSV or IPNV and incubated with 10 μg/ml MPA. At 24 h, 48 h and 72 h p.i. total RNA was extracted to detect VHSV (left) or IPNV (right) RNA. (■) Non-treated controls; (□) MPA added at 0 h; (△) MPA added 16 h before infection and maintained during infection. The relative amount of viral RNA was calculated by comparison to RNA expression in virus-infected cell monolayers at 1 h p.i. Error bars represent standard deviations from three PCR reactions.

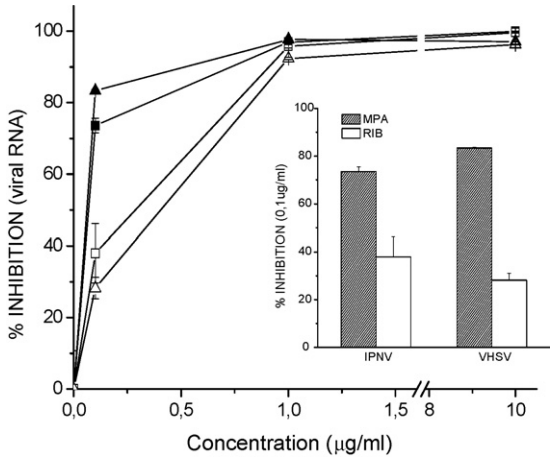

Fig. 4. Comparison of the antiviral potency of MPA and ribavirin (RIB). CHSE cells monolayers infected with IPNV (■, □) or VHSV (▲, △), and incubated with 0.1 μg/ml, 1 μg/ml or 10 μg/ml MPA (■,▲) or RIB (□, △) for 24 h. Total RNA was extracted and subjected to qRT-PCR. The % inhibition was calculated by the comparison to mock (medium) treatment. Mean values and standard deviations of three PCR reactions are represented. Inserted graph: detailed bar plot comparing the percent inhibition of MPA (black) and RIB (white) on viral RNA synthesis at 0.1 μg/ml drug concentration.

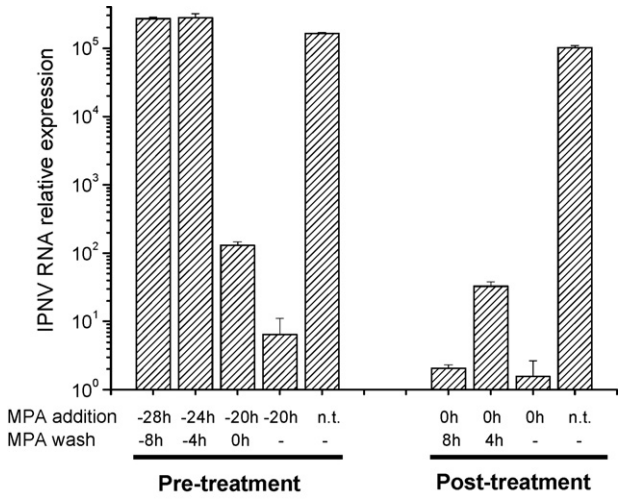

Fig. 6. Reversibility of the inhibitory effect of MPA on IPNV RNA synthesis. Total RNA relative to IPNV A genomic segment was quantitated by real-time RT-PCR at 24 h post-infection. MPA (10 μg/ml) was added and washed out at different times before and after infection (indicated in the figure). Two independent experiments are presented. Left: addition and removal of MPA before IPNV infection (pre-treatment). Right: addition of MPA at the onset of infection (0 h) and removal at a later time of infection (post-treatment).

10 μg/ml of both compounds viral RNA synthesis in IPNV and VHSV-infected cells was reduced by > 90%, in agreement with previous results.

3.6. Effect of time of application of MPA on virus RNA expression

To determine at which point during the virus replication cycle MPA exerts its antiviral effect, the drug was added at various time points pre- and post-infection and viral RNA was quantitated by qRT-PCR at 24 h and 48 h post-infection and compared with non-treated samples (Fig. 5). Reduction of IPNV and VHSV RNA synthesis was maximal when MPA was added at the time of infection (up to a 4-log reduction of IPNV RNA and a 2-log reduction of VHSV RNA at 48 h p.i.). When the compound was added at later times (4 h p.i. or 8 h p.i.), there was still a 10–1000-fold reduction of viral RNA expression in the infected cells. Consistently with previous results, MPA exhibited a higher antiviral activity against IPNV.

3.7. Reversibility of the inhibition of IPNV RNA synthesis by MPA

Since MPA appeared to have a greater effect on IPNV than on VHSV replication we chose IPNV to evaluate the reversibility of the MPA-dependent inhibition of viral RNA synthesis, so partial reversion situations could be more easily assessed (Fig. 6). It was observed that pre-treatment with MPA, addition of MPA at 0 h p.i. or continuous presence of MPA from −20 h to 24 h p.i. greatly reduced IPNV RNA levels. In contrast, we found that removing the compound 8 h or 4 h before virus infection restored IPNV RNA synthesis to non-treated control levels. Thus, indicating that the MPA block to IPNV RNA synthesis is reversible, even after a 20-h long incubation period. In contrast, when MPA was added at 0 h p.i. and removed 4 μg/ml or 8 h later, it still retained the inhibitory effect on IPNV RNA accumulation in the infected cells (Fig. 6).

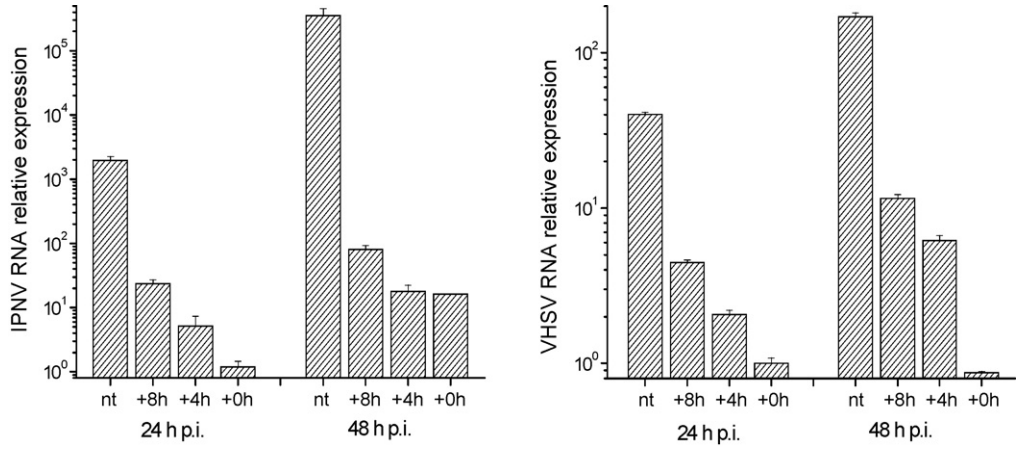

Fig. 5. Time course of the effect of MPA treatment on IPNV and VHSV infection. MPA (10 μg/ml) was added to CHSE cells at 0 h, 4 h or 8 h post-infection with IPNV or VHSV. Virus-infected cells were harvested at 24 h and at 48 h p.i., and virus replication was measured using the qRT-PCR assay. Arbitrary units are relative to viral RNA at 24 h p.i. in MPA-treated cells from time 0 of infection (value = 1). Error bars represent standard deviations from three PCR reactions. nt: not-treated.

4. Discussion

The aim of this study was to evaluate the antiviral activity of MPA, a nonnucleoside inhibitor of the enzyme inosine monophosphate dehydrogenase (IMPDH), against two important rainbow trout viral pathogens with worldwide distribution: IPNV (Birnaviridae) and VHSV (Rhabdoviridae). The potential of MPA as an antiviral agent has been shown for a number of RNA viruses, including hantaviruses, flaviviruses and reoviruses (Chung et al., 2007; Diamond et al., 2002; Robertson et al., 2004; Stuyver et al., 2002; Sun et al., 2007; Takhampunya et al., 2006). By using a cell viability assay we demonstrated that MPA prevented the VHSV-induced cytopathic effect on infected cells in a dose-dependent fashion. MPA-induced toxicity was not observed within the effective concentration range of MPA, in three fish cell lines tested. Release of infectious virus to the cell culture medium was greatly reduced by MPA, exhibiting a more potent effect on IPNV than on VHSV replication. Immunoblot analysis revealed that viral protein synthesis was strongly inhibited by MPA. Furthermore, we assessed the effect of MPA on the synthesis of IPNV and VHSV RNA by relative quantitation of viral RNA in real-time RT-PCR assays: qRT-PCR analysis showed that MPA prevents the accumulation of viral RNA in the infected cells. Time of addition experiments indicated that the antiviral effect of MPA occurs at a post-entry step of virus replication, since MPA was effective when added at 8 h after infection. Likewise, in the reovirus/L929 cell system, the production of infectious progeny was reduced by addition of MPA 12 h after infection (Hermann and Coombs, 2004).

Since ribavirin, another IMPDH inhibitor, has been shown to be effective against VHSV and IPNV (Marroquí et al., 2007; Migus and Dobos, 1980), it was interesting to compare the effects of the two drugs. Both IPNV and VHSV replication is halted in the presence of either ribavirin or MPA, but real-time RT-PCR analysis showed a more potent effect of MPA on viral RNA synthesis at the lower concentration tested (0.1 μg/ml). MPA has been previously reported to be a more potent inhibitor than ribavirin on the replication of some RNA viruses (Leyssen et al., 2005, 2006; Sun et al., 2007). Another compound, VX-497, also an uncompetitive inhibitor of IMPDH, was also found to be a more potent antiviral compound than ribavirin against all RNA viruses tested, with the exception of influenza A virus (Markland et al., 2000). The reason for the greater antiviral potency of MPA could be due to different modes of MPA and ribavirin to inhibit the IMPDH enzyme. On this respect, extracellular guanosine treatment has been shown to reverse the antiviral activity of ribavirin and MPA against viruses (Leyssen et al., 2005; Markland et al., 2000; Marroquí et al., 2007; Smee et al., 2002; Sun et al., 2007). However, we have not achieved reversal of MPA inhibition by exogenous GTP (not shown), suggesting that an inhibitory mode other than inhibition of IMPDH may be playing a role. The distinct efficacy of MPA and ribavirin against IPNV and VHSV could also be explained by a direct effect of MPA on viral RNA polymerase. In fact, the results of the wash-out experiments suggest a different mechanism of action of MPA before and after infection: inhibition by MPA pre-treatment is reversible after compound removal; in contrast, removal of MPA after virus infection did not reverse the inhibitory effect of MPA. It remains unclear what other mode of action in addition to IMPDH inhibition contributes to the antiviral activity of MPA. A direct action on the viral RNA polymerase is one possibility that has been suggested for the mechanism of action of ribavirin against a number of viruses (Rankin, 1989; Toltzis et al., 1988). Treatment of the cells with MPA prior to infection is also effective in preventing virus replication, although the inhibition is lost when the compound is washed out 4 h before adding the virus. Taken together, our results suggest that pre-infection treatment of MPA induces a reversible antiviral state on the cells, while post-infection treatment with MPA appears to have an irreversible effect on virus replication. This may be consistent with a dual target for MPA: a cellular target (IMPDH) during pretreatment, and a viral target (RNA polymerase) when MPA is present from the onset of infection.

In summary, it can be concluded that MPA has anti-VHSV activity as well as a very potent anti-IPNV activity: MPA can completely abrogate the production of infectious virus in vitro, likely as a consequence of the inhibition of IPNV RNA synthesis, which dropped 10^5-fold at 72 h p.i. in the presence of 10 μg/ml (≈3 μM) MPA. This is in accordance with prior studies showing the inhibitory effect of MPA on the replication of double-stranded RNA viruses (Robertson et al., 2004). MPA is also a more potent inhibitor of the VHSV rhabdovirus replication than ribavirin. Activity against two unrelated viruses supports the notion that MPA exhibits broad range antiviral properties. In addition, since MPA does not require cell metabolism for phosphorylation or other chemical transformations (as ribavirin does) it should be less sensitive than ribavirin to cell/species variation as well.

It is intriguing to consider the therapeutic use of MPA for fish viral diseases. Since MPA has shown antiviral activity when added to cultures before infection, it may be effective as a prophylactic treatment in an in vivo situation. In a preliminary trial in vivo using a low MPA dosage (50 μg per 2.5 g fish), we did not find any significant protective effect on VHSV immersion challenged rainbow trout. However, the results were not conclusive since a positive control of antiviral activity was lacking in that experiment. Further studies with higher concentrations of MPA will be required to assess the activity of MPA in vivo. Nevertheless, the data presented in this study strongly indicate that MPA can be useful as a broad-spectrum agent to eradicate or attenuate viral disease in rainbow trout.

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