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IN VITRO EFFECT OF METHISOPRINOL ON SALMONID RHABDOVIRUSES REPLICATION

ANDRZEJ K. SIWICKI[1*], FRANÇOISE POZET[2], MARC MORAND[2], BARBARA KAZUŃ[3] AND SYLWIA TRAPKOWSKA[1]

[1]Department of Microbiology and Clinical Immunology,
Faculty of Veterinary Medicine, University of Olsztyn, 10-957 Olsztyn, Poland
[2]Laboratoire Departemental d'Analyses,
Conseil General du Jura, Lons le Saunier Cedex, France
[3]Department of Fish Pathology and Immunology,
Inland Fisheries Institute, Olsztyn, Poland

The *in vitro* influence of methisoprinol on the replication of viral haemorrhagic septicaemia (VHS) and infectious haematopoetic necrosis (IHN) viruses by measuring their RNA synthesis was examined. EPC cell cultures containing various concentrations of methisoprinol (0, 100, 200, 300, 400 and 500 μg/ml of medium) were inoculated with VHS or IHN virus suspension containing 10^7 $TCID_{50}$/ml. The inhibition of [^{3}H]-uridine incorporation into VHSV and IHNV RNA propagated in cell cultures with methisoprinol was demonstrated. The highest percentage of viral RNA inhibition was observed when a dose of 500 μg/ml was used. The results indicated that methisoprinol inhibits the replication of salmonid rhabdoviruses and possibly may be effective in prevention of viral infection in aquaculture.

Key words*:* fish, VHSV, IHNV, methisoprinol, replication.

Rhabdoviridae, identified as bullet-shaped, enveloped viruses with a single-stranded RNA genome of negative polarity, are frequently isolated from wild and cultured fish. Several rhabdoviruses have been isolated from many fish species, but only some of them have been extensively investigated. Viral haemorrhagic septicemia virus (VHSV) and infectious haematopoietic necrosis virus (IHNV) have been most extensively studied due to their significant economic impact on the salmonid culture (1, 2). Aquaculture, just as other intensive farming methods, is constantly at risk of viral diseases. While good husbandry and therapeutics can substantially decrease the losses due to infectious agents, prevention is still the best form of control. Significant progress has been made towards controlling the major bacterial fish diseases using vaccines, but this approach has not been totally successful in preventing VHS and IHN diseases in fish culture (11). However, for an ideal prevention approach, specific drugs should be developed to inhibit selectively virus replication or stimulate the antiviral protection. Several adenine-nucleoside analogue antiviral drugs inhibit the replication of viruses (12).

Methisoprinol is a synthetic compound formed from the p-acetamidobenzoate salt of N-N dimethylamino-2-propanol and inosine in a 3:1 molar ratio. It exerts antiviral and antitumour activities *in vitro* and *in vivo*, which are secondary to an immunomodulating influence on both non-specific and specific defence mechanisms (5, 8, 9). Methisoprinol presents low toxicity and has been shown to act *in vitro* and *in vivo* by inhibiting the replication of various single-stranded and double-stranded RNA-containing viruses (4, 13, 14).

The aim of the present study was to determine quantitatively the *in vitro* influence of different concentrations of methisoprinol on the replication of two salmonid rhabdoviruses VHS and IHN by measuring viral RNA synthesis.

Material and Methods

Epithelioma papulosum cyprini (EPC) cell line (7) in minimum essential medium (BHK21 Medium, Glasgow MEM, Life Technologies) containing 10% tryptose phosphate broth (DIFCO), 10% foetal calf serum (FCS, Life Technologie) and antibiotics (penicillin 100 IU/ml, streptomycin 0.1 mg/ml and kanamycin 0.1 mg/ml) were used. Viral haemorrhagic septicaemia virus (VHSV) and infectious haematopoietic necrosis virus (IHNV) isolated from diseased fish from a French trout farm in Laboratoire Veterinaire Departemental (LDA39) Lons le Saunier were propagated in EPC cells as described by Dorson *et al.* (6). After inoculation, the cells were incubated at 15^{o}C in medium buffered at pH 7.4 and supplemented with 2% FCS and antibiotics (penicillin 100 IU/ml, streptomycin 0.1 mg/ml and kanamycin 0.1 mg/ml). The viruses were harvested when the cytopathic effect was complete. Each virus was quantified by titration against EPC cells in 96-well microtitre plates in tissue culture infective doses of 50%/ml ($TCID_{50}$).

Methisoprinol (Polfa Grodzisk, Poland) was used in the study. The stock solution was prepared in medium (Glasgow MEM) at the concentration of 1 000 µg/ml and stored at 4^{0}C for no more than 3 days.

The monolayers of EPC cell cultures in tissue culture plates (Multiwell, 24 wells, Becton Dickinson, USA) were refed with 0.75 ml of maintenance medium, free of FCS, containing 0, 100, 200, 300, 400 and 500 µg/ml of methisoprinol. After that the cultures were inoculated with 100 µl of VHS or IHN virus suspension containing 10^{7} $TCID_{50}$/ml. The negative control (uninoculated either with VHSV or IHNV) was used in each concentration of methisoprinol. Four culture plates for each virus and sixteen wells for each concentration of methisoprinol were used. After 48 h (2 plates with each virus) and 72 h (2 plates with each virus) following inoculation, the EPC cell cultures were submitted to one hour starvation period by replacing medium with pre-heated PBS, at pH 7.4, followed by two-hour incubation with 10 µCi/ml of [^{3}H]-uridine (27 Ci/mmol; Amersham Int., UK) in maintenance medium, according to the method presented by Lanhares *et al.* (13). Cultures were submitted to three freezing and thawing cycles. Culture homogenates of each isolate were incubated with phenol/chloroform to extract RNA, followed by slab polyacrylamide gel electrophoresis for 2 h at 25 mA, using a 7% separation polyacrylamide gel. The silver nitrate staining was used for the detection of RNA bands. The gel strips were dissolved in a solution containing 30% hydrogen peroxide and 0.9 N ammonium hydroxide,

according to the method of Bonner and Laskey (3). After that, the scintillation fluid for aqueous samples (Sigma Chemicals, USA) was added and the counts per minute (cpm) were evaluated in a scintillation counter (Beckman LS).

Statistical analyses were performed using Student's *t*-test and the data were reported as means ± SD as percentage inhibited by methisoprinol of VHS or IHN virus RNA synthesis compared to inoculated methisoprinol-free EPC cells.

Results

In the preliminary study, the influence of different concentrations of methisoprinol on the *in vitro* replication of salmonid rhabdoviruses VHSV and IHNV was examined. These effects were observed 48 and 72 h after inoculation. The replicative cycle of the viruses in EPC cell cultures was rapid. In the control group inoculated only by VHS virus, the incorporation of [^{3}H]-uridine counted by scintillation was 175 000 ± 2 250 cpm at 48 h and 440 800 ± 3 550 cpm at 72 h. Similar results were observed in the control group with IHN virus, where 164 200 ± 3 200 cpm and 410 500 ± 4 250 cpm were recorded after 48 and 72 h, respectively. However, a cytopathic effect was observed in virus-infected cultures without methisoprinol (control) at 24 h after inoculation. Cell cultures exposed only to methisoprinol did not show any morphological change until the end of experimental time. VHSV and IHNV-inoculated cultures exposed to methisoprinol at the concentration of 100 and 200 µg/ml showed a small (maximum 20% at the dose 100 µg/ml and 10% at the dose 200 µg/ml) cytopathic effect. However, no cytopathic effect was observed in cultures treated with methisoprinol at doses 300, 400 and 500 µg/ml. The *in vitro* influence of different concentrations of methisoprinol on the percentage of inhibition of VHSV RNA synthesis compared to inoculated cells not exposed to methisoprinol and examined at 48 h and 72 h after inoculation are presented in Fig. 1. The percentage of inhibition of VHS viral RNA labelled under influence of methisoprinol ranged from 15% to 70% depending on drug concentration and time when the cultures were harvested. The highest percentage of inhibition was observed 72 h after inoculation at the dose 500 µg/ml. The *in vitro* influence of different concentrations of methisoprinol on the percentage of inhibition of IHNV RNA synthesis compared to inoculated cells not exposed to methisoprinol and examined 48 and 72 h after inoculation are presented in Fig. 2. The percentage of inhibition of IHN viral RNA labelled under influence of methisoprinol ranged from 12% to 75% depending on concentration of tested product and time when the cultures were harvested. The highest percentage of inhibition also 72 h after inoculation was observed at the dose 500 µg/ml.

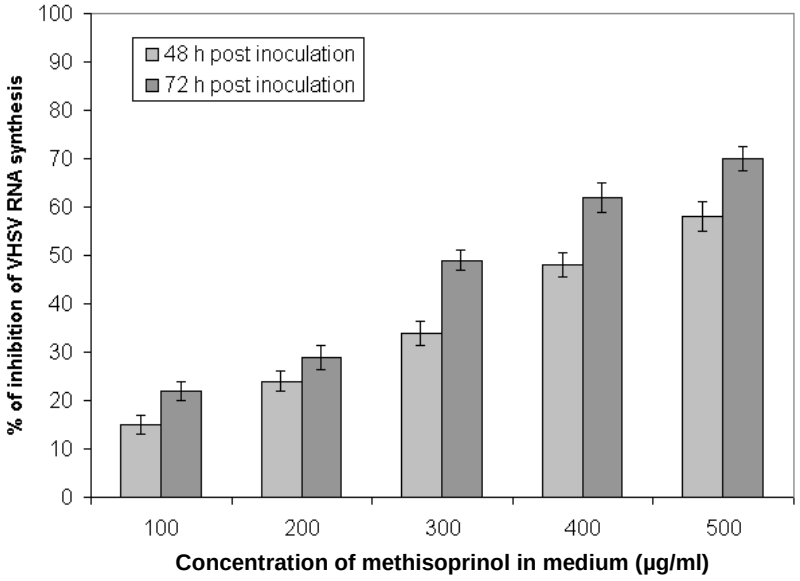

Fig. 1. The *in vitro* influence of different concentrations of methisoprinol on the percentage of inhibition of VHSV RNA synthesis compared to inoculated cells not exposed to methisoprinol at 48 h and 72 h post inoculation of EPC cells (mean ± SD, n=7).

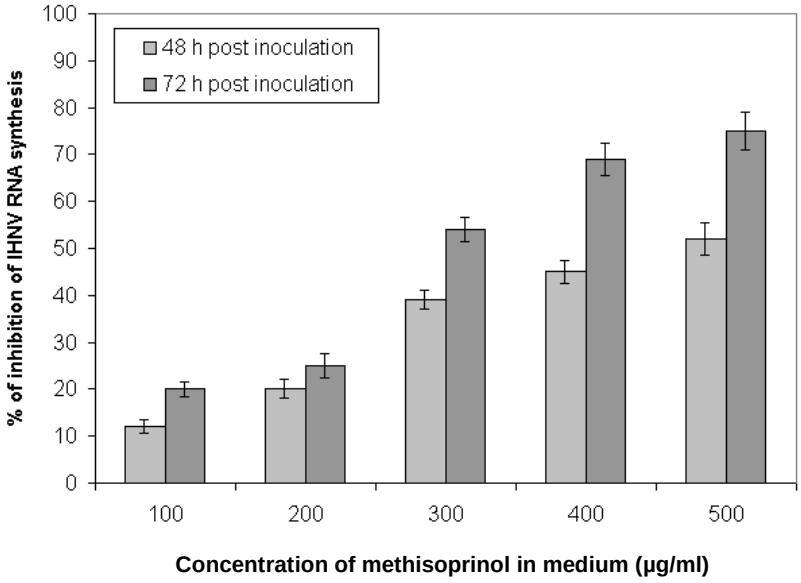

Fig. 2. The *in vitro* influence of different concentrations of methisoprinol on the percentage of inhibition of IHNV RNA synthesis compared to inoculated cells not exposed to methisoprinol at 48 h and 72 h post inoculation of EPC cells (mean ± SD, n=7).

Discussion

The results of this experimental study show the inhibition of incorporation (cpm) of [^{3}H]-uridine into VHS and IHN viral RNA in cell cultures exposed to methisoprinol at various concentrations. The highest percentage of inhibition of viral RNA was observed 72 h after exposition to the dose of 500 μg/ml of medium. These results are comparable to the study with other viruses. In tissue cultures, methisoprinol has been reported to inhibit the replication of several RNA and DNA viruses, including *Herpes simplex*, cytomegalovirus, adenovirus, rotavirus, rabies virus, encephalomyocarditis and Eastern equine encephalitis viruses (4, 9, 10, 13, 15, 17, 18).

Ronsen and Gordon (16) demonstrated that isoprinosine (methisoprinol) stimulates the synthesis of cellular protein and, if absorbed by a cell, is rapidly metabolized. Linhares *et al.,* (13) suggested that prior to metabolization it is dissociated into its constituents, which are three molecules of N,N-dimethylaminoisopropanol-p-acetamidobenzoate and one molecule of inosine. Inosine in the form of inosinate is an active inhibitor of the synthesis of phosphoribosylpyrophosphate, an intermediate in the biosynthesis of purine nucleotides such as adenylate and guanylate. Since inosinate inhibits the conversion of ribose phosphate to phosphoribosylpyrophosphate, isoprinosine may act by blocking the synthesis of viral RNA and this process is faster than the cellular RNA synthesis.

The present study demonstrates that methisoprinol at various concentrations inhibits the replication of salmonid rhabdoviruses. The results suggested that additional *in vitro* and *in vivo* experimental study must be carried out for the possibility of application of methisoprinol in prevention or therapy of viral infection in salmonid fish culture.

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