Anti-Birnavirus Activity of Methisoprinol – i*n vitro* Study with Infectious Pancreatic Necrosis Virus (IPNV)

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Received November 23, 2001
Accepted November 18, 2002

Abstract

Siwicki A. K., M. Morand, F. Pozet, B. Kazun: *Anti-Birnavirus Activity of Methisoprinol – in vitro Study with Infectious Pancreatic Necrosis Virus (IPNV)*. Acta Vet. Brno 2002, 71: 543-547.

This study was conducted to evaluate the influence of methisoprinol, synthetic anti-viral product, on the IPNV replication *in vitro* by measuring viral RNA synthesis. The monolayers of RTG-2 cells in tissue culture plates (Multiwell, 24 wells, Becton Dickinson, USA) was cultivated with different concentrations of methisoprinol (Polfa, Poland) 0, 100, 200, 400, 500 and 1 000 µg/ml of medium and were followed by infection with 100 µl of IPN virus suspension containing 10^7 $TCID_{50}$/ml, in triplicate. At 24, 48 and 72 h after infection, the IPN virus-infected and methisoprinol-applicated RTG-2 cell cultures were submitted to one hour starvation and after two hours incubation with 10 µCi/ml of [^{3}H]-uridine. Culture homogenates of each isolate were incubated with phenol/chloroform to extract RNA and followed by slab polyacrylamide gel electrophoresis for 2 h. The gel strips were dissolved and the counts per minute (cpm) evaluated in a scintillation counter. The replicative cycle of IPN virus in RTG-2 cell culture was rapid. In control group (only infected by IPNV), the incorporation of [^{3}H]-uridine was 45 000 ± 1 500 cpm at 24 h, 186 000 ± 2 450 cpm at 48 h and 554 500 ± 4 550 cpm at 72 h. The percent of inhibition of IPN viral RNA labelling under methisoprinol application ranged from 5% at 24 h to 85% at 72 h depending on concentration of tested product and time when cultures were harvested. The highest percent of inhibition at 72 h after infection was observed at the dose 1000 µg/ml. The results of these experimental studies show the inhibition of incorporation (cpm) of [^{3}H]-uridine into IPN viral RNA in cell cultures exposed with methisoprinol at various concentrations.

Fish, IPNV, methisoprinol, replication

The family *Birnaviridae* presents the smallest RNA viruses and consists of three genera: Aquabirnavirus, Avibirnavirus and Entomobirnavirus. The aquatic birnaviruses are the largest group of viruses including several strains from different fish species and invertebrates. The *Birnaviridae* are the most extensively studied fish viruses, because they are ubiquitous in aquatic organisms, since they have been isolated all over the world from both freshwater and marine fish of different species, and are responsible for severe losses in aquaculture.

Infectious pancreatic necrosis virus (IPNV), the genus Aquabirnavirus, is the prototype of the birnavirus infecting fish, molluscs and crustaceans. The single-shelled, naked, icosahedral viral particles of birnaviruses containing 2 segments (A and B) have double-stranded RNA. Their dsRNA genome is easily purified and is resistant to common RNase. Very little is known about the replication strategy of birnaviruses. A viral protein 1 (VP1) is found in the viral particle both as a free form and bound to the RNA genome-linked protein (VPg) and is thought to be the viral

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RNA dependent RNA polymerase (Duncan and Dobos 1986; Duncan et al. 1987; Duncan et al. 1991).

IPNV has been detected for years in several places of the world with high mortality rates of rainbow trout fry at the temperatures of 8-12 ºC. This virus is spread by shipment of contaminated fish eggs and it is probably present in all trout-farming countries. Actually, one of the very important questions is to develop some prevention methods against IPNV infection (Okamoto et al. 1993). Protection of fish against viral diseases by vaccine has been developed without relevant success. Therefore, research is concentrated on how to improve the anti-viral protection and develop some anti-viral products to selectively inhibit virus replication (Bradshaw and Sumner 1977; Ginsberg and Glasky 1977; Berkman et al. 1979; Ballet et al. 1982; Zagni and Cannarozzo 1982; Linhares et al. 1996). 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. The *in vitro* and *in vivo* studies show antiviral and antitumour activities of methisoprinol secondary to an immunomodulating influence on cell-mediated immunity and anti-viral immune responses (Delogu et al. 1982; Fudenberg and Whitten 1984). Methisoprinol has been shown to act *in vitro* and *in vivo* by inhibiting the replication of various single-stranded and double-stranded RNA-containing viruses (Chang and Weinstein 1973; Linhares et al. 1989; Linhares et al. 1996). In our preliminary *in vitro* study, we demonstrated that methisoprinol at different concentrations inhibited the replication of the two salmonid rhabdoviruses VHSV and IHNV (Siwicki et al. 2002).

In this study, we continue to examine the *in vitro* influence of methisoprinol on the infectious pancreatic necrosis virus (IPNV) replication by measuring viral RNA synthesis.

Materials and Methods

The cell line, rainbow trout gonad (RTG-2) was used for the isolation, propagation and identification of IPN virus. RTG-2 cell line was propagated in Glasgow medium (BHK21 Medium, Life Technologies), buffered to pH 7.45 with 0.16 M Tris-HCL (Sigma) and supplemented with 10% foetal calf serum (FCS, Life Technologie) and antibiotics (penicillin 100 IU/ml, streptomycin 0.1 mg/ml and kanamycin 0.1 mg/ml). IPN virus isolated from diseased fish from a French trout farm in Laboratoire Veterinaire Departemental (LDA39) Lons le Saunier (Siwicki et al. 1998) were propagated in RTG-2 cell line. After infection, the RTG-2 cells were incubated at 15 ºC in medium and supplemented with 2% FCS and antibiotics (penicillin 100 IU/ml, streptomycin 0.1 mg/ml and kanamycin 0.1 mg/ml). The virus was harvested when the cytopathic effect was complete and quantified by titration against RTG-2 cells in 96-well microtitre plates in tissue culture infective doses of 50%/ml ($TCID_{50}$).

Methisoprinol (series 33-03-00; Polfa Grodzisk, Poland) was used in this study. The stock solution was prepared in medium (Glasgow MEM) at the concentration 2 000 µg/ml and stored at 4 ^{0}C for not more than 3 days.

The monolayers of RTG-2 cells in tissue culture plates (Multiwell, 24 wells, Becton Dickinson, USA) were cultivated with 0.75 ml of maintenance medium, free of FCS, containing 0, 100, 200, 400, 500 and 1 000 µg/ml methisoprinol. The cell cultures containing different concentration of methisoprinol were followed by infection with 100 µl of IPN virus suspension containing 10^7 $TCID_{50}$/ml, in triplicate. The negative control (without infection by IPNV) was used in each concentration of methisoprinol. At 24, 48 and 72 h after infection, the IPN virus-infected and methisoprinol-applicated RTG-2 cell cultures were submitted to one hour starvation period by replacing medium with pre-heated PBS, at pH 7.4, followed by a 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 Linhares et al. (1996). Cultures were submitted to three freezing and thawing cycles. Culture homogenate 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 presented by Bonner and Laskey (1974). After, the scintillation fluid for aqueous samples (Sigma Chemicals, USA) was added and the counts per minute (cpm) evaluated in a scintillation counter (Beckman LS).

Statistical analyses were performed using the Student's *t*-test. Data are reported as means ± SD for 4 experiments in triplicate as percent inhibition of virus RNA synthesis by methisoprinol compared to infected RTG-2 cells not exposed with methisoprinol.

Results and Discussion

In the present study, we continued the *in vitro* experiments to examine the influence of different concentrations of methisoprinol on the replication of infectious pancreatic necrosis virus, aquatic birnavirus, responsible for high mortality rates of trout fry and fingerlings in all major trout-farming countries. We examined the anti-viral influence of methisoprinol by determination of the inhibition the viral RNA synthesis at the 24, 48 and 72 h after infection. Monitoring the influence of different concentrations of methisoprinol on the synthesis of IPN virus RNA in RTG-2 cells culture by radioisotope labelling permitted the quantitative determination of the inhibition and provided information about the early stage of infection since the method permitted the detection of minute quantities of viral RNA. The replicative cycle of IPN virus in RTG-2 cell culture was rapid. In control group, only infected by IPN virus, the counted level after incorporation of [^{3}H]-uridine was 45 000 ± 1500 cpm at 24 h, 186 000 ± 2450 cpm at 48 h and 554 500 ± 4550 cpm at 72 h. However, a cytopathic effect was observed in virus-infected cultures without methisoprinol (control) at 24 h after infection. Cell cultures exposed only to the methisoprinol did not show any morphological change to the end of experimental time. IPNV-infected culture exposed with methisoprinol at the concentration of 100 and 200 µg/ml showed a small (maximum 25% at dose 100 µg/ml and about 10% at dose 200 µg/ml) cytopathic effect and no cytopathic effect was observed in cultures treated with 400, 500 and 1 000 µg/ml of the methisoprinol. Fig. 1 presents the *in vitro* influence of different concentrations of methisoprinol on the percent of inhibition of IPNV RNA synthesis compared to infected cells not exposed on methisoprinol at 24, 48 and 72 h after infection of RTG-2 cells line. The percent of inhibition of IPN viral RNA labelling under methisoprinol application ranged from 5% at 24 h to 85% at 72 h depending on concentration of tested product and time when cultures were harvested. The highest percent of inhibition at 72 h after infection was observed at the dose of 1000 µg/ml.

The results of this experimental study shows the inhibition of incorporation (cpm) of [^{3}H]-uridine into IPN viral RNA in cell cultures exposed with methisoprinol at various concentrations. A low level of incorporation occurred at 24 h after infection, with a non-significant rate of viral replication in methisoprinol-exposed cultures, which was comparable to that of unexposed cultures. The percent of inhibition of viral RNA at 24 h after infection was low, probably because of the low incorporation of [^{3}H]-uridine as a result of the non-significant quantity of viral RNA synthesized. These results are in agreement with the study with other fish viruses. The results of experimental study show the inhibition of incorporation of [^{3}H]-uridine into VHS and IHN viral RNA in cell cultures exposed to methisoprinol at various concentrations (Siwicki et al. 2002). The highest percentage of inhibition of viral RNA was observed 72 h after exposition to the dose of 500 g/ml of medium. In tissue cultures methisoprinol has been reported to inhibit the replication of several RNA and DNA viruses in animals (Chang and Weinstein 1973; Ginsberg and Glasky 1977; Hernandez-Jauregui et al. 1980; Pompidou et al. 1985; Tsang and Fudenberg 1982; Zagni and Cannarozzo 1982; Linhares et al. 1996). Linhares et al. (1996) observed also a very low percentage of

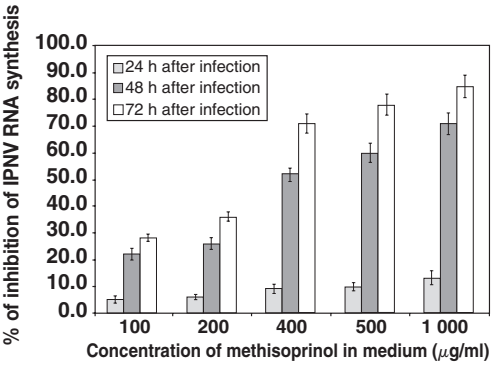

Fig. 1. The *in vitro* effect of different concentrations of methisoprinol on the percent of inhibition of IPNV RNA synthesis compared to infected cells not exposed to methisoprinol at 24 h, 48 h and 72 h after infection of RTG-2 cells (mean SD, n=12)

inhibition of rotavirus 24 h post infection and suggested that this observation is a result of the non-significant quantity of viral RNA synthesis.

In our *in vitro* study we observed the anti-viral activity of methisoprinol, which at different concentrations, inhibits the IPN virus replication. The results suggested that additional *in vivo* study must be carried out for the possibility of application of methisoprinol in prevention or therapy of IPNV infection in intensive fish culture.

Vliv působení methisoprinolu na birnaviry – *in vitro* studie viru infekční nekrózy pankreatu (IPNV)

Virus infekční nekrózy pankreatu (IPNV), obsahující RNA, je prototypovým virem skupiny birnavirů, zodpovědných za infekce ryb, měkkýšů a korýšů. IPNV byl izolován z různých druhů jak sladkovodních, tak i mořských ryb v řadě zemí z celého světa. Nejvyšší mortalita byla však zaznamenána u plůdků pstruha duhového. Snaha zabránit této infekci formou vakcinace nepřinesla žádný výrazný úspěch. Tato studie měla za cíl posoudit účinek methisoprinolu, syntetického antivirového preparátu, na replikaci IPNV v podmínkách *in vitro* měřením syntézy virové RNA. Monolayery buněčné linie RTG-2 narostlé na destičkách určených pro kultivaci tkáňových kultur (Multiwell, 24 jamek, Becton Dickinson, USA) byly kultivovány v přítomnosti různých koncentrací methisoprinolu (Polfa, Poland), 0, 100, 200, 400, 500 a 1.000 μg/ml kultivačního média. Poté následovala inokulace monolayerů suspenzí viru IPN v dávce 100 l, obsahující 10^7 $TCID_{50}$ /ml, ve trojím provedení (v tripletu). Za 24, 48 a 72 h po inokulaci byly kultury buněčné linie RTG-2, infikované virem IPN a ošetřené methisoprinolem, podrobeny po dobu 1 h hladovění a po 2 h inkubaci v přítomnosti 10 μCi/ml [^{3}H]-uridinu. Homogenizované kultury každého izolátu byly inkubovány ve směsi fenolu s chloroformem za účelem extrakce RNA, po níž následovala elektroforéza v polyakrylamidovém gelu po dobu 2 h. Proužky gelů byly nejprve rozpuštěny a poté vyhodnoceny scintilačním přístrojem (počty za minutu [cpm]). Replikační cyklus viru IPN na buněčné linii RTG-2 byl velmi rychlý. U kontrolní buněčné linie (infikované pouze virem IPN) byly zjištěny následující hodnoty inkorporace [^{3}H] / uridinu: 45 000 1 500 cpm za 24 h, 186 000 2 450 cpm za 48 h a 554 500 4 550 cpm za 72 h. Procento inhibice syntézy RNA viru IPN, značeného methisoprinolem, se pohybovalo v rozmezí 5% za 24 h až 85% za 72 h v závislosti na koncentraci testovaného produktu a čase, ve kterém byly buněčné kultury sklízeny. Nejvyšší procento inhibice bylo pozorováno po aplikaci dávky 1 000 μg/ml za 72 h po infekci. Z výsledků této experimentální studie je zřejmé, že vlivem různých koncentrací methisoprinolu, došlo k inhibici inkorporace (cpm) [^{3} H]-uridinu do RNA viru IPN kultivovaném na buněčných kulturách.

References

BALLET, JJ, MORIN, A, SCHMITT, C, AGRAPART, M 1982: Effect of isoprinosine on *in vitro* proliferative responses of human lymphocytes stimulated by antigen. Inter J Immunopharmacol **4**: 151-157

BERKMAN, N, LEGOIX, H, MOUBRI, M, DE SAXE, E 1979: Action favorable de l'isoprinosine au cours des affections oculaires virales et inflamatoires. Nouvelle Presse Medicale **8**: 3829-3830

BONNER, WM, LASKEY, RA 1974: A film detection method for tritium-labeller proteins and nucleic acids in polyacrylamide gels. Eur J Bioch **46**: 83-88

BRADSHAW, LJ, SUMNER, HL 1977: *In vitro* studies on cell-mediated immunity in patients treated with inosiplex for herpes virus infection. Ann New York Acad Sc **284**: 190-196

CHANG, TW, WEINSTEIN, C 1973: Antiviral activity of isoprinosine *in vitro* and *in vivo*. Am J Med Sci **265**: 143-146

DELOGU, G, LOZZI, A, CAMPANELLI, A, DE RITIS, G, PIETROPAOLI, P 1982: Cell-mediated immunity and immunomodulatory drugs in critically ill patients. Acta Anaes Ital **33**: 619-625

DUNCAN, R, DOBOS, P 1986: The nucleotide sequence of infectious pancreatic necrosis virus (IPNV) ds. RNA segment A reveals one large ORF encoding a precursor polyprotein. Nucleic Acids Res **14**: 5934-5938

DUNCAN, R, NAGY, E, KRELL, PJ, DOBOS, P, 1987: Synthesis of the infectious pancreatic necrosis virus polyprotein, detection of a virus-encoded protease, and fine structure mapping of genome segment A coding regions. J Virol **61**: 3655-3664

DUNCAN, R, MASON, CL, NAGY, E, LEONG, JA, DOBOS, P 1991: Sequence analysis of infectious pancreatic necrosis virus genome segment B and its encoded VP1 protein: a putative RNA-dependent RNA polymerase lacking the Gly-Asp motif. Virology **181**: 541-552

FUDENBERG, HH, WHITTEN, HD 1984: Immunostimulation: synthetic and biological modulators of immunity. Ann Rev Pharm Toxic **24**: 147-174

GINSBERG, T, GLASKY, AJ 1977: Inosiplex: an immunomodulation model for the treatment of viral disease. Ann New York Acad Sci **284**: 128-138

HERNANDEZ-JAUREGUI, P, GONZALEZ-VEGA, D, CRUZ-LAVIN, E, HERNANDEZ-BAUMGARTEN, E 1980: *In vitro* effect of isoprinosine on rabies virus. Am J Vet Res **41**: 1475-1478

KITAOKA, S, KONNO, T, DE CLERQ, E 1986: Comparative efficacy of broat-spectrum antiviral agents as inhibitors of rotavirus replication *in vitro*. Antivir Res **6**: 57-65

LINHARES, REC, REBELLO, MA, NOZAWA, CM 1996: Effect of isoprinosine on rotavirus replication *in vitro*. Bras J Med Biol Res **29**: 219-222

LINHARES, REC, WIGG, MD, LAGROTA, MHC, NOZAWA, CM 1989: The *in vitro* antiviral activity of isoprinosine on simian rotavirus (SA-11). Bras J Med Biol Res **22**: 1095-1103

OKAMOTO, N, TAYAMA, T, KAWANOBE, M, FUJIKI, N, YASUDA, Y, SANO, T 1993: Resistance of rainbow trout strain to infectious pancreatic necrosis. Aquaculture **117**: 71-76

POMPIDOU, A, DELSAUX, MC, TELVI, L, MACE, B, COUTANCE, F 1985: Isoprinosine and imuthol, two potentially active compounds in patients with AIDS-related complex symptoms. Cancer Res **45**: 4671-4673

RONSEN, B, GORDON, P 1976: Methisoprinol enhancements of nucleocytoplasmic transport of putative messenger RNA in rat liver. Bioch Pharmacol **25**: 707-715

SIWICKI, AK, MORAND, M, KLEIN, P, KICZKA, W 1998: Treatment of infectious pancreatic necrosis virus (IPNV) disease using dimerized lysozyme (KLP-602). J Appl Ichthyol **14**: 229-232

SIWICKI, AK, POZET, F, MORAND, M, KAZUN, B 2002: *In vitro* effect of methisoprinol on salmonid rhabdoviruses replication. Bull Vet Inst Pulawy **46**: 53-58

TSANG, KY, FUDENBERG, HH 1982: *In vitro* modulation of virus susceptibility by isoprinosine and NPT 15392. Clin Res **30**: 564A

ZAGNI, G, CANNAROZZO, C 1982: Clinical trial on the topical application of methisoprinol in some cutaneous viruses. Clin Europea **21**: 3-7