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Short communication

Inhibitors of infectious pancreatic necrosis virus (IPNV) replication

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Abstract

In attempts to detect inhibitors of infectious pancreatic necrosis virus (IPNV) replication, we have evaluated, by an IPNV plaque inhibition assay, a group of compounds that have broad spectrum antiviral activity for both single- and double-stranded RNA viruses. The inosine monophosphate dehydrogenase (IMP dehydrogenase) inhibitors 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) and 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR), and the orotidine monophosphate decarboxylase (OMP decarboxylase) inhibitor 4-hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamide (pyrazofurin), were found to inhibit IPNV replication. For EICAR and pyrazofurin the concentrations that inhibited the IPNV plaque formation by 50% (EC_{50}) were 0.01 μg/ml and 0.5 μg/ml, respectively. The cytotoxic concentrations required to reduce cell viability by 50% (CC_{50}) were 50 μg/ml and 100 μg/ml, respectively, and the concentrations that reduced [*methyl*-^{3}H] thymidine incorporation by 50% (IC_{50}) were 0.5–1 and 50 μg/ml. Thus, for both compounds the IPNV-inhibitory concentration was 50–100 times lower than the concentration that affected DNA synthesis in growing cells. EICAR and pyrazofurin seem to be good candidates for further evaluation in an in vivo model of IPNV infection.

Keywords: Salmonid virus; Infectious pancreatic necrosis virus (IPNV); Antiviral; In vitro evaluation

Infectious pancreatic necrosis virus (IPNV) is a member of the *Birnaviridae* family that causes one of the most serious diseases in trout and salmon farms around the world. It is also found in other fish species and shellfish. The losses among hatchery-reared salmonid fry caused by IPNV has stimulated efforts to control this disease.

With the purpose of establishing an efficient IPNV therapy, we evaluated a group of compounds that, based on structural and functional IPNV characteristics, could have antiviral activity. Therefore, some compounds known to have broad-spectrum antiviral activity for both single-

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Table 1
Effects of antiviral agents on IPNV replication in CHSE-214 cells

Antiviral agent	Virus-plaque inhibition assay		Cytotoxic assays	
	Range assay concentration (μg/ml)	EC^a_{50} (μg/ml)	CC^b_{50} (μg/ml)	IC^c_{50} (μg/ml)
Ribavirin	0.05–40	0.5	100	0.5
EICAR	0.01–20	0.01	50	0.5–1
Pyrazofurin	0.5–50	0.5	>50	>50
5′-Noraristeromycin	1–100	N.I.	N.D.	N.D.
6′-β-Fluoroaristeromycin	0.01–50	N.I.	N.D.	N.D.
3-Deazaneplanocin A	1–200	N.I.	N.D.	N.D.
Foscarnet	15–90	N.I.	N.D.	N.D.
Brefeldin A	0.1–50	N.I.	N.D.	N.D.
Sodium carbenoxolone	30–600	N.I.	N.D.	N.D.

[a]Effective antiviral concentration required to inhibit IPNV plaque formation by 50%.
[b]Cytotoxic concentration required to reduce cell viability by 50%, as measured in stationary cells.
[c]Inhibitory concentration required to reduce [*methyl*-^{3}H]thymidine incorporation by 50%.
N.I.: not inhibitory; N.D.: not determined.

and double-stranded RNA viruses were selected for testing. We included the inosine monophosphate dehydrogenase (IMP dehydrogenase) inhibitors 1-D-ribofuranosyl-1,2-4-triazole-3-carboxamide (ribavirin) and 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR), the orotidine monophosphate decarboxylase (OMP decarboxylase) inhibitor pyrazofurin, the *S*-adenosyl-homocysteine hydrolase (SAH hydrolase) inhibitors 5′-noraristeromycin, 6′-β-fluoroaristeromycin and 3-deazaneplanocin A, and the pyrophosphate analog phosphonoformic acid (foscarnet). We also tested compounds that produce alterations of cellular membranes necessary for some virus replication, such as brefeldin A and sodium carbenoxolone (Dargan and Subak-Sharpe, 1992; Dargan et al., 1992; Maynell et al., 1992).

The effect of the antivirals was evaluated by a virus plaque inhibition assay at various concentrations. We tested concentrations previously reported to have antiviral effect against other viruses (Migus and Dobos, 1980; De Clercq, 1985, 1993; Hudson et al., 1988; De Clercq et al., 1991; Crumpacker, 1992; Dargan and Subak-Sharpe, 1992; Dargan et al., 1992; Maynell et al., 1992). The compounds were tested at varying concentrations up to the cytotoxicity limit. The assays were done in triplicate and were each repeated at least three times.

Monolayers of the Chinook salmon embryo cell line (CHSE-214) were grown in MEM supplemented with 10% bovine fetal serum (BFS). The cells were infected with 100–200 plaque forming units (PFU) of the IPNV VR-299 strain. After 1 h incubation at 15°C the cells were overlaid with 0.5% of low gelling temperature agarose in MEM with 10% BFS and incubated for 3 days at 15°C. The antiviral compound was added in the agarose overlay and, in some cases, it was also added during the viral adsorption step. Afterwards the cells were formaldehyde-fixed and stained with a 0.5% crystal violet solution. The anti-IPNV activity was determined as the EC_{50} or the effective antiviral concentration required to inhibit IPNV plaque formation by 50%.

The results are shown in Table 1. Ribavirin was included as the reference compound for its derivative EICAR. Ribavirin was tested at a concentration range of 0.05–40 μg/ml and the EC_{50} was 0.5 μg/ml. This results agrees with previous data obtained in IPNV-infected RTG-2 cells (Hudson et al., 1988). EICAR was tested over a concentration range of 0.01–20 μg/ml, and the EC_{50} was 0.01

μg/ml. This value is 20–400 times lower than the EC_{50} for other RNA viruses (De Clercq et al., 1991).

The SAH hydrolase inhibitors 5′-noraristeromycin and 6′-β-fluoroaristeromycin were tested over the 1–100 and 0.01–50 μg/ml concentration range. However, they did not inhibit IPNV replication. Moreover, the latter compound turned out to be cytotoxic (i.e. cytopathic) in our system. Similar results were found for 3-deazaneplanocin A, which was tested at concentrations up to 200 μg/ml. It did not make any difference whether these antivirals that are assumed to interact with mRNA 5′-cap formation were added during the adsorption step or thereafter as part of the agarose layer. These results reinforce the assumption that IPNV mRNA lacks a cap structure, as was suggested by in vitro transcription studies (Mertens et al., 1982).

For the OMP decarboxylase inhibitor pyrazofurin an EC_{50} of 0.5 μg/ml was obtained. When foscarnet was added at concentrations of 15–90 μg/ml, no inhibition was observed, just as previously reported (Hudson et al., 1988). Higher concentrations proved to be cytotoxic. However, because it was previously reported that cellular incorporation of foscarnet requires cells to be preincubated with this compound (Helgstrand et al., 1978), this compound was also tested by adding it during the viral adsorption step or preincubating the cells for 2 h at 18°C with this antiviral. However, in all these cases no inhibition was observed.

Although IPNV has a double-stranded RNA as genome, it appears to resemble poliovirus, not only by the presence of VPg but also by the protein synthesis mechanism which starts with a polyprotein (Duncan et al., 1987); we determined if, as for poliovirus, IPNV is sensitive to brefeldin A (Maynell et al., 1992). As shown in Table 1, when brefeldin A was used at concentrations of from 0.1 to 50 μg/ml, no IPNV inhibition was observed. Moreover, brefeldin A turned out to be cytotoxic (i.e. cytopathic) for the CHSE-214 cells at a concentration of 1 μg/ml.

We also tested a triterpene derivative, sodium carbenoxolone, (Dargan and Subak-Sharpe, 1992) at a concentration range of 30–600 μg/ml; however, it did not inhibit IPNV replication under the conditions used. We added the compound also during the adsorption and in a cell preincubation step, but we were unable to see any antiviral effect.

For the compounds that showed inhibitory activity, the cytotoxic effect on CHSE-214 was studied. First, the effect on cellular viability in stationary cells was measured by the trypan blue exclusion assay. Different concentrations of the antiviral agent were added and after 3 days of incubation at 18°C the cellular viability were measured. We determined the CC_{50}, the cytotoxic concentration required to reduce cell viability by 50%. The results are shown in Table 1. For ribavirin and EICAR the concentration ranges tested were 1–100 μg/ml and 10–400 μg/ml, respectively. We obtained a CC_{50} of 100 μg/ml for ribavirin and of 50 μg/ml for EICAR. For pyrazofurin we did not observe a marked alteration of cell viability even at concentrations up to 100 μg/ml, the highest concentration tested.

In addition, the antivirals agents were tested for their effects on cellular DNA synthesis, which was monitored by the incorporation of [*methyl*-^{3}H]thymidine. Cells were grown up to approximately 50% of confluence to make sure active growth occurs. Then, different concentrations of the antiviral agent and 1 μCi/ml [*methyl*-^{3}H]thymidine (specific activity 67 Ci/mmol, ICN Biomedicals) were added, and the cells were incubated for 18–20 h at 18°C. Afterwards the radioactivity in the acid insoluble material was determined. Table 1 lists the IC_{50} values, the inhibitory concentrations required to reduce [*methyl*-^{3}H]thymidine incorporation by 50%. The concentration ranges tested were 0.01–50 μg/ml, 0.01–10 μg/ml and 0.1–50 μg/ml for ribavirin, EICAR and pyrazofurin, respectively. Pyrazofurin did not affect cellular DNA synthesis even at concentrations 100 times higher than the EC_{50}. The IC_{50} for EICAR was 50–100 times higher than the EC_{50}. Similar IC_{50} values were previously found for other cell lines exposed to EICAR (De Clercq et al., 1991). For ribavirin, the IC_{50} was of the same magnitude as the EC_{50}. This is in agreement with results published previously (Migus and Dobos, 1980), where the ribavirin concentra-

tion required to reduce IPNV titers by 3–4 log units also inhibited DNA synthesis in CHSE-214 cells. Moreover, it should be kept in mind that both EICAR and ribavirin may be less cytotoxic than revealed by the [*methyl*-^{3}H]thymidine uptake assays since the compounds have been shown to interfere directly with thymidine uptake (Drach et al., 1981).

From the test compounds, EICAR and pyrazofurin emerged as promising candidates for further evaluation in an in vivo model. Both compounds achieved inhibition of IPNV replication at concentrations that were 50–100 times lower than the concentration required to inhibit DNA synthesis in growing cells. We now plan to evaluate the antiviral efficacy of pyrazofurin and EICAR in experimentally IPNV infected fishes.

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