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Short Communication

The efficacy of amantadine and other antiviral compounds against two salmonid viruses in vitro

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Summary

Various compounds, with known clinical efficacy against human viruses, were evaluted for their ability to inhibit the growth of infectious hematopoietic necrosis virus (IHNV, a rhabdovirus), and infectious pancreatic necrosis virus (IPNV, a birnavirus), in rainbow trout cell cultures. Amantadine inhibited the plaque forming ability of IHNV, at concentrations which did not affect cell growth or morphology, although it was not active against IPNV. Metisazone and bis-benzimidazole were also effective against IHNV; but they were slightly cytotoxic. Ribavirin, as expected, was active against IPNV, but was also equally effective against IHNV, although it was cytotoxic.

Several other compounds were also tested but they were not inhibitory to either virus. The attraction of amantadine is the fact that relatively easy administration should be feasible.

Amantadine; Salmonid virus; Infectious hematopoietic necrosis virus; Infectious pancreatic necrosis virus

Virus infections are important causes of morbidity and mortality in fishes in many parts of the world, especially in farmed populations. The birnavirus infectious pancreatic necrosis virus (IPNV) is a constant threat to many fish species throughout North America, and the rhabdovirus infectious hematopoietic necrosis virus (IHNV) is often associated with disease outbreaks among salmonids (especially

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sockeye) in the Pacific Northwest. At present there are no effective preventive or therapeutic measures. Consequently IHNV epidemics have frequently resulted in the loss of millions of fish (Amend, 1976; Wolf, 1976,1984; Pilcher and Fryer, 1980; Leong and Barila, 1982; Hudson, 1985; Traxler, 1986; Mulcahy and Wood, 1986).

In human medicine there have been some notable successes in limited trials with antiviral compounds, e.g. amantadine against influenza A infections (Oxford and Galbraith, 1980, 1984), metisazone against smallpox (Bauer, 1965; McLean, 1977), and more recently a variety of nucleotide analogues or pyrophosphate analogues against herpes simplex virus infections (Shannon, 1984). Also, bis-benzimidazoles have been shown to have potent activity against enteroviruses and rhinoviruses in vitro and in vivo (Roderick et al., 1972; Shipkowitz et al., 1972).

In contrast there have been very few reports on successful control of virus infections in experimental fish (Savan and Dobos, 1980; Kimura et al., 1983; Hasobe and Saneyoshi, 1985). For practical reasons, compounds intended for use in fish would have to be administered to the water directly, e.g. in a holding tank, or in feed pellets. These routes are feasible, however, since it was shown some time ago that amantadine could be successfully administered to influenza-infected turkeys or mice in feed pellets or in drinking water (McGahen and Hoffman, 1968; Lang et al., 1970).

We instigated a survey of potential antiviral chemicals for activity against IHNV and IPNV, by means of plaque assays in fish cell cultures. We also examined the compounds for possible cytotoxicity against both growing and non-growing cells. The objective was to find chemicals which were capable of inactivating one or both viruses at non-cytotoxic concentrations. Any successful compounds should then be tested subsequently in live fish.

The following compounds were tested for antiviral activity: amantadine (adamantanamine hydrochloride, ICN Pharmaceuticals); bis-benzimidazole = (*S,S*)-1,2-bis(5-methoxy-benzimidazolyl)-1,2-ethanediol, (Abbot 36683); the following three beta diketones from Sterling-Winthrop: WIN 38020 (arildone), WIN 41258-3, WIN 42202; metisazone (methylisatin thiosemicarbazone), from Aldrich Chemical Co.; ribavirin (virazole); 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, from ICN Pharmaceuticals; phosphonoacetic acid and phosphonoformic acid from Helix Biotech. Several other antiviral chemicals, and other chemicals, were obtained from Sigma Chemical Company.

The rainbow trout gonad cell line, RTG-2, was used for propagation of the viruses and for the tests with chemicals. These cells were originally obtained from Dr. G. Traxler (Pacific Biological Station, Nanaimo, BC). They were cultivated in a modified form of Dulbecco MEM, obtained as a powder from Grand Island Biological Co. (GIBCO catalogue no. H20), supplemented with 3.34 g/l HEPES, 0.75 g/l $NaHCO_3$ and sufficient NaOH to increase pH to 7.2–7.3. In addition the medium received 25 μg/ml gentamicin sulfate (Sigma Chemical Company), and 10% by volume of fetal bovine serum (GIBCO). Cultures were maintained in 9 cm diameter tissue culture dishes or 250 ml flasks (Falcon or Corning), at 15°C in a humidified incubator. Cultures intended for plaque assays were sub-cultured (by trypsinization) in 5 cm dishes, and those intended for toxicity evaluation were sub-cultured in 24-well tissue culture trays (Linbro).

Infectious hematopoietic necrosis virus (IHNV), Great Central Lake strain, was obtained from Dr. G. Traxler, Pacific Biological Station Nanaimo. Infectious pancreatic necrosis virus (IPNV), Jasper strain, was obtained from Dr. T. Yamamoto, University of Alberta. Both viruses were propagated in RTG-2 cells, and the cell-free culture fluids were obtained from the lysed cells and stored in aliquots at −70°C.

In order to carry out antiviral tests serial ten-fold dilutions of virus were prepared. These were mixed with the test compound and inoculated onto cell monolayers. After incubation at 15°C for 60 min the inocula were removed and replaced by an overlay consisting of 0.5% agarose, 5% fetal bovine serum, and the same concentration of test compound, in standard medium. The cultures were incubated at 15°C until plaques became visible (5 days for IPNV, 10–12 days for IHNV), at which time they were fixed in formaldehyde-saline and stained with crystal violet for enumeration of plaques (Wolf et al., 1984).

Cell growth estimations were made by plating trypsinized RTG-2 cells into 24-well tissue culture plates, 10^4 cells per well, together with the appropriate concen-

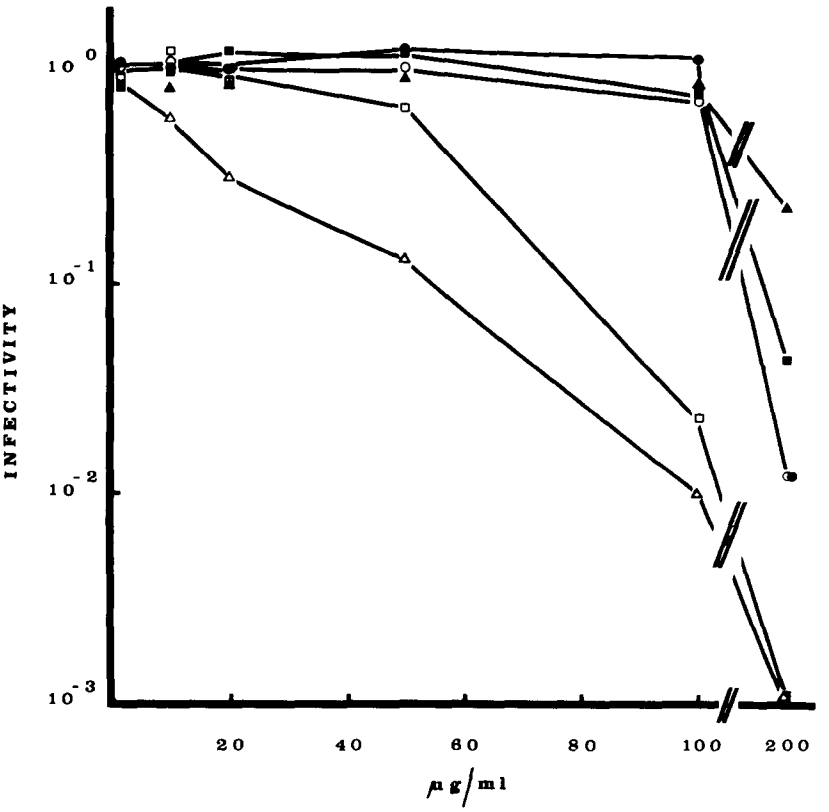

Fig. 1. Antiviral activity of amantadine, bis-benzimidazole and metisazone. Amantadine vs. IPNV (▲) and IHNV (△); benzimidazole vs. IPNV (■) and IHNV (□); metisazone vs. IPNV (●) and IHNV (○).

tration of compound (in quadruplicate). Cell counts were made microscopically (in ten random fields per well) every second day. After 7 days, cultures were replenished with fresh medium without compound. In other experiments confluent cultures (approximately 5×10^5 cells per well) were observed for cytopathic effects for 7 days.

Fig. 1 illustrates the effect of three of the compounds, amantadine, bis-benzimidazole and metisazone, against the two viruses, in plaque assays in RTG-2 cells. None of these compounds were active against IPNV (solid symbols) up to 100 μg/ml, although they were all inhibitory to some degree at 200 μg/ml.

In contrast amantadine showed a concentration dependent effect, between 10 and 200 μg/ml, against IHNV, while bis-benzimidazole was effective above 50 μg/ml. Metisazone was only active at 200 μg/ml.

Several other compounds were evaluted for antiviral effects. Ribavirin was inhibitory to both viruses in RTG-2 cells, at 0.5 μg/ml or greater (Fig. 2), although

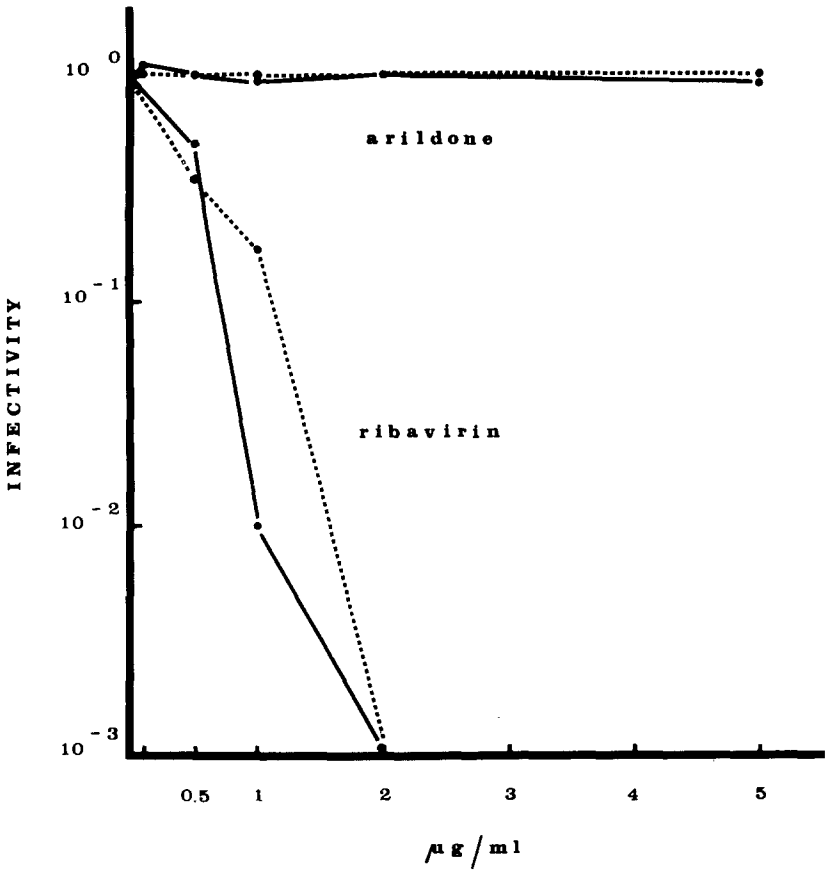

Fig. 2. Antiviral tests with ribavirin and diketones. Solid lines, IPNV; broken lines, IHNV. The arildone data are shown; the other two diketones were identical, and have been omitted for clarity.

TABLE 1

Effect of compounds on RTG-2 cells

Compound (μg/ml)	Growing cells	Confluent cultures
Amantadine 1–100	no effect	no effect
Amantadine 200	toxic	toxic
Bis-benzimidazole 1–100	inhibitory (ID_{50}~20 μg/ml)	change in cell morphology – not toxic
Bis-benzimidazole 200	inhibitory (97%)	change in cell morphology – not toxic
Metisazone 1–100	inhibitory (ID_{50}~5 μg/ml)	no effect
Metisazone 200	inhibitory (95%)	no effect
Ribavirin 0.5–5.0	inhibitory (ID_{50}~4 μg/ml)	no effect
Ribavirin 10–50	toxic	no effect
Ribavirin 100	toxic	toxic

ID_{50} = inhibitory dose of compound giving 50% reduction in cell numbers (extrapolated from concentrations of compound giving > 50% and < 50% reduction in cell numbers).
Toxic = all cells killed.

it has been reported to be ineffective in controlling IPNV infection in rainbow trout fry (Migus and Dobos, 1980; Savan and Dobos, 1980). Arildone and the other two diketones (Diana et al., 1977) were completely ineffective (Fig. 2). Deoxyglucose, phosphonoacetate, phosphonoformate, and several arabinoside analogues were also ineffective in these tests (data not shown).

Those compounds with demonstrable antiviral activity (against IHNV or IPNV) were examined for possible cytotoxic or cytostatic effects against RTG-2 cells. This was done both by microscopic examination of confluent cell monolayers and by cell counts on growing cells over a period of 14 days. The results derived from several such experiments are summarized in Table 1. Inhibition denotes a decrease in the number of growing cells in the culture; toxic implies that all cells were killed.

Amantadine had little effect on growing cells up to 100 μg/ml, although 200 μg/ml was definitely toxic (i.e., the cells were killed). Confluent cultures were not visibly affected until 200 μg/ml, in which the cells remained attached to the dish and were apparently viable but displayed extensive cytoplasmic vacuolation.

Ribavirin was inhibitory to growing cells at concentrations greater than 1 μg/ml and was cytotoxic at 10 μg/ml; whereas it had no visible effect on confluent cells at 50 μg/ml, although at 100 μg/ml it was cytotoxic.

Bis-benzimidazole and metisazone showed gradually increasing inhibition of cell growth between 10 and 200 μg/ml without obvious cytotoxicity. Confluent monolayers were not adversely affected, except that the benzimidazole showed a concentration-dependent alteration in cellular morphology, in which the cells changed from their normal spindle shape to a more polygonal shape.

The objective of this study was to find a suitable antiviral compound which could

be used safely in fish. The primary requirement for a successful chemical is that it must be able to inhibit virus replication without adversely affecting the host cells. It appears that amantadine fulfills this condition, and is therefore worthwhile investigating in more detail. The chances of its success in the whole animal are reasonable since the compound has been used clinically in humans and various other animals for the control of influenza infections (Oxford and Galbraith, 1980, 1984). The mechanism of action is not completely understood, although an early stage in virus replication appears to be the target. Amantadine is also effective against a few other enveloped viruses, although the majority of those tested (including, surprisingly, a Rhabdovirus, vesicular stomatitis virus), are resistant (Oxford and Galbraith, 1980).

In the present context it is of interest that amantadine has been administered therapeutically in drinking water to mice (McGahen and Hoffman, 1968), and in feed pellets to turkeys (Lang et al., 1970). Therefore it seems feasible to consider similar practical methods of administering the compound to fish populations.

The other three compounds which did show some antiviral activity, ribavirin, bis-benzimidazole and metisazone, would probably be less useful in vivo since there is much less difference between effective antiviral concentration and cell growth inhibitory concentrations. Thus Migus and Dobos (1980) showed that ribavirin could block IPNV replication in RTG-2 and Chinook salmon embryo (CHSE-214) cultures, but in another study Savan and Dobos (1980) were unable to demonstrate protection of rainbow trout fry against IPNV by this compound, although the fish evidently tolerated the compound.

Hasobe and Saneyoshi (1988) demonstrated that several nucleoside analogues could interfere with IHNV replication in vitro, but could only afford partial protection in steelhead fry.

We believe that it would be worthwhile to evaluate amantadine against IHNV in similar animal infections.

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