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In vitro antiviral effect of 9-(2-hydroxyethoxymethyl) guanine on the fish herpesvirus, *Oncorhynchus masou virus* (OMV)

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The antiviral activity of 9-(2-hydroxyethoxymethyl) guanine (Acyclovir, ACV) on the salmon herpesvirus, *Oncorhynchus masou virus* (OMV), was studied in vitro. ACV showed high efficacy against the fish herpesvirus OMV, *Herpesvirus salmonis* and channel catfish virus (CCV). Cytopathic effect (CPE) induced by 100 $TCID_{50}$/ml of OMV in rainbow trout gonad (RTG-2) cells was inhibited by 2.5 μg/ml of ACV. ACV was more effective than other compounds such as 9-β-D-arabinofuranosyladenine (Ara-A), 5-iodo-2'-deoxyuridine (IUdR) and phosphonoacetate (PA). Growth of RTG-2 cells was considerably inhibited by ACV at 25 μg/ml, but no morphological changes were observed in the cells. Replication of OMV in RTG-2 cells inoculated with 100 $TCID_{50}$/ml was completely suppressed by 2.5 μg/ml of ACV. Addition of ACV within 4 days post infection was effective in reducing OMV replication. In order to be effective, ACV had to be present continuously .

Acyclovir; antiviral activity; *Oncorhynchus masou virus*

Introduction

The anti-herpesvirus compound 9-(2-hydroxyethoxymethyl) guanine (Acyclovir, ACV) was first reported by Elion et al. [9]. The antiviral activity of this compound has been confirmed in vitro by Crumpacker et al. [6], Collins and Bauer [5], Centifanto and Kaufman [3], De Clercq et al. [7,8] and Colby et al. [4] and in vivo by Field et al. [10], Klein et al. [15], Park et al. [18], Bauer et al. [2] and Shiota et al. [19]. These workers reported that the compound had high antiviral activity but very little effect on a normal metabolic system.

Because of their prevalence and economic importance, viral diseases of fish in aquaculture have received much attention. However, in spite of extensive research, no effective antiviral therapy has been developed.

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Oncorhynchus masou virus (OMV) is a recently discovered fish virus and a member of the herpesvirus group. It is also the causative agent of infectious hepatitis and a potent tumor inducer in salmonid fish [12–14].

In this paper, the antiviral activity of ACV against OMV in vitro was compared with the other anti-herpesvirus compounds 9-β-D-arabinofuranosyladenine (ARA-A) [16], phosphonoacetate (PA) [17] and 5-iodo-2'-deoxyuridine (IUdR). Two other fish herpesviruses, *Herpesvirus salmonis* and channel catfish virus (CCV), and the fish rhabdovirus of infectious hematopoietic necrosis virus (IHNV), were used for comparison. The toxicity of ACV for RTG-2 cells was also investigated.

Materials and methods

Cell lines and viruses

Rainbow trout gonad (RTG-2) cells for OMV, *H. salmonis* and infectious hematopoietic necrosis virus, and brown bullhead (BB) cells for channel catfish virus were used in this study. These cells were maintained in Eagle's minimum essential medium (Gibco) supplemented with 10% fetal bovine serum (MEM_{10}).

The salmonid herpesvirus, OMV, was isolated from the ovarian fluids of landlocked masu salmon (*O. masou*) in 1978 in Hokkaido, Japan. *Herpesvirus salmonis* was kindly provided by K. Wolf of the National Fish Health Research Lab., WV, U.S.A. and CCV by B.J. Hill, Fish Diseases Lab., Weymouth, U.K. The authors isolated IHNV from chum salmon (*O. keta*). The viruses were propagated in RTG-2 or BB cells and stored at -80°C until used.

Chemicals

The ACV (sodium salt) was supplied by the Wellcome Foundation Ltd., London, U.K. It was dissolved in distilled water to a concentration of 1000 μg/ml by warming at 30°C and then decontaminated by passage through a 0.45-μm Millipore filter membrane, and stored at -20°C. Solutions of Ara-A (Ajinomoto Co. Inc., Japan), PA (Abbott Laboratory, U.S.A.) and IUdR (Wako Pure Chemical Industries Ltd., Japan) were prepared by the same method as ACV.

Antiviral activity

Monolayers of cells seeded in 96 well tissue culture plates (Flow) were infected with 100 $TCID_{50}$/ml (MOI 5×10^{-3}) of virus. Each of the chemicals to be tested was then added in 0.5 $\log_{10}$ dilutions. The OMV and IHNV plates were incubated at 15°C for 10 days, the *H. salmonis* plates at 10°C for 14 days and CCV plates at 25°C for 7 days. The cytopathic effect (CPE) caused by each virus was observed after staining the cells with crystal violet. The antiviral activity was expressed as a virus rating (VR). The minimal discernible virus inhibitory concentration of the compounds at which CPE was depressed by $> 50\%$ (MIC) was determined by the modified method described by Sidwell and Huffman [20].

Effect of antiviral compounds on RTG-2 cell growth

RTG-2 cells were seeded in 60-mm plastic dishes (Falcon) with MEM_{10}. After 3 days at 15°C, various concentrations of ACV, PA, Ara-A or IUdR were added to the cultures, and incubation was continued. At the time these compounds were added and at 2, 7, and 12 days thereafter, the cells were dispersed with trypsin, suspended in 5 ml of Hanks' balanced salt solution (BSS, Gibco) and the number of cells was determined with a hemocytometer.

Effect of ACV on replication of OMV

RTG-2 cells were seeded in 25-cm^2 culture flasks (Falcon). After 3 days of incubation at 15°C, the monolayers were inoculated with OMV at 100 $TCID_{50}$/ml. After adsorption for 2 h, the cultures were incubated with medium containing ACV. The final drug concentrations were 0.5, 1.0 and 2.5 μg/ml. The virus titer in the culture fluid was determined at selected intervals by standard methods using RTG-2 cells.

To determine the effect on OMV replication when ACV was added to the culture at various times post infection, 2.5 μg/ml of ACV (final concentration) was added to the culture at 0, 0.5, 1, 2, 3, 4 and 6 days.

Effect of ACV removal from infected cultures

The monolayers of RTG-2 cells in 25-cm^2 flasks were inoculated with 100 $TCID_{50}$/ml of OMV. Following virus adsorption, growth medium containing 2.5 μg/ml of ACV was added and the cultures incubated at 15°C. At 1,2 and 4 days post infection, the medium was replaced with medium without ACV. The cultures were observed for the appearance of CPE for 3 days after the removal of the drug. The cell-associated virus titer was determined after the disruption of the cultures by the freeze–thaw method described by Goodrich et al. [11].

Results

CEP inhibition

Among the 4 compounds tested, ACV was the most effective against OMV, followed by IUdR and PA in that order (Table 1). Additional studies revealed that the CPE induced by OMV was inhibited by 2.5 μg/ml of ACV. Morphological changes in RTG-2 cells caused by those compounds were only observed at 100 μg/ml of PA.

Ara-A had no effect on the fish herpesvirus tested up to a concentration of 100 μg/ml. The virus rating for ACV was above 1.0 for all three herpesviruses. In CCV and BB cells, PA was not effective within the concentration range tested. In a further study, 300 μg/ml of PA inhibited the CPE caused by CCV. The rhabdovirus, IHN, was resistant to all four compounds (Table 2).

TABLE 1

Effect of Ara-A, PA, IUdR and ACV on OMV infection in RTG-2 cells

Drug conc. (μg/ml)	CPE[a]							
	Ara-A	Ct	PA	Ct	IUdR	Ct	ACV	Ct
100	4 4	N	0 0	St	0 0	N	0 0	N
32	4 4	N	0 0	N	0 0	N	0 0	N
10	4 4	N	4 4	N	0 0	N	0 0	N
3.2	4 4	N	4 4	N	4 4	N	0 0	N
1.0	4 4	N	4 4	N	4 4	N	4 4	N
0	4 4	N	4 4	N	4 4	N	4 4	N
VR[b]	0.0		0.6		1.2		1.6	
MIC[c]	>100		32		10		3.2	

[a] Numbers of CPE positive well per each 4 wells. 0 = all four negative, 4 = all positive.

[b] Virus rating:

$$VR = \frac{\Sigma\,(\text{total virus control score} - \text{treated score of each drug concentration})}{10 \times (\text{times of test})},$$

$2 \geqslant VR \geqslant 0$. Difference in virus control and treated scores divided by 2 at partially toxic drug levels.

[c] Minimal inhibitory concentration (μg/ml); see Materials and Methods.

Ct = cytotoxic; N = not toxic; St = slightly toxic.

TABLE 2

Antiviral activities of Ara-A, PA, IUdR and ACV against fish viruses

Compound	Virus							
	OMV (RTG-2)		*Herpesvirus salmonis* (RTG-2)		CCV (BB)		IHNV (RTG-2)	
	VR[a]	MIC[b]	VR	MIC	VR	MIC	VR	MIC
Ara-A	0.0	>100	0.0	>100	0.0	>100	0.0	>100
PA	0.6	32	0.6	32	0.0	>100	0.0	>100
IUdR	1.2	10	1.4	3.2	0.85	10	0.0	>100
ACV	1.6	3.2	1.6	3.2	1.1	10	0.0	>100

[a] Virus rating; see Table 1.

[b] Minimal inhibitory concentration (μg/ml); see Table 1.

Effect on RTG-2 cell growth

The growth of RTG-2 cells was unaffected by ACV at low concentrations that were in the inhibitory range for OMV. However, cell growth was inhibited at higher (25 and 50 μg/ml, Fig. 1) concentrations. For PA, RTG-2 cell growth was inhibited 50% at a concentration of 30 μg/ml. The addition of Ara-A (40 μg/ml) and IUdR (16 μg/ml) to culture media also inhibited RTG-2 cell growth.

Effect of drug concentration on replication of OMV

In the virus control flask, after an eclipse stage of 2 days, the virus titer in the culture fluid reached 10^6 $TCID_{50}$/ml by 10 days post infection. In the culture treated with 0.5 μg/ml of ACV, the maximum titer reached 10^5 $TCID_{50}$/ml within 13 days. When 1.0 μg/ml of the ACV was added to the culture, a further decrease in titer was observed reaching only 10^4 $TCID_{50}$/ml within 10 days. When 2.5 μg/ml of the ACV was added to the culture, no released virus was detected throughout the incubation period (Fig. 2).

Effect of delayed addition of ACV on replication of OMV

The time of addition of ACV following infection influenced replication of OMV. Addition of ACV at 0, 0.5 or 1 day after virus inoculation prevented CPE, and virus was not detected in culture fluids throughout the incubation period (Fig. 3). When the drug was added at 2, 3 and 4 days post inoculation, the virus titer in the culture fluid decreased. As a result, no virus was detected at 17 days. When ACV was added after 6 days, the decrease in virus titer after drug addition demonstrated a pattern similar to that of the virus control.

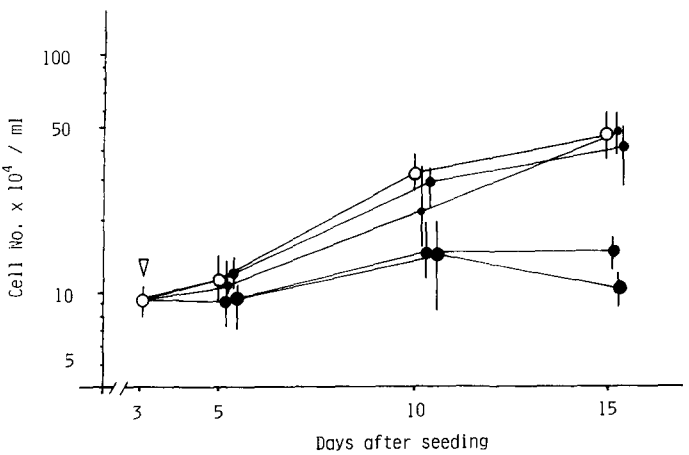

Fig. 1. Effect of various concentrations of ACV on RTG-2 cell growth. O——O = Control (without drug), ●——● = ACV 1.0 μg/ml, ●——● = ACV 2.5 μg/ml, ●——● = ACV 25 μg/ml, ●——● = ACV 50 μg/ml.

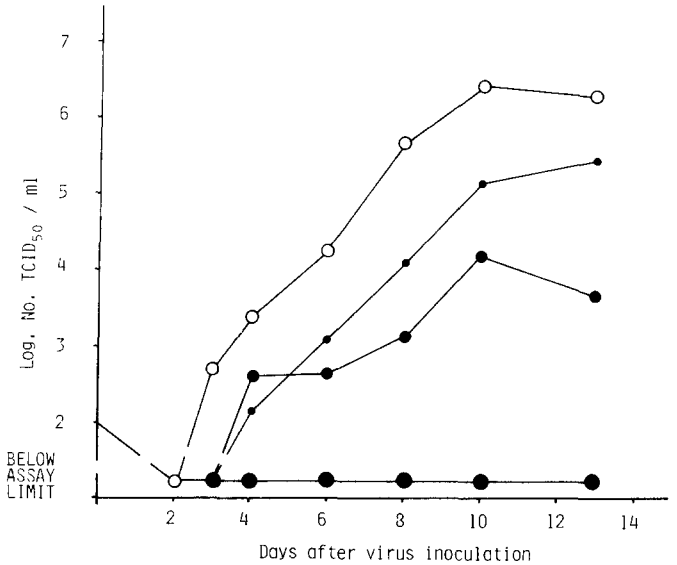

Fig. 2. Effect of ACV on the replication of OMV at various concentrations. Virus inoculum: 100 $TCID_{50}$/ml. O———O = virus control (without drug), •———• = ACV 0.5 µg/ml, ●———● = ACV 1.0 µg/ml, ●———● = ACV 2.5 µg/ml.

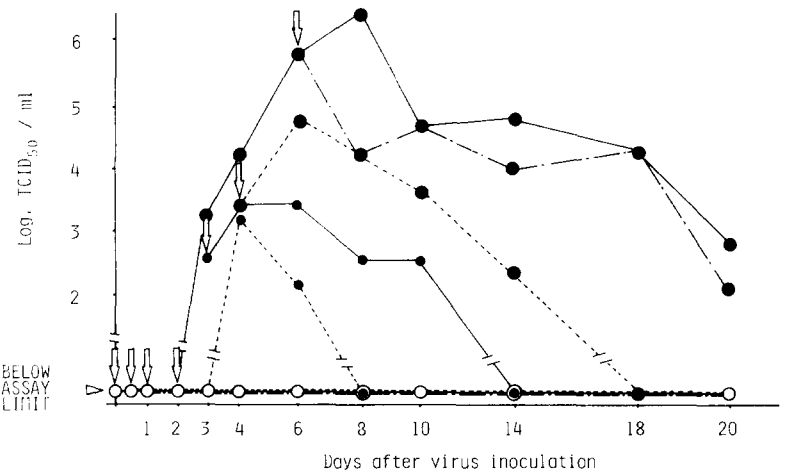

Fig. 3. Effect of ACV addition to culture media on the replication of OMV at different periods post infection. Virus inoculum: 100 $TCID_{50}$/ml. ACV added at days 0, 0.5 and 1 gave the same results and are all represented by O———O; virus control = ●———●; ACV added 2 days after virus inoculation = •-----•, ACV added 3 days after virus inoculation = •———•, ACV added 4 days after virus inoculation = ●-----●, ACV added 6 days after virus inoculation = ●—-—●. ⇨ = time of addition of ACV.

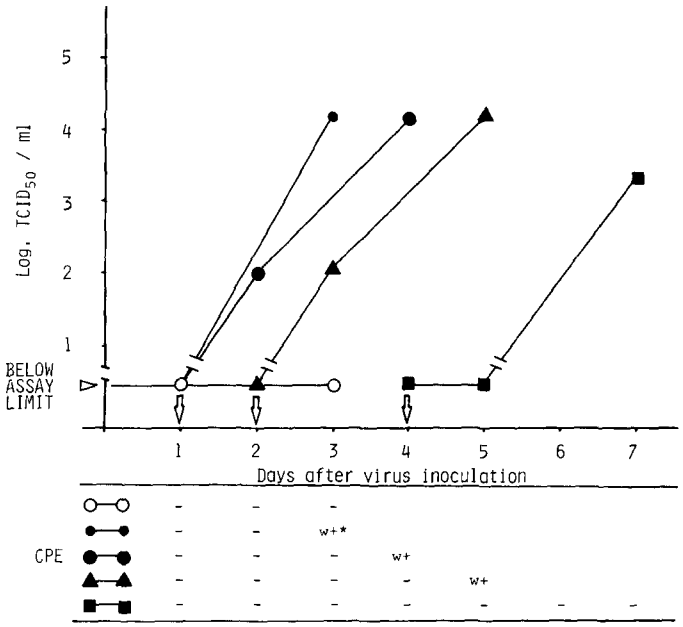

Fig. 4. Effect on OMV replication of ACV removal from media at different periods. Virus inoculum: 100 $TCID_{50}$/ml. O———O = control (ACV added 0 day after inoculation), ●———● = virus control (without drug), ●———● = ACV removed 1 day after virus inoculation, ▲———▲ = ACV removed 2 days after virus inoculation, ■———■ = ACV removed 4 days after virus inoculation, ⇨ = removal of ACV, * = weakly positive.

Effect of removal of drug on replication and CPE formation by OMV

Replication of OMV and the appearance of CPE were dependent on the length of time that ACV was in contact with the cultures (Fig. 4). If ACV was removed at 1 or 2 days post infection, cell-associated virus could be detected 1 day following the removal of the drug and CPE was observed at 3 days after removal. When ACV was removed after 4 days, no virus could be detected the following day but the virus was detected 3 days later.

Discussion

It has been reported that ACV is selectively active against herpesvirus and is more effective against HSV-1 in Vero cells than IUdR, Ara-A and PA in that order [5]. Our results likewise indicate that against fish herpesviruses, ACV is the most effective of the 4 compounds. Ara-A was found not to be effective up to 100 μg/ml against the three fish herpesviruses tested. This may indicate that, in fish cells the phosphorylation of Ara-A into its active form is different from that of mammalian cells. The efficacy of the four drugs against OMV and *H. salmonis* was similar. This suggests that

OMV and *H. salmonis* have some similar properties. All four compounds tested were ineffective against the fish rhabdovirus, IHNV. This result is in agreement with reports that these drugs are selectively active against herpesviruses.

At 10 times the effective concentration (25 μg/ml) of ACV for OMV, the growth of RTG-2 cells was inhibited but the cell morphology was not affected. Collins and Bauer [5] reported that the replication of Vero cells was inhibited by ACV in concentrations > 50 μM. However, they also observed that no cell death occurred nor did gross toxicity appear at that concentration level. These findings indicate the low toxicity of ACV for normal cells.

The replication of OMV in RTG-2 cells, inoculated with 100 $TCID_{50}$/ml of OMV was completely suppressed by 2.5 μg/ml of ACV. Moreover, the delayed addition of ACV within 4 days post infection was effective in reducing OMV replication. Barahona et al. [1] reported that the CPE induced by *Herpesvirus saimiri* in OMK cells regressed if PA was added shortly after virus infection. A similar phenomenon was observed in our system. These results suggest that ACV has a potential for curing herpesvirus infections in vitro.

If ACV was removed early after infection, a number of uninfected cells would have been present in the culture and it may have been these that then supported OMV replication. Consequently, in order to maintain the antiviral effect, continuous presence of the drug was necessary.

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