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Antiviral activity of various 1-β-D-arabinofuranosyl-*E*-5-halogenovinyluracils and *E*-5-bromovinyl-2′-deoxyuridine against salmon herpes virus, *Oncorhynchus masou* virus (OMV)*

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Summary

1-β-D-Arabinofuranosyl-*E*-5-bromovinyluracil (BVaraU), 1-β-D-arabinofuranosyl-*E*-5-iodovinyluracil (IVaraU), 1-β-D-arabinofuranosyl-*E*-5-chlorovinyluracil (CVaraU) and 1-β-D-arabinofuranosyl-5-vinyluracil (VaraU) were examined for antiviral activity against salmon herpesvirus, *Oncorhynchus masou* virus (OMV) in vitro using Yamame (*Oncorhynchus masou*) kidney cells (YNK). BVaraU, IV araU, CVaraU and VaraU were highly active against OMV; 50% inhibitory concentration (IC_{50}): 0.01, 0.003, 0.003, 0.003 μg/ml, respectively. The IC_{50} of 5-bromovinyl-2′-deoxyuridine (BVDU) was 0.3 μg/ml. The lower activity may be due to cleavage of it *N*-glycosyl linkage by pyrimidine nucleoside phosphorylases (i.e. thymidine phosphorylase) during the incubation period. The arabinofuranosyl counterparts are resistant to this (these) enzyme(s). Both OMV-induced DNA polymerase and cellular DNA polymerase α were strongly inhibited by BVaraU 5′-triphosphate (BVaraUTP). In an in vivo study, daily immersion of OMV-infected chum salmon (*Oncorhynchus keta)* fry into aqueous solution of BVaraU (5 μg/ml, 30 min/day, 30 times) did not increase the life span of infected fish.

BVaraU; Halogenovinyluracil arabinoside; *Oncorhynchus masou* virus (OMV); BVDU; Degradation in cells; Thymidine phosphorylase

* This paper is dedicated to Professor Morio Ikehara on the occasion of his retirement from Osaka University in March 1986.

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Introduction

Oncorhynchus masou virus (OMV), one of the herpes viruses, is known to be a pathogenic for salmonids (Kimura et al., 1981). Because of their practical importance, viral diseases of fish in aquaculture have received considerable attention. 9-(2-hydroxyethoxymethyl)guanine (acyclovir, ACV) exhibit antiviral activity against OMV in vitro and in vivo (Kimura et al., 1983a, b). In this paper, we describe the comparative antiviral study of several 1-β-D-arabinofuranosyl-5-vinyluracil derivatives and *E*-5-bromovinyl-2′-deoxyuridine (BVDU) against OMV in vitro. We also describe the inhibitory effects of 1-β-D-arabinofuranosyl-*E*-5-bromovinyluracil 5′-triphosphate (BVaraUTP) on OMV-induced DNA polymerase and host cellular DNA polymerase α.

Materials and Methods

Cells and Virus

Rainbow trout (*Salmo gairdnerii)* gonadal cell line (RTG-2) (Wolf and Quimby, 1962) was used for propagation of OMV. Cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS) (growth medium). For the plaque reduction assay, Yamame (*Oncorhynchus masou*) kidney cells (YNK) (Watanabe et al., 1978) were used. Both cells were cultured at 15°C during incubation. *Oncorhynchus masou* virus (OMV) was kindly provided by Professor Takahisa Kimura, Laboratory of Microbiology, Faculty of Fisheries, Hokkaido University, Hakodate.

Chemicals and Nucleoside Analogues

The following compounds were examined for their activity against OMV: 1-β-D-arabinofuranosyl-*E*-5-bromovinyluracil (BVaraU), 1-β-D-arabinofuranosyl-*E*-5-chlorovinyluracil (CVaraU) (Machida et al., 1981), 1-β-D-arabinofuranosyl-*E*-5-iodovinyluracil (IVaraU) (Machida and Sakata, 1984), 1-β-D-arabinofuranosyl-5-vinyluracil (VaraU) (Machida et al., 1980) and BVDU (De Clercq et al., 1979). 5-Iodo-2′-deoxyuridine (IUdR) and 1-β-D-arabinofuranosylthymine (AraT) were used as positive controls. 1-β-D-arabinofuranosyl-*E*-5-bromovinyluracil 5′-triphosphate (BVaraUTP) was synthesized from BVaraU by conventional phosphorylation methods. [*methyl*-^{3}H]Thymidine 5′-triphosphate (77.9 Ci/mmol) was purchased from New England Nuclear (U.S.A.). Natural 2′-deoxyribonucleoside 5′-triphosphate (dNTPs) were purchased from Yamasa Shoyu Co. Ltd.

Plaque reduction method

Confluent monolayers of YNK cells grown in a 24 multiwell plate (Coaster) were infected with approximately 100 plaque-forming units (PFU) of OMV. After a 1-h absorption period at 15°C, the virus solution was discarded and the culture washed with MEM and 1 ml of maintenance medium (MEM supplemented with 10% FBS) containing an appropriate amount of test compound in serial half-$\log_{10}$ dilutions.

After 72 to 96 h of incubation at 15°C, the infected culture was fixed with 5% formaldehyde and stained with 0.1% crystal violet. The number of plaques was counted under a dissecting microscope at ×40 magnification. The 50% inhibitory concentration (IC_{50}) of each drug represents the dose at which the number of plaques was reduced by at least 50% when compared with that of the control culture. It was previously established that prior to 96 h of incubation, no plaques were induced by secondary infection with OMV. Therefore, the cultures were not overlayed with agarose.

Nucleoside analysis by high performance liquid chromatography (HPLC)

Monolayers of YNK cells grown in 35-mm dishes were infected with 3×10^4 PFU of OMV. To the dish was added 2 μg/ml of BVaraU or BVDU in MEM supplemented with 10% FBS. After 72 h of incubation at 15°C, the concentrations of BVaraU, BVDU and *E*-5-bromovinyluracil (BVUra) in the culture media were determined by HPLC on a reverse phase chemopak 300-10Cl8 in a Shimadzu LC-5A system. The minimum amount of detectable nucleoside in this assay was about 0.06 μg/ml for each compound. The conditions of analysis were as follows. The mobile phase was 0.01 M KH_2PO_4-methanol (5:1); the flow rate was 1 ml/min; detection was at 290 nm and injection volume was 10 μl. The retention time of BVUra, BVaraU and BVDU in this system were 7.94 min, 9.64 min and 11.04 min, respectively.

Assay for DNA polymerase activity

OMV-induced DNA polymerase (OMV-pol) was partially purified from OMV-infected Kokanee (*Oncorhynchus nerka adonis*) ovary cells (KO-6) as described previously (Suzuki et al., 1986). OMV-induced DNA polymerase activity was assayed in a 25-μl reaction mixture containing 50 mM Tris-HCl, pH 8.0, 0.5 mM dithiothreitol, 4 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 70 μM each of dATP, dGTP, dCTP and 7 μM of [*methyl*-^{3}H]dTTP (9 cpm/pmol), 2.5 μg of activated salmon sperm DNA and enzyme preparation (0.3 units). Incubation was performed for 20 min at 25°C. The reaction was chilled and 20 μl of aliquots were washed with 5% Na_2HPO_4 six times, twice with ethanol and dried. The remaining radioactivity was measured in a Packard Tri-Carb liquid scintillation counter.

Inhibition of cell growth by the analogues

YNK cells were seeded in a 35 mm dish and incubated at 15°C. The cell numbers were approximately 10^5 cells per plate after 3 days of incubation. At that time, an appropriate amount of test compound was added to duplicate cultures. After an additional 5 days-incubation period, the cells were dispersed by trypsin and the cell numbers were counted with an hemocytometer. Inhibition of cell growth was determined by comparison of the cell numbers in the drug-treated and control cultures.

Effect of BVaraU on the survival of OMV-infected chum salmon (Oncorhynchus keta) in vivo

Chum salmon (*Oncorhynchus keta)* fry (average body weight 0.5 g), kindly provided by Sapporo Salmon Science Center, were divided into five groups of 58 each and three groups were infected by immersion in water containing 200 PFU per ml of OMV (Kimura et al., 1983b). The fish were held in 12°C running water. Drug treatment was also performed using the immersion method described previously (Kimura et al., 1983b). The fish were immersed at either daily or 2 daily intervals for 30 min in an aqueous solution containing 5 μg/ml BVaraU. Uninfected and OMV-infected controls underwent the same handling stress without drug treatment. The treatment was continued for 30 days after infection.

Results

Antiviral activity of the analogues in vitro

Antiviral activity of the nucleoside analogues was determined by the plaque reduction method (Table 1). Of all the test compounds, VaraU, CVaraU and IVaraU

TABLE 1

In vitro antiviral effects of the compounds against OMV in YNK cells

Compound	IC_{50} (μg/ml)
BVaraU	0.01
CVaraU	0.003
IVaraU	0.003
VaraU	0.003
BVDU[a]	0.3
BVDU[b]	0.01
IUdR	1.0-3.2
AraT	0.032

[a] BVDU added once, immediately after virus infection.

[b] MEM containing BVDU was replaced with fresh medium containing BVDU every 24 h.

TABLE 2

Concentration of BVDU, BVaraU and BVUra in the culture media after 72 h incubation

Sample	Nucleoside added	Concentration (μg/ml)		
		BVaraU	BVDU	BVUra
Mock-infected	BVDU		<0.06	0.976
Mock-infected	BVaraU	1.592		<0.06
OMV-infected	BVDU		<0.06	1.153
OMV-infected	BVaraU	1.584		<0.06
Medium only[a]	BVDU		1.309	<0.06
Medium only	BVaraU	1.784		<0.06

[a] Medium (MEM) supplemented with 10% FBS.

TABLE 3

Inhibition of DNA polymerases by BVaraUTP

	OMV-pol[a]	Cellular pol α[b]
dTTP (K_m)	5.7 μM	7.4 μM
BVaraUTP (K_i)	2.3 μM	1.9 μM
K_i/K_m	0.40	0.26

[a] Specific activity (817 units/mg protein).

[b] Purified from cherry salmon tissues, specific activity (35000 units/mg protein).

were the most effective for OMV (IC_{50}: 0.003 μg/ml). BVaraU was slightly less active than the other vinyl analogues (IC_{50}: 0.01 μg/ml). BVaraU was however, approximately 30 times more active than BVDU (IC_{50}: 0.3 μg/ml). When fresh medium containing BVDU was added every 24 h after virus infection, BVDU had a similar activity against OMV as BVaraU (Table 1).

Degradation of BVDU during incubation

Before and after incubation (72 h), the concentration of BVaraU, BVDU and BVUra in the culture media were determined by HPLC. As shown in Table 2, BVUra, but not BVDU, was detected in the media of the mock- and OMV-infected cultures treated with BVDU. In contrast, BVUra could not be detected in the media of BVaraU-treated groups. These findings clearly indicate that BVDU is easily cleaved to BVUra by cellular thymidine phosphorylase or pyrimidine nucleoside phosphorylases in general, whereas BVaraU is completely resistant to these enzymes.

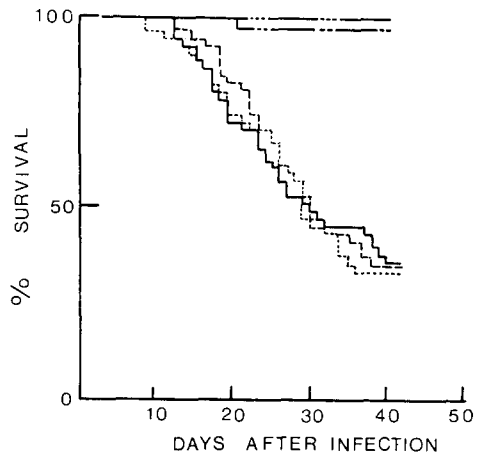

Fig. 1 Effect of BVaraU treatment by the immersion method on the survival of chum salmon fry following infection with OMV. OMV infection by 200 PFU/ml, —·--·—, negative control; —··—, drug control; ———, virus control; ---------, drug treatment (at daily intervals); and -------, drug treatment (at 2-daily intervals).

Inhibition of DNA polymerases by BVaraUTP

The inhibitory effects of BVaraUTP on OMV-pol and host cellular DNA polymerase are summarized in Table 3. Analysis by Lineweaver-Burk plots revealed that BVaraUTP inhibited OMV-pol as well as cellular DNA polymerase α purified from cherry salmon (*Oncorhynchus masou*) tissue competitively with respect to dTTP (data not shown). The calculated K_m value for dTTP was 5.7 μM and K_i value for BVaraUTP was 2.3 μM.

Inhibition of cell growth

The nucleoside analogues tested showed little or no inhibitory effect on YNK cell growth (data not shown). Specifically, VaraU, IVaraU and BVaraU did not inhibit cell growth at concentrations up to 100 μg/ml.

Experimental chemotherapy using BVaraU on OMV-infected chum salmon fry

The survival rate of OMV-infected chum salmon fry is shown in Fig. 1. Using the immersion method in selected conditions BVaraU treatment failed to extend the life-span of infected fish. There was no decrease in survival rate due to the toxicity of the drugs.

Discussion

BVaraU is known to be a highly selective antiviral agent against herpes viruses such as Herpes simplex type 1 and Varicella-zoster virus (VZV) (Machida et al., 1981, 1982, 1984). Herein, we have examined the antiviral effect of BVaraU and related 5-vinyl AraU derivatives against the salmon herpes virus, OMV. The order of efficacy of these analogues was CVaraU ≅ IVaraU ≅ VaraU > BVaraU > AraT > BVDU > IUdR. BVDU has also been reported to be a selective inhibitor of herpesvirus replication (De Clercq et al., 1979, De Clercq and Walker, 1984). However, in the present study, BVDU was found to be 1/30 less active than BVaraU and 1/100 less active than IVaraU, CVaraU and VaraU, respectively. The decreased activity of BVDU seems to be caused by the degradation of BVDU to BVUra by thymidine phosphorylase or pyrimidine nucleoside phosphorylases in cells. The antiviral activity of BVDU was equal to that of BVaraU following 'refeeding' at 24 h intervals with medium containing BVDU. That BVDU was cleaved to BVUra could be confirmed by HPLC analysis. It has been reported that BVDU is a good substrate for thymidine phosphorylase (Desgranges et al., 1983). Our present findings confirm this observation. It is noteworthy in this context that Desgranges et al. (1985) have recently reported that the combination of BVDU with 6-aminothymine as an inhibitor of thymidine phosphorylase significantly increases the half-life of BVDU (Desgranges et al., 1985). Allaudeen et al. (1981) have reported that BVDU 5′-triphosphate inhibits the herpes virus DNA polymerase more effectively than cellular DNA polymerases α, β or γ. Ruth and Cheng confirmed this observation (Ruth and Cheng, 1981). We have now shown that BVaraUTP strongly inhibits OMV-induced DNA polymerase and host cellular DNA poly-

merase α to a similar extent. Thus, BVaraUTP is a non-selective inhibitor of the OMV-DNA polymerase, although cytotoxicity of BVaraU or related analogues was not observed in mock-infected cells. Thus the selective activity of BVaraU and related compounds against OMV may entirely depend on a specific phosphorylation by virus-induced thymidine kinase. In vivo, treatment of OMV-infected chum salmon (*Oncorhynchus keta)* fry with BVaraU did not extend the life span of the fish. Using similar treatment methods and schedules, ACV proved effective in suppressing OMV infection in chum salmon fry (Kimura et al., 1983b). These findings suggest differences in the bioavailability or in vivo metabolism of BVaraU and ACV. Additional studies, i.e. different treatment schedules or procedures, with antiviral agents are needed to establish an effective and convenient chemotherapy of viral diseases of fish in aquaculture.

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