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Mechanism of Selective Inhibition of Varicella Zoster Virus Replication by 1-β-D-Arabinofuranosyl-*E*-5-(2-bromovinyl)uracil

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SUMMARY

To investigate the mechanism of action of 1-β-D-arabinofuranosyl-(*E*)-5-(2-bromovinyl)uracil (BV-araU) on varicella zoster virus (VZV) replication, we examined the metabolism of the drug in VZV-infected cells using ^{14}C-labeled BV-araU. [^{14}C]BV-araU was taken up by the cells infected with thymidine kinase-positive (TK^+)VZV, but not so much by TK^- VZV-infected or mock infected cells. Most of the radioactivity in TK^+ VZV-infected cells that were incubated with [^{14}C]BV-araU was recovered from their acid-soluble fraction, and little from their acid-insoluble fraction. By high performance liquid chromatographic assay of the acid-soluble fraction, it was proved that BV-araU was metabolized to its 5′-monophosphate, diphosphate, and triphosphate only in TK^+ VZV-infected cells. The radioactivity was not detected in VZV nucleocapsids or in VZV DNA and cellular DNA isolated from TK^+ VZV-infected cells, even if BV-araU was added at a 1000 times higher concentration than the 50% inhibitory dose for VZV replication *in vitro*. Furthermore, it was enzymatically proved that [^{14}C]BV-araU was selectively and effectively phosphorylated to BV-araU monophosphate by VZV TK and that affinity of BV-araU triphosphate for VZV DNA polymerase was the quite strong. From these results, it can be concluded that marked inhibition of VZV replication by BV-araU is due to selective phosphorylation of BV-araU in the TK^+ VZV-infected cells and strong inhibition of VZV DNA synthesis by BV-araU triphosphate, without detectable incorporation into VZV DNA.

A number of pyrimidine nucleoside analogues exhibit potent antiherpes activity *in vitro* and *in vivo*. Among them, 5-substituted pyrimidine nucleosides having a halogenovinyl residue, such as BV-araU BVDU, and IVDU, have been reported to be the most potent and selective antiherpes agents (1–6). They inhibit not only HSV-1 but also VZV replication in cell culture at a very low concentration, without any significant toxicity for the host cells. Especially, BV-araU is regarded as one of the most promising agents against VZV infection. The 50% inhibitory dose (ID_{50}) of BV-araU for several VZV strains including clinical isolates ranged from 0.0003 to 0.01 μM (6–8). Although BV-araU is the most potent anti-VZV agent so far examined and VZV is much more sensitive to the drug than HSV-1, the mechanism of action of BV-araU against VZV replication has not been precisely investigated. The basis for the selective action of BVDU and IVDU against HSV-1 and VZV replication resides in their high affinity for the virus-encoded TK, which is essential for the selective antiviral activity of the nucleoside analogues (9–13). Triphosphate derivatives of BVDU and IVDU are competitive inhibitors of HSV-1 and VZV DNA polymerases (14–16). Moreover, BVDU and IVDU are incorporated into DNA in HSV-1- and VZV-infected cells (9, 10, 17). The selective and strong action of BV-araU against HSV-1 also seems to depend on its high affinity for viral TK and the strongly competitive inhibition by BV-araU triphosphate of DNA polymerase (14, 18, 19).

To assess the effect of BV-araU on VZV replication, we have investigated the metabolic fate of BV-araU in VZV-infected cells and the incorporation of BV-araU into viral and cellular DNA in both TK^+ and TK^- VZV-infected cells, using ^{14}C-labeled BV-araU. In marked contrast to the results with BVDU and IVDU, it appeared that detectable incorporation of BV-araU into VZV DNA was not observed, even if TK^+ VZU-infected cells, although BV-araU was efficiently taken up and phosphorylated in the infected cells.

Materials and Methods

VZV. The parent strain (Kanno, TK^+ VZV) of VZV, which had been isolated from a patient with herpes zoster, was cloned and cultivated in HEF cells. A mutant strain (Kanno-Kohmura, TK^- VZV),

ABBREVIATIONS: BV-araU, 1-β-D-arabinofuranosyl-(*E*)-5-(2-bromovinyl)uracil; VZV, Varicella zoster virus; IVDU, (*E*)-5-(2-iodovinyl)-2′-deoxyuridine; HSV-1, Herpes simplex virus type 1; TK, thymidine kinase; HPLC, high performance liquid chromatography; HEF, human embryonic fibroblasts; BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; ACV, 9-[(2-hydroxyethoxy)methy]guanine; HSV-2, Herpes simplex virus type 2; BV-uracil; (*E*)-5-(2-bromovinyl)uracil.

which is resistant to BVDU, IVDU, and BV-araU, was isolated by repeated passage of the parent VZV strain in the presence of 5-bromodeoxyuridine and 5-iododeoxyuridine (15, 16). Cell-associated viruses, obtained from monolayers of HEF cells infected with VZV, were employed throughout the present experiments.

Chemicals. 1-β-D-Arabinofuranosyl-(*E*)-5-(2-bromo-[2-^{14}C]vinyl)-uracil (specific activity, 4.12 mCi/mmol) was synthesized from 5-formyl-arabinofuranosyluracil and ^{14}C-labeled malonic acid, as described elsewhere (20).

Inhibitory effect of BV-araU for VZV. Minimum inhibitory concentrations of compounds that reduce the number of plaques of virus to 50% of the control (ID_{50}) were examined by the procedures described previously (8).

Cellular uptake of [^{14}C]BV-araU. Confluent monolayers of HEF cells grown in Falcon tissue culture flasks (75 cm^2/flask) were infected with TK$^+$ VZV or TK$^-$ VZV (5×10^4 focus forming units/flask) or were mock-infected. Each infection was performed in triplicate, and 30 ml of the fresh growth medium were added to each flask. After 24 to 36 hr of incubation at 37°, [^{14}C]BV-araU was added to the cell cultures at the indicated concentrations. At appropriate times after the addition of [^{14}C]BV-araU, the cells were detached by gentle scraping and harvested by low speed centrifugation. The harvested cells were washed five times with phosphate-buffered saline and the radioactivity of the total cells was counted with a liquid scintillation counter.

Acid-soluble and acid-insoluble fractions. The harvested cell pellet, obtained from 10^7 cells as described above, was suspended in 0.2 ml of 0.5 N perchloric acid. After the mixture was vortexed for 1 min and allowed to stand on ice for 5 min, the supernatant was obtained by centrifugation (acid-soluble fraction). The residual pellet was washed twice with 0.2 ml of the perchloric acid solution (acid-insoluble fraction). The radioactivity of the acid-soluble and acid-insoluble fractions was determined with a liquid scintillation counter.

HPLC analysis of the acid-soluble fraction. Twenty-four hours after infection with TK$^+$ and TK$^-$ VZV or mock infection, [^{14}C]BV-araU was added, at a final concentration of 1 μM, and incubated at 37°. After 5, 10, 30, and 60 min of incubation, cells were treated with perchloric acid. The acid-soluble fractions were neutralized with a mixture of 2 N KOH and 0.5 M K_2HPO_4 and centrifuged at 4° for 10 min. The supernatants were subjected to HPLC analysis to examine the phosphorylation of BV-araU to BV-araU-5′-monophosphate, -diphosphate, and -triphosphate. HPLC was performed on a Hitachi gel No. 3013-N (strongly basic macroreticular anion exchange resin) column with a Hitachi 630-50 system. The conditions of analysis were as follows: the mobile phase was isocratic, consisting of 0.39 M NH_4Cl, 0.1 M KH_2PO_4, 0.1 M K_2HPO_4, and 10% acetonitrile in water; the flow rate was 0.5 ml/min; the temperature was 70°, and the UV detector was set at 250 nm. The employed macroreticular resin was made of styrene-divinylbenzene copolymer, to which bromovinyl residues showed higher affinity than phosphate residues. The elution times of BV-uracil, BV-araU, BV-araU monophosphate, BV-araU diphosphate, and BV-araU triphosphate were 44.66, 22.44, 11.82, 14.14, and 18.28 min, respectively. The peaks of radioactivity on HPLC were identified using the corresponding unlabeled authentic compounds, which were simultaneously injected into the column with the labeled sample in each assay.

VZV nucleocapsid and VZV DNA. VZV nucleocapsids were isolated from VZV-infected HEF cells as previously described by Martin *et al.* (21). TK$^+$ VZV DNA and TK$^-$ VZV DNA were purified from the VZV nucleocapsids by the method of Straus *et al.* (22).

Viral and cytosolic TKs. VZV TK was obtained from TK$^+$ VZV-infected HEF cells. TKs of HSV-1 and HSV-2 were obtained from HSV-1- and HSV-2-infected HeLa cells, respectively. Cytosolic TK was obtained from noninfected HEF cells. VZV, HSV-1, and HSV-2 TKs were purified by affinity chromatography with thymidine-coupled carboxyhexyl-Sepharose, as described by Kowal and Markus (23). The TK assay was performed according to the method of Cheng *et al.* (11).

VZV DNA polymerase assay. VZV DNA polymerase was isolated from HEF cells that were infected with VZV and was purified by chromatography on a phosphocellulose column, as previously described (16). The methods for the VZV DNA polymerase assay were described previously (16). Briefly, the incorporation of tritium-labeled dTTP into an acid-precipitable product was examined with activated calf thymus DNA as template. Enzymatic kinetic analysis of TK and DNA polymerase was done from the Lineweaver-Burk plots of the results.

Results

Inhibitory effect of BV-araU for TK$^+$ and TK$^-$ VZV strains. The ID_{50} values of BV-araU for TK$^+$ and TK$^-$ VZV strains were 0.0037 μM and more than 170 μM, respectively (Table 1). BV-araU was more inhibitory to VZV than any other antiherpes drugs tested.

Dose response of cellular uptake of [^{14}C]BV-araU. To measure uptake of [^{14}C]BV-araU by VZV-infected or mock-infected cells, [^{14}C]BV-araU at a wide range of concentrations (from 10^{-4} to 10^2 μM) was added to the cultures of HEF cells (1×10^7 cells) at 24 hr after virus inoculation. After an additional 12 hr of incubation, the cells were harvested, and the cell pellets were then examined for radioactivity. As shown in Table 2, the cellular uptake of [^{14}C]BV-araU by TK$^+$ VZV-infected cells was detected even at a concentration of BV-araU as low as 10^{-4} μM. The amount of [^{14}C]BV-araU taken up increased proportionally with the concentration of the drug added, although raising the drug concentration 1000-fold resulted in only a 6- to 15-fold increase in uptake. In contrast, [^{14}C]BV-araU was little taken up by mock- or TK$^-$ VZV-infected cells. Even when the concentration of [^{14}C]BV-araU was increased to 10^2 μM and the cells were treated, the amount of BV-araU taken up was limited.

Incorporation of [^{14}C]BV-araU into acid-soluble and acid-insoluble fractions and into VZV nucleocapsid and

TABLE 1

Inhibitory effects of BV-araU and several antiherpes drugs against TK$^+$ and TK$^-$ VZV replication *in vitro*

Drugs	ID_{50}	
	TK$^+$ VZV	TK$^-$ VZV
	μM	
BV-araU	0.0037	>170
ACV	2.0	>170
BDU[a]	4.2	33
BVDU	0.0068	>130
IDU[b]	3.9	49
IVDU	0.0047	>130

[a] BDU, 5-bromodeoxyuridine.
[b] IDU, 5-iododeoxyuridine.

TABLE 2

Uptake of [^{14}C]BV-araU by VZV-infected or mock-infected HEF cells

[^{14}C]BV-araU Added	Uptake of [^{14}C]BV-araU		
	TK$^+$ VZV-infected cells	TK$^-$ VZV-infected cells	Mock-infected cells
μM	cpm/10^7 cells		
10^{-4}	452	NT[a]	NT
10^{-3}	1,065	NT	NT
10^{-2}	2,010	NT	NT
10^{-1}	4,140	<300	<300
10^0	6,720	<300	<300
10^1	17,720	534	540
10^2	63,205	555	570

[a] NT, not tested.

VZV DNA. As shown in Table 3, the amount of [^{14}C]BV-araU recovered from the acid-soluble fraction of TK$^+$ VZV-infected cells increased gradually with increasing concentrations of added [^{14}C]BV-araU, as was seen in total cellular uptake. On the other hand, radioactivity in the acid-insoluble fraction was not detected when the concentration of [^{14}C]BV-araU was 0.1 μM or less. The level of BV-araU detected in the acid-insoluble fraction at concentrations of 1 to 100 μM was still very low. Less than 5% of the total radioactivity in VZV-infected cells was fractionated into the acid-insoluble fraction.

To examine whether BV-araU is utilized as an alternate substrate for VZV DNA synthesis and is incorporated into VZV DNA, nucleocapsid and VZV DNA were isolated from TK$^+$ VZV-infected, [^{14}C]BV-araU-treated cells. No radioactivity was detected from nucleocapsid or VZV DNA even when several micrograms of VZV DNA were purified from the nucleocapsids and examined (data not shown). In mock-infected HEF cells also, BV-araU was never incorporated into cellular DNA. Under the corresponding condition, in a marked contrast, it was proved that both BVDU and IVDU were incorporated into VZV DNA and cellular DNA in the infected cells (17).

TABLE 3

Incorporation of [^{14}C]BV-araU into acid-soluble and acid-insoluble factions of TK$^+$ VZV-infected HEF cells

[^{14}C]BV-araU Added	Total Uptake	Acid-Soluble Fraction		Acid-Insoluble Fraction	
μM	cpm/10^7 cells	cpm/10^7 cells	% of total	cpm/10^7 cells	% of total
10^{-4}	452	410	90	<100	
10^{-3}	1,065	950	89	<100	
10^{-2}	2,010	1,830	91	<100	
10^{-1}	4,140	3,810	92	<100	
10^{0}	6,720	6,380	95	190	2.8
10^{1}	17,720	17,270	97	720	4.1
10^{2}	63,205	61,940	98	2,850	4.5

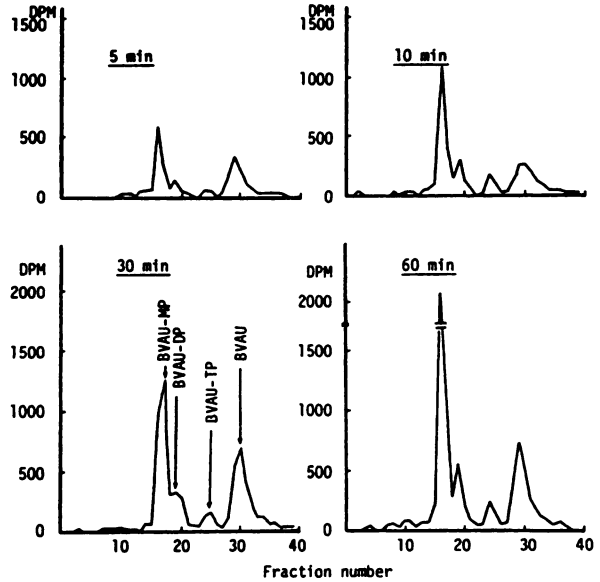

Fig. 1. HPLC analyses of the acid-soluble fractions of TK$^+$ VZV-infected cells. [^{14}C]BV-araU was added to the infected cultures 24 hr after inoculation. The acid-soluble fractions were obtained from the cells after 5, 10, 30, and 60 min of incubation with 1 μM [^{14}C]BV-araU and were analyzed by HPLC as described in Materials and Methods. *BVAU*, BV-araU; *BVAU-MP*, BV-araU monophosphate; *BVAU-DP*, BV-araU diphosphate; *BVAU-TP*, BV-araU triphosphate.

Analysis of the acid-soluble fraction by HPLC. Fig. 1 shows HPLC profiles of the acid-soluble fraction of TK$^+$ VZV-infected cells obtained at 5, 10, 30, and 60 min after exposure to 1 μM [^{14}C]BV-araU. The incorporated [^{14}C]BV-araU was proven to be immediately phosphorylated to the monophosphate derivative. The peak of BV-araU monophosphate was clearly found as early as 5 min after addition of [^{14}C]BV-araU. At that time, smaller amounts of BV-araU diphosphate and BV-araU triphosphate were also detected. BV-araU monophosphate rapidly increased with increasing exposure times until 60 min, whereas BV-araU diphosphate and BV-araU triphosphate increased gradually until 30 min, resulting in accumulation of [^{14}C]BV-araU monophosphate in the infected cells. This observation differs from the results on BVDU phosphorylation in HSV-1-infected cells, in which the phosphorylation rates of BVDU to monophosphate, to diphosphate, and to triphosphate were practically identical (24).

In contrast to the phenomenon observed in TK$^+$ VZV-infected cells, phosphorylated products of [^{14}C]BV-araU were hardly recovered from either mock-infected or TK$^-$ VZV-infected cells (Fig. 2). In particular, both BV-araU diphosphate and BV-araU triphosphate were completely absent in these cell fractions at any time tested. [^{14}C]BV-araU was the only radioactive compound found in these cells, which might be taken up nonspecifically. BV-uracil was not found in these cells, including TK$^+$ VZV-infected cells, or in the culture medium employed for incubation of TK$^+$ VZV-infected cells (data not shown). BV-araU is thought not to be degraded to BV-uracil by cellular pyrimidine phosphorylase(s).

Kinetics of TKs and VZV DNA polymerase. The kinetic studies on purified VZV, HSV-1, HSV-2, and cytosol TKs are summarized in Table 4. BV-araU, BVDU, and 5-bromodeoxyuridine showed high affinity for VZV TK. On the other hand, ACV showed the lowest affinity for VZV TK. BV-araU showed much higher affinity for VZV TK than for HSV-1 and HSV-2

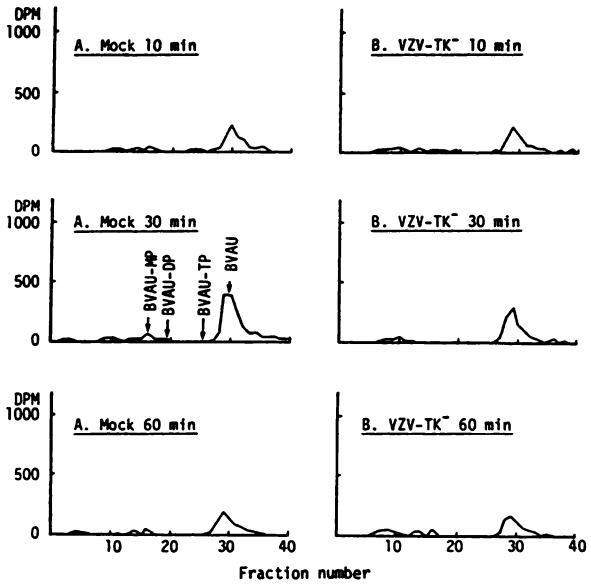

Fig. 2. HPLC analyses of the acid-soluble fractions of TK$^-$ VZV-infected (A) and mock-infected (B) cells. [^{14}C]BV-araU wad added to the infected cultures 24 hr after inoculation. The acid-soluble fractions were obtained from the cells after 10, 30, and 60 min of incubation with 1 μM [^{14}C]BV-araU and were analyzed by HPLC as described in Materials and Methods. Abbreviations as in Fig. 1.

TABLE 4

Kinetic constants (K_m) of purified VZV TK for nucleoside analogues as a substrate and K_i values of nucleoside analogues for viral TKs and cytosol TK activity

Compound	VZV TK K_m	K_i			
		VZV	HSV-1	HSV-2	Cytosol[a]
	μM	μM			
BV-araU	0.19	0.19	8	100	274
BVDU		0.08	0.2	4.1	150
IVDU	0.07[b]	0.06	0.06	0.05	
BDU[c]	0.23			0.63	
ACV		820	47	180	>1000

[a] Prepared from HEF.
[b] ^{125}I-labeled IVDU was used.
[c] BDU, 5-bromodeoxyuridine.

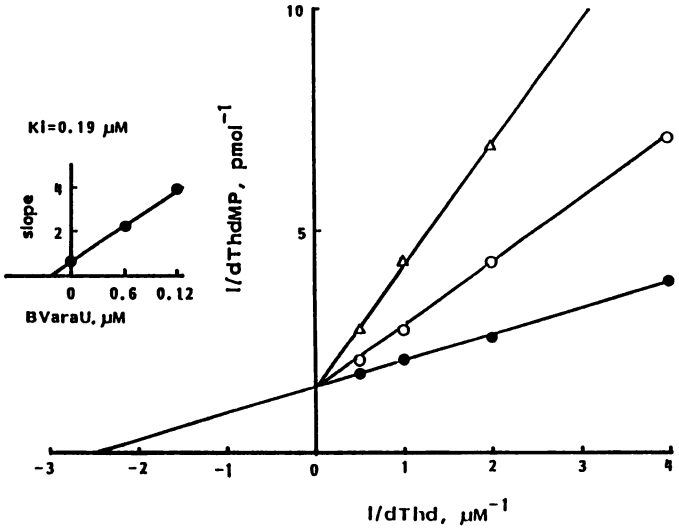

Fig. 3. Inhibitory effect of BV-araU on thymidine phosphorylation by purified VZV TK. ●, No inhibitor; ○, 0.3 μM BV-araU; △, 0.6 μM BV-araU.

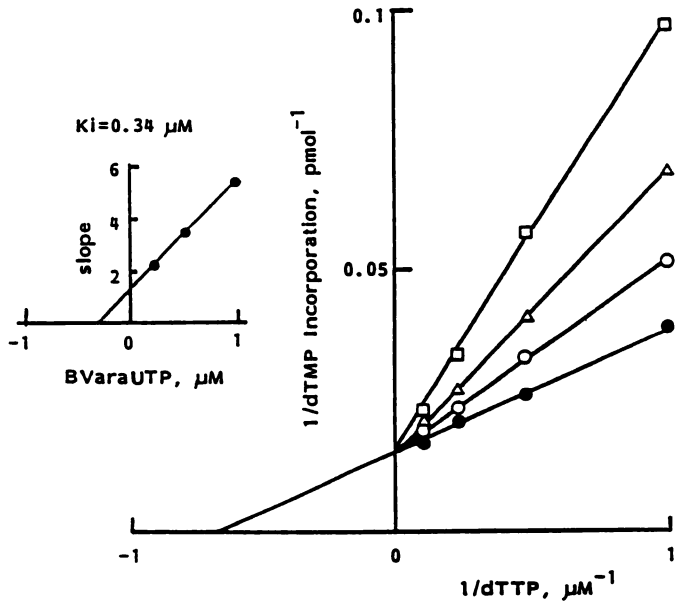

Fig. 4. Inhibitory effect of BV-araUTP on VZV DNA polymerase activity with activated calf thymus DNA as template, in the presence of different concentration of [^{3}H]dTTP. ●, No inhibitor; ○, 0.2 μM BV-araUTP; △, 0.5 μM BV-araUTP; □, 1.0 μM BV-araUTP.

TABLE 5

Kinetic constant (K_m) of VZV DNA polymerase for dTTP and K_i values of triphosphate derivatives of nucleoside analogues for the DNA polymerase activity

Compound	K_m	K_i
	μM	μM
dTTP	1.43	
BV-araU triphosphate		0.34
BVDU triphosphate		0.55
ACV triphosphate		0.29

TKs. From Lineweaver-Burk plots (Fig. 3), BV-araU acted as a competitive inhibitor in the phosphorylation of thymidine. As shown in Fig. 4, BV-araU triphosphate strongly competed with dTTP in DNA synthesis by VZV DNA polymerase. The apparent K_i value of BV-araU triphosphate for VZV DNA polymerase was 0.34 μM. This value was similar to the K_i values of BVDU triphosphate and ACV triphosphate and was much lower than the K_m value of the enzyme for dTTP as a substrate (Table 5).

Discussion

Several reports have pointed out the relationship between the incorporation of nucleoside analogues into HSV-1 or VZV DNA and the suppression of virus replication by treatment with the analogues (9, 10, 17, 25). However, Descamps *et al.* (19) found that there was no qualitative change in the densities of viral and cellular DNAs isolated from HSV-1-infected cells that were incubated with BV-araU, suggesting that BV-araU was not internally incorporated into replicating DNA in the virus-infected cells. Although BV-araU is more inhibitory to VZV than any other antiherpes nucleosides so far examined (4, 6–8), no evidence has so far been presented on whether BV-araU is incorporated into VZV DNA and/or changes the physical properties of VZV DNA.

In this study, we have demonstrated that [^{14}C]BV-araU is selectively taken up by TK$^+$ VZV-infected cells but not appreciably by mock- or TK$^-$ VZV-infected cells, even when the cells were incubated with BV-araU at a concentration 1000 times higher than the ID_{50} for VZV replication *in vitro*. This selective uptake of BV-araU in TK$^+$ VZV-infected cells is similar to the case of IVDU (17). Thus, one may infer that the marked uptake of BV-araU in VZV-infected cells depends on the presence of viral TK, as the uptake of IVDU depends on viral TK in the infected cells. In kinetic studies on purified VZV, HSV-1, HSV-2, and cytosol TKs, BV-araU showed a high affinity for VZV TK and low affinity for both HSV-2 and cytosol TKs. On the other hand, ACV showed a low affinity for VZV TK. This result is compatible with the finding of Karlström *et al.* (26). Interestingly, when we compared the K_i values of various nucleotide triphosphates for VZV DNA polymerase, both BV-araU triphosphate and ACV triphosphate showed a similarly high affinity for the enzyme. Therefore, the basis for the selective inhibition of VZV replication by BV-araU seems to mainly reside in the high affinity of BV-araU for VZV TK rather than the inhibition of DNA polymerase activity by BV-araU triphosphate.

Ayisi *et al.* (24) reported that both BV-araU and BVDU were metabolized to the triphosphate derivatives in HSV-1-infected cells, but not in HSV-2-infected cells. They have shown that the high activity of these compounds against HSV-1 and low activity against HSV-2 parallels the ability of the virus-infected

cells to produce the diphosphate and triphosphate derivatives. Recently, Suzutani *et al.* (27, 28) have confirmed this using [^{14}C]BV-araU. We also proved that BV-araU was metabolized to its mono-, di-, and triphosphates in TK$^+$ VZV-infected cells but not in TK$^-$ VZV-infected cells. This finding suggests that phosphorylation of BV-araU is catalyzed by VZV TK and is involved in the anti-VZV action of BV-araU. Accumulation of [^{14}C]BV-araU triphosphate in TK$^+$ VZV-infected cells was much higher than that of [^{14}C]BV-araU diphosphate. Probably, the phosphorylation rate of BV-araU diphosphate to BV-araU triphosphate by cellular enzyme exceeds that of BV-araU monophosphate to BV-araU diphosphate by viral TK-associated dTMP kinase (13). The conversion of BV-araU monophosphate to BV-araU diphosphate seems to be the step limiting the phosphorylation of BV-araU to BV-araU triphosphate.

We did not detect any radioactivity in VZV nucleocapsids, or in VZV DNA from the nucleocapsids, isolated from TK$^+$ VZV-infected cells that were incubated in the presence of [^{14}C] BV-araU. Moreover, the radioactivity recovered from the acid-insoluble fraction was limited. Ruth and Cheng (14) reported that BV-araU triphosphate did not act as an alternate substrate to dTTP for DNA synthesis by HSV-1 DNA polymerase but BVDU triphosphate did and that BV-araU triphosphate competed with dTTP for both HSV-1 and cellular DNA polymerase activity. Our data on the metabolism of [^{14}C]BV-araU in VZV-infected cells and on the inhibition of VZV DNA polymerase are in agreement with their view. At a marked contrast, as we previously reported, IVDU was incorporated into VZV DNA and did not appear to inhibit VZV DNA elongation (17). From our findings and those of the other researchers, it can be concluded that BV-araU is selectively incorporated and phosphorylated in VZV-infected cells and that BV-araU triphosphate formed in the infected cells strongly inhibits viral DNA synthesis without incorporation into replicating DNA strands. It should be the subject for further study whether BV-araU triphosphate acts as a chain terminator or simply as a direct inhibitor for DNA polymerase.

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