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Metabolism and Mode of Inhibition of Varicella-Zoster Virus by L-β-5-Bromovinyl-(2-hydroxymethyl)-(1,3-dioxolanyl)uracil Is Dependent on Viral Thymidine Kinase

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ABSTRACT

A nonnaturally occurring L-configuration nucleoside analog, L-β-5-bromovinyl-(2-hydroxymethyl)-1,3-(dioxolanyl)uracil (L-BVOddU) selectively inhibited varicella-zoster virus growth in human embryonic lung (HEL) 299 cell culture with an EC_{50} of 0.055 μM, whereas no inhibition of CEM and HEL 299 cell growth or mitochondrial DNA synthesis was observed at concentrations up to 200 μM. L-BVOddU was phosphorylated by viral thymidine kinase but not by human cytosolic thymidine kinase, and the antiviral activity of this compound is dependent on the viral thymidine kinase. Unlike other D-configuration bromovinyl deoxyuridine analogs, such as E-5-(2-bromovinyl)-2′-deoxyuridine and 1-β-arabinofuranosyl-E-5-(2-bromovinyl)uracil, this compound was metabolized only to its monophosphate metabolite. The di- or triphosphate metabolites were not detected. This suggested that the inhibitory mechanism may be unique and different from other anti-herpesvirus nucleoside analogs.

(E)-5-(2-bromovinyl)-2′-deoxyuridine (D-BVDU) and its derivatives are the most potent and selective compounds against herpesviruses in vitro and in vivo, especially against varicella-zoster virus (VZV). These groups of compounds have been shown to be preferential substrates for herpesvirus-induced thymidine kinases (TK) but not for human cytosolic TK (Cheng et al., 1981a,b; Zou et al., 1984; Balzarini et al., 1993). Once these compounds are phosphorylated by viral TKs, the monophosphate metabolites are subsequently converted to di- and triphosphate metabolites (Cheng et al., 1981a,b; Zou et al., 1984; Balzarini et al., 1993). It is generally believed that the triphosphate metabolites of these compounds are the active metabolites. The antiviral action of the metabolites may not be identical (Ruth and Cheng, 1981; Yokota et al., 1984, 1989; Balzarini et al., 1993). For instance, D-BVDU triphosphate is an alternative substrate for herpes simplex virus 1(HSV-1) DNA polymerase and can be incorporated into viral DNA (Ruth and Cheng, 1981; Yokota et al., 1984; Balzarini et al., 1993). However, D-BVDU monophosphate can act as an inhibitor of cellular thymidylate synthase (Ruth and Cheng, 1981; Balzarini et al., 1993). This action may facilitate the incorporation of D-BVDU triphosphate into DNA by depleting dTTP in virus-infected cells. In contrast, the antiviral action of acyclovir (ACV) was quite different in spite of the formation of ACV monophosphate by viral TKs. Among those compounds shown to have selective activity against VZV, 1-β-arabinofuranosyl-E-5-(2-bromovinyl)uracil (BV-araU) is the most potent one against VZV. It has a unique antiviral profile and has been examined for the treatment of patients with VZV infection. The viral polymerase has a high affinity for the triphosphate metabolite of this BV-araU as a substrate, and its incorporation subsequently inhibits viral DNA synthesis (Ruth and Cheng, 1981; Yokota et al., 1989).

Because naturally occurring nucleosides in mammalian cells are all in the D-configuration, it was generally thought that, given the enzymatic steps required for nucleosides to be incorporated into nucleic acid, that the nonnaturally occurring L-nucleoside counterparts might not have biological ac-

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ABBREVIATIONS: D-BVDU, (E)-5-(2-bromovinyl)-2′-deoxyuridine; VZV, varicella-zoster virus; EBV, Epstein-Barr virus; L-BVOddU, L-β-5-bromovinyl-(2-hydroxymethyl)-1,3-(dioxolanyl)uracil; L-BVDU, L-(E)-5-(2-bromovinyl)-2′-deoxyuridine; D-BVOddU, D-β-5-bromovinyl-(2-hydroxymethyl)-1,3-(dioxolanyl)uracil; BV-araU, 1-β-arabinofuranosyl-E-5-(2-bromovinyl)uracil; ACV, acyclovir; TK, thymidine kinase; HSV, herpes simplex virus; dThd, thymidine; 5′-Et dThd, 5′-ethynyl thymidine; HEL 299, human embryonic lung cell; DPD, dihydropyrimidine dehyrogenase; RT, retention time; 5-FU, 5-fluorouracil; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; BVU, bromovinyl uracil; L-BVOddUMP, L-BVOddU monophosphate; D-BVDUTP, D-BVDU triphosphate; D-BVDUMP, D-BVDU monophosphate.

tivity. This concept is no longer true since the discovery from this laboratory and other groups that some L-nucleosides have exhibited antiviral and anticancer activity (Chang et al., 1992; Chu et al., 1995; Grove et al., 1995; Lin et al., 1996, 1999; Ma et al., 1996; Dutschman et al., 1998). Compared to their D-configuration counterparts, some of the L-nucleosides have good selectivity with potent antiviral activity and low toxicity (Chang et al., 1992; Chu et al., 1995; Grove et al., 1995; Lin et al., 1996, 1999; Ma et al., 1996; Dutschman et al., 1998). L-Nucleoside analogs have been found to have unique spectra of activities against various viruses, for instance, L-2′-fluoro-5-methyl-β-L-arabinofuranosyluracil against human hepatitis B virus and Epstein-Bar virus (EBV) (Ma et al., 1996, 1997; Yao et al., 1996), L-2′,3′-dideoxy-2′,3′-didehydro-β-L(−)-5-fluorocytidine against human immunodeficiency virus and hepatitis B virus (Dutschman et al., 1998), and L-IOddU against EBV (Lin et al., 1999). L-IOddU also has shown weak activity against VZV with an EC_{50} of 17 μM.

L-β-5-Bromovinyl-(2-hydroxymethyl)-1,3-(dioxolanyl)uracil (L-BVOddU), initially synthesized by Bednarski et al. (1994), has demonstrated antiviral activity against HSV-1 but not HSV-2. In this paper, we report that L-BVOddU has selective anti-VZV activity with a potency about 80-fold greater than ACV. The action of this compound was found to be dependent on VZV TK. The metabolism of L-BVOddU is different from that of other anti-VZV nucleoside analogs.

Materials and Methods

Cells and Virus. Human embryonic lung cells (HEL 299) were grown and maintained in Eagle's minimal essential medium supplemented with 10% dialyzed fetal bovine serum at 37°C in a 5% CO_2 humidified atmosphere. VZV, Ellen strain, was obtained from American Type Culture Collection (Rockville, MD). Cell-associated virus was prepared from HEL 299 infected with VZV. The cell monolayer was infected at a MOI of 0.1 for 3 to 4 days. Then, the cell monolayer was washed with phosphate-buffered saline and detached by treatment with trypsin. The suspended cells were washed twice and stored at −80°C in complete medium containing 5% dimethyl sulfoxide.

Compounds. L-BVOddU and its analogs were synthesized in the laboratory of Dr. C.K. Chu (College of Pharmacy, University of Georgia, Athens, GA) (Choi et al., 2000). 5′-Ethynyl thymidine (5′-Et dThd) was obtained from Dr. M. Bobek (Roswell Park Memorial Institute, Buffalo, NY) (Nutter et al., 1987). ACV and D-BVDU were obtained from Sigma (St. Louis, MO).

Virus Inhibition Assay. Confluent HEL 299 cells in 12-well tissue culture plates were inoculated with the virus at 30 to ~40 plaque-forming units/well. After a 1-h adsorption at 37°C, the inoculum was replaced with the medium containing 10% dialyzed fetal bovine serum and various concentrations of compounds to be tested. Each dose was tested in duplicate. The infected cells were harvested 72 h postinfection. The cells were lysed by three cycles of "freeze-thaw," and then the lysate was treated with proteinase K for 3 h and RNase A for 30 min. The DNA was then subjected to "slot-blot" analysis as described previously (Lin et al., 1999). The viral DNA was probed with a ^{32}P-labeled DNA fragment encoding the VZV-specific TK gene. Autoradiography data were then quantitated by Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA). The same membrane was stripped and rehybridized with human [^{32}P]β-actin DNA to control for loading variation. The drug concentration, which inhibits 50% of the viral DNA synthesis compared with untreated infected control, was designated EC_{50}. The virus inhibition assay for EBV was performed as previously described (Lin et al., 1999).

TK Assay. The enzymatic assay of TK was performed as previously described (Cheng et al., 1979). Briefly, 10 μl of crude cellular extract containing about 0.15 unit/mg TK activity was mixed with 75 μl of the reaction mixture containing 100 μM [^{14}C]dThd (75 μCi/mmol), 2 mM ATP-Mg^{2+}, 12.5 mM NaF, 2 mM dithiothreitol (DTT), 4.5 mM phosphocreatine, 6 units/ml creatine kinase, and 1% bovine serum albumin in 100 mM Tris-HCl, pH 7.5. In the 5′-Et dThd inhibition assay, 5′-Et dThd at various concentrations was included in the reaction mixture. After a 60-min incubation at 37°C, 50 μl of the reaction mixture were spotted onto a 2.3-cm disc of Whatman DE 81 anion exchange paper (Whatman, Inc., Clifton, NJ). The discs were washed three times in 95% ethanol. They were then dried and placed in a vial containing 7.5 ml of scintillation liquid (American Bioanalytical, Natick, MA). A unit is defined as the amount of enzyme that converts 1 nmol of dThd to TMP per minute under the standard conditions.

Cytotoxicity. Cytotoxicity was assayed essentially as previously described (Cheng and Cheng, 1989). For HEL 299 cells, the protocol was modified. HEL 299 cells at 10^5 cells/ml in the logarithmic growth phase were treated with drugs at various concentrations. The cells were then maintained for three generations. The cell monolayer was washed twice with phosphate-buffered saline and stained with 0.5% methylene blue containing 50% ethanol. The cells were rinsed with water, and the cellular proteins were solubilized with 1% *N*-lauroylsarcosine (Sigma). The protein concentration was determined by reading the A_{595} using an automated microplate reader (Bio-Tek Instrument, Inc., Winooski, VT). The toxicity of the compound was then determined by comparing the treated cells with the untreated control.

mtDNA Content. The mtDNA was analyzed as described previously (Cheng and Cheng, 1989). Briefly, human T-lymphoid CEM cells were incubated with the drug at various concentrations for 3 days. The cells were counted and resuspended in fresh medium with drugs at the same concentration at 5×10^4 cells/ml. On day 6, the cells were harvested, and the cellular DNA was extracted by the same procedure described above. The DNA was then transferred to a membrane by using the slot-blot analysis, and the mtDNA was detected by using a ^{32}P-labeled mtDNA-specific probe. Then, the same membrane was stripped and probed with a ^{32}P-labeled *alu* DNA as a control for loading variation. The DNA was quantitated with a Personal Densitometer SI.

High-Performance Liquid Chromatography (HPLC) Analysis of L-BVOddU Nucleoside and Its Metabolites. The mock- or VZV-infected cells were detached by trypsin treatment. The cells were then lysed in buffer containing 10 mM Tris-HCl, pH 7.4, 2 mM ATP-Mg^{2+}, and 2 mM DTT. The cellular extracts were dialyzed against the same buffer. L-BVOddU at 100 μM was incubated with the extract from either the mock- or VZV-infected cells at 37°C for 0, 15, 30, 45, or 60 min. At the end of incubation, the reaction mixtures were terminated with 3 volumes of 80% ice-cold methanol and then were incubated on ice for 15 min. After the sample was centrifuged for 5 min at 12,000*g*, the supernatants were removed and dried in a Speed-Vac apparatus (Savant Instruments, Farmingdale, NY). The dried samples were then resuspended in 100 μl of water and analyzed by anion exchange HPLC using a Partisil 10/25 SAX column (Whatman) as previously described (Dutschman et al., 1998). A UV channel was set at 300 nm to monitor the metabolite of L-BVOddU based on the unique spectral absorbance. Each peak believed to be related to L-BVOddU was confirmed by its UV spectrum, and the amounts of L-BVOddU and its metabolites were normalized by comparing the total amount of ATP and ADP in each sample.

Results

Antiviral Activity of L-BVOddU in HEL Cells. The inhibitory activities of L-BVOddU, D-BVOddU, ACV, and D-BVDU against VZV growth in HEL 299 cells were examined.

The dose responses of L-BVOddU and D-BVDU are shown in Fig. 1. L-BVOddU displayed an inhibitory activity with an EC_{50} of 0.055 μM. This concentration is about 80-fold more potent than ACV (EC_{50}: 4 μM) but is comparable to D-BVDU (EC_{50}: 0.06 μM; Table 1). A concentration of 0.3 μM L-BVOddU inhibited more than 90% of the virus growth. The relative potency of antiviral activity is in the order: L-BVOddU $\approx$ D-BVDU $>$ ACV $>$ D-BVODDU $>$ L-BVDU. L-BVOddU also exhibits low cytotoxicity at high concentrations. The ID_{50} of HEL 299 or CEM cells is more than 200 μM. The cell culture selective antiviral index (the ratio of ID_{50} to EC_{50}) of L-BVOddU is greater than ACV but similar to BVDU. In addition, L-BVOddU has inhibitory activity against EBV (EC_{50}: 1.3 μM; Table 1).

Metabolism of L-BVOddU. The metabolism of L-BVOddU was studied using the protein extracts from mock- or VZV-infected cells. In the presence of ATP, incubation of 100 μM L-BVOddU with the cellular extract of mock-infected cells generated one peak corresponding to L-BVOddU with a retention time (RT) of 4.5 min in the HPLC profile (Fig. 2A). No other L-BVOddU metabolites could be detected using this methodology. In contrast, after L-BVOddU was incubated with the cellular extract of VZV-infected cells, two peaks with the L-BVOddU specific UV absorbance spectrum were detected in the HPLC profile. In addition to the nucleoside peak at RT 4.5 min, a second one appeared at RT 13.2 min. The RT of the second peak suggested that this could be L-BVOddU monophosphate (Fig. 2A). This peak was collected and treated with alkaline phosphatase. The RT of the resultant peak and its UV spectrum was consistent with L-BVOddU in the HPLC profile. Therefore, we believed that the second peak was L-BVOddUMP; however, no additional L-BVOddU metabolites were detected. Thus, L-BVOddU was phosphorylated to L-BVOddUMP by the VZV-infected cells but not by the mock-infected cells. As a control to test HPLC sensitivity, D-BVDU was subjected to the same experiment, and both D-BVDUMP and D-BVDUTP were detected by this methodology (Fig. 2A).

We further determined whether L-BVOddUMP was the only phosphorylated metabolite in VZV-infected cells. The heavily infected cells were treated with 100 μM L-BVOddU for 3 h. The intracellular nucleosides and nucleotides were extracted with 80% ice-cold methanol. The methanol-soluble fraction was dried and resuspended in 100 μL of water. The samples were then subjected to HPLC analysis as described under *Materials and Methods*. The results are shown in Fig. 2B. We did not detect any peaks with the same UV spectrum as L-BVOddU in untreated infected cells. Two peaks relating to L-BVOddU were detected in the HPLC profile from the extract of the infected cells treated with L-BVOddU. The RTs of these two peaks corresponded to those observed in the study with cell-free extracts. Similarly, we did not detect any

TABLE 1
Antiviral activity and cytotoxicity of 5-bromovinyl analogs

	ID_{50}	EC_{50}	
		VZV	EBV
	μM	μM	
L-BVOddU	>200	0.05 ± 0.03	1.1
D-BVOddU	>200	21 ± 4	>100
L-BVDU	>200	>100	N.D.
D-BVDU	>200	0.06 ± 0.007	N.D.
ACV	>200	4 ± 1.06	19

N.D., not determined; ID_{50}, drug concentration required to inhibit 50% of cell growth; EC_{50}, drug concentration that inhibits 50% of virus growth compared to the untreated control.

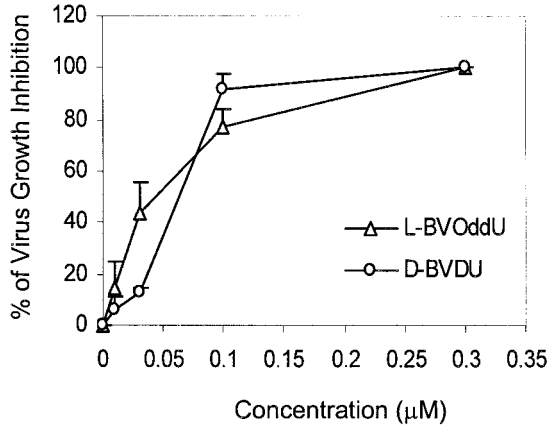

Fig. 1. Dose-response curve of L-BVOddU and BVDU. The virus growth inhibition assay was performed as described under *Materials and Methods*. The infected cells were treated with L-BVOddU (△) or BVDU (○) at various concentrations. The infected cells were harvested 72 h post-treatment. The viral DNA was detected with a ^{32}P-labeled DNA probe from the viral TK-specific gene.

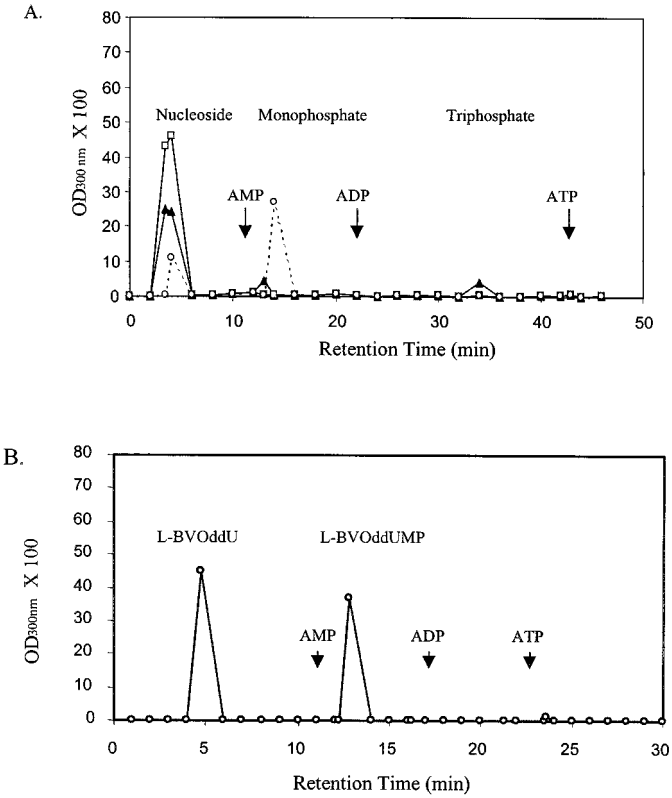

Fig. 2. HPLC chromatogram of D-BVDU and L-BVOddU and their metabolites. L-BVOddU was treated with the crude extracts from either mock-infected cells or VZV-infected cells. The nucleoside metabolites were analyzed as described under *Materials and Methods*. A, HPLC profiles of L-BVOddU and L-BVOddUMP. L-BVOddU was treated with the extracts from mock-infected cells (□) or VZV-infected cells (○). BVDU and its metabolites were used as controls (▲). B, metabolism of L-BVOddU in tissue culture. The cells heavily infected with VZV were treated with 300 μM L-BVOddU. Four hours later, the cells were harvested, and nucleoside metabolites were analyzed by HPLC as described under *Materials and Methods*. OD, absorbance.

phosphorylated derivatives other than the monophosphate metabolite, suggesting that the monophosphate form could be the active drug metabolite.

Phosphorylation of L-BVOddU by VZV TK. It is known that VZV induces a viral specific TK and this kinase has a substrate specificity different from human cytosolic TK (Cheng et al., 1979). It is conceivable that the formation of L-BVOddUMP by the cellular extract of VZV-infected cells is due to the action of VZV TK. To test this hypothesis, we included a VZV TK inhibitor in our assay.

5′-Et dThd was shown by our laboratory to be a potent inhibitor specific for HSV-1 and HSV-2 TKs (Nutter et al., 1987). Our unpublished results also suggested that it could have a potent inhibitory effect on VZV TK. 5′-Et dThd was added at various concentrations to TK assays, for which the concentration of dThd was 100 μM. The cytosolic TK and mitochondrial TK in the extract of the mock-infected cells were not sensitive to 5′-Et dThd treatment at the concentrations studied. The addition of 25 and 100 μM 5′-Et dThd reduced host TK activity by 5 and 18%, respectively (Fig. 3A), and concentrations up to 400 μM could not achieve an IC_{50} effect on host TK activity (data not shown). This is consistent with the previously published observation (Nutter et al., 1987). In contrast, VZV TK behaved differently with this compound. As shown in Fig. 3A, the total TK activity in the extract of the VZV-infected cells decreased dramatically with increased amounts of 5′-Et dThd from 1 to 10 μM. The addition of 1 and 3 μM 5′-Et dThd reduced the total TK activity by 20 and 38%, respectively, and 30 μM inhibited total TK activity by 70%. Increasing the drug concentrations to more than 30 μM did not further reduce the TK activity. The dose response from 30 to 100 μM was similar to that of the host TK, suggesting that the residual TK activity that was resistant to the drug treatment was contributed by cellular TK. The residual TK activity from the extract of the infected cells treated with 100 μM 5′-Et dThd was approximately 30% of the total TK activity of the untreated infected cells. Therefore, the host TK activity in the extracts was about 30% of the total TK activity. After the deduction of the host TK activity, 3 μM 5′-Et dThd inhibited 50% of viral TK activity, whereas 30 μM inhibited more than 90%. Based on the published VZV K_m value for dThd (Cheng et al., 1979), the K_i of VZV TK for 5′-Et dThd was calculated to be about 0.01 μM. By using this VZV specific inhibitor, we were able to study the effect of VZV TK on the metabolism of L-BVOddU.

When 30 μM 5′-Et dThd, a dose inhibiting more than 90% of the viral TK activity (Fig. 3A), was included in the reaction, the formation of L-BVOddUMP was completely blocked, even with a prolonged incubation time (Fig. 3B). The results indicated that the VZV TK was involved in the formation of L-BVOddUMP.

Behavior of L-BVOddU toward VZV TK. Reaction mixtures containing 100 μM L-BVOddU, D-BVDU, or dThd were incubated with the same amount of the extract from VZV-infected cells at various times. The phosphorylated products of these nucleosides were determined by HPLC. The conversion of dThd into TMP, TDP, and TTP was observed with HPLC, and the formation of these phosphorylated metabolites was time dependent (Fig. 4). Similar results were obtained with D-BVDU with the major products of D-BVDUMP and D-BVDUTP. The lack of D-BVDUDP suggests a rapid and efficient conversion to D-BVDUTP (Fig. 4). In contrast, only the formation of L-BVOddUMP was detected. After the contribution by cellular TK was deducted, we found that the phosphorylation of L-BVOddU by viral TK was more than 9-fold greater than that of dThd and D-BVDU. No putative L-BVOddUDP and L-BVOddUTP could be detected even when the cellular extract was supplemented with additional recombinant human thymidylate kinase, uridylate/cytidylate kinase, and nucleoside diphosphate kinase (data not shown).

The binding affinity of L-BVOddU to viral TK was estimated by determining its ability to inhibit viral TK activity in VZV-infected cells. For comparison, D-BVOddU, D-BVDU, and L-BVDU were also included in this study. K_i values of these compounds for VZV TK were calculated based on the previously published K_m value (0.4 μM) of dThd for VZV TK and the Michaelis-Menten equation (Cheng et al., 1979). Table 2 shows that L-BVOddU and D-BVOddU have similar K_i values (125 and 78 μM) that are much higher than that for D-BVDU and L-BVDU.

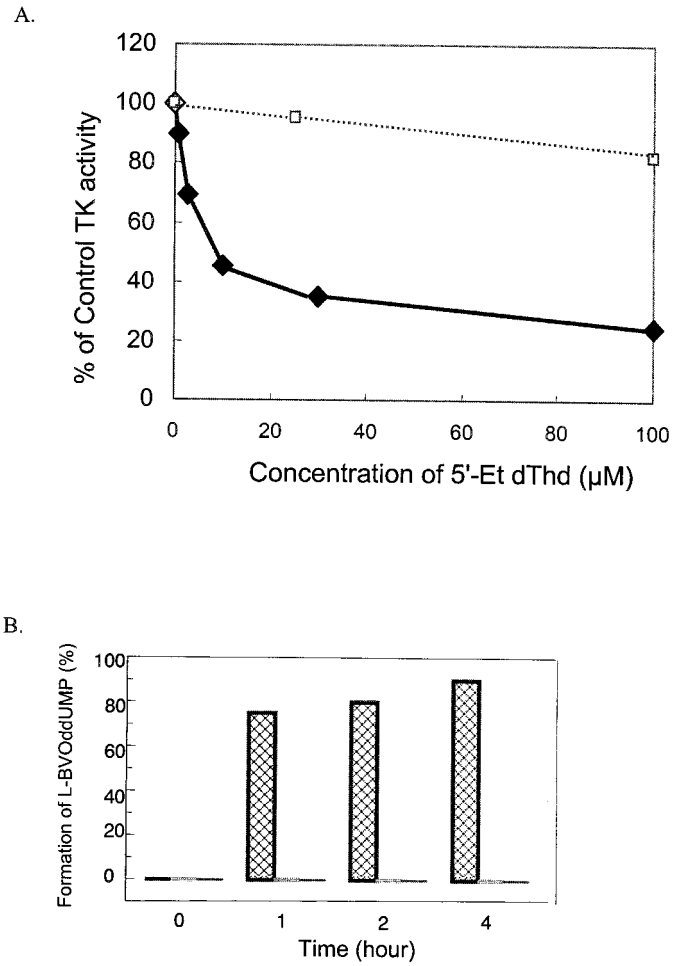

Fig. 3. The inhibitory activity of 5′Et dThd on VZV TK activity. A, mock- or VZV-infected cells were lysed with the buffer containing 50 mM Tris-HCl, pH 7.4, 2 mM ATP-$MgCl_2$, and 2 mM DTT. The TK activity in the crude extracts was examined as described under *Materials and Methods*. 5′-Et dThd at various concentrations was included in each reaction. The control TK activity had no 5′-Et dThd treatment. ♦, VZV infection; □, mock infection. B, formation of L-BVOddUMP in the presence or absence of 5′-Et dThd. L-BVOddU was treated with crude extracts from mock infection (open columns), VZV infection (cross-hatched columns), or VZV infection with addition of 30 μM 5′-Et dThd (slashed columns). The reaction times were 0, 1, 2, and 4 h.

Dependence of the Antiviral Action of L-BVOddU on VZV TK. The role of viral TK on the action of L-BVOddU against VZV growth was assessed by measuring the effect of the viral TK inhibitor, 5′-Et dThd, on the anti-VZV activity of L-BVOddU. The results are depicted in Fig. 5. 5′-Et dThd, which has little effect on VZV virus growth up to 1 μM (Fig. 5), could prevent the antiviral action of L-BVOddU in a dose-dependent manner. More than 95% of virus growth was inhibited by 0.3 μM L-BVOddU alone (Fig. 1). The addition of 0.1 and 3 μM 5′Et dThd resulted in the loss of 20 and 80% of antiviral activity of L-BVOddU, respectively. This suggests the important role of viral TK in mediating the antiviral action of L-BVOddU. A similar result was observed when 1 μM L-BVOddU was coincubated with 5′-Et dThd, except that a higher amount of 5′-Et dThd was required to achieve similar prevention of L-BVOddU antiviral activity.

Discussion

L-BVOddU is the first L-nucleoside that shows potent anti-VZV activity in cell culture. The antiviral spectrum is different from other biologically active L-nucleosides. L-BVOddU is more potent than D-BVOddU, whereas D-BVDU is more potent than L-BVDU. Because they contain the same base and sugar, L-configuration nucleosides are not always more potent than their D counterparts. Our studies have demonstrated that L-BVOddU is converted into its phosphorylated form by only VZV TK and the anti-VZV activity of L-BVOddU is VZV TK dependent. This finding is consistent with the findings that bromovinyl nucleoside analogs, such as BVDU and BV-araU, are preferential substrates of the viral TK (Cheng et al., 1981b).

L-BVOddU has a K_i value (125 μM) that is about 250- and 1400-fold greater than dThd (K_i: 0.4 μM) (Cheng et al., 1979) and D-BVDU (K_i: 0.09 μM), respectively, suggesting that it has a low affinity for VZV TK and, therefore, is a very weak inhibitor. As an alternative substrate of VZV TK, L-BVOddU can be phosphorylated by VZV TK into its monophosphate metabolite. Because both K_m and K_i often reflect the enzyme substrate-binding affinity, we assume that K_m and K_i values of each compound for VZV TK are almost equal if K_{cat} is insignificant. The relative V_{max} of L-BVOddU, D-BVOddU, and D-BVDU compared with dThd for VZV TK was, therefore, calculated to be 22, 2.78, and 0.84, respectively. The estimated relative V_{max}/K_m or K_i of these three compounds to dThd is 0.18, 0.04, and 9.3, respectively. Thus, the phosphorylation efficiency of L-BVOddU and D-BVOddU with VZV TK is about 7 and 1% of that of dThd, whereas the efficiency of D-BVDU is 4-fold greater than dThd (Table 2). Although L-BVOddU has a low affinity for VZV TK, it may have a high V_{max} so that the efficiency for the enzyme may be improved.

BVDU and BV-araU monophosphate can be further phosphorylated to their di- and triphosphate metabolites in the virus-infected cells (Yokota et al., 1984, 1987; Ayisi et al., 1987). Previous studies have demonstrated that the cells transfected with VZV TK were susceptible to the inhibition by BVDU and BV-araU, suggesting that cellular proteins could be involved in the formation of the di- and triphosphate (Yokota et al., 1984, 1987). BV-araUTP has strong affinity to viral DNA polymerase, suggesting that its antiviral action could be due to the inhibition of viral DNA polymerase (Ruth and Cheng, 1981). The antiviral action of BVDU resides not only in its triphosphate as a competitive inhibitor of viral

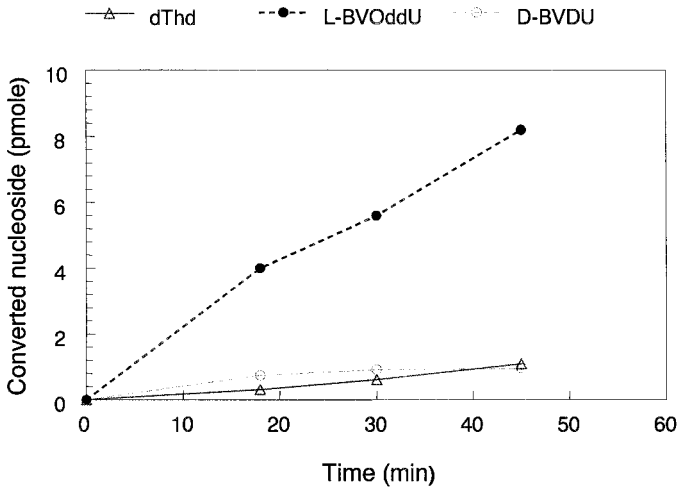

Fig. 4. Time-dependent formation of L-BVOddU, D-BVDU, and dThd metabolites by VZV TK. L-BVOddU, D-BVDU, or dThd were incubated with the extract from VZV-infected cells for 0, 15, 30, and 45 min in the presence or absence of 30 μM 5′-Et dThd. 5′-Et dThd was included to determine the host TK activity. The host TK activity for dThd was then deducted. The products were analyzed as described under *Materials and Methods*. The mono, di, and triphosphate metabolites were totaled, and the conversion rate of each nucleoside was calculated.

TABLE 2
The K_i of bromovinyl analogs for VZV TK and relative velocity to dThd

	K_i	Relative Velocity to dThd[a]
	μM	
L-BVOddU	125 ± 20	9.8 ± 2.7
D-BVOddU	78 ± 30	1.56 ± 0.71
L-BVDU	1.34 ± 0.47	N.D.
D-BVDU	0.09 ± 0.03	0.9 ± 0.09
dThd	0.4[b]	1.0

[a] In assay, the concentration of nucleosides used was 100 μM, and ATP-Mg was 2 mM. N.D., not determined.
[b] K_m value of dThd for VZV TK is adopted from the paper by Cheng et al. (1979).

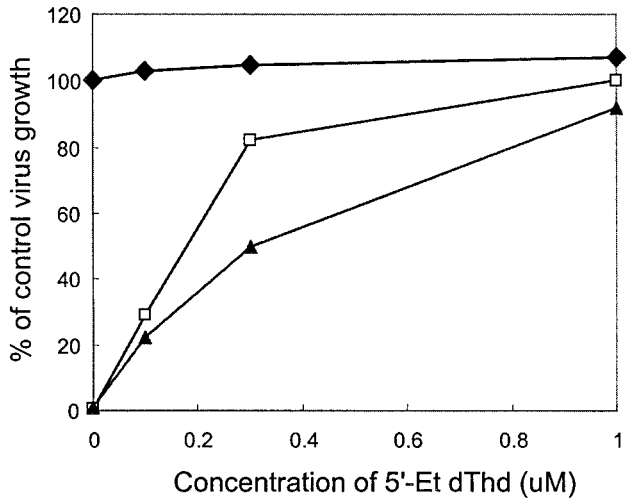

Fig. 5. The effect of 5′-Et dThd on the anti-VZV activity of L-BVOddU. The VZV-infected cells were treated with 5′-Et dThd in the absence or presence of L-BVOddU. The virus growth was determined as described under *Materials and Methods*. The cells were treated with 5′-Et dThd only (♦), 5′-Et dThd combined with 0.3 μM L-BVOddU (□), or 5′-Et dThd combined with 1 μM L-BVOddU (▲).

polymerase but also in its monophosphate metabolite serving as an inhibitor of thymidylate synthase (Ruth and Cheng, 1981; Yokota et al., 1984; Balzarini et al., 1993). In contrast to these two compounds, L-BVOddU is not further converted to the di- and triphosphate. Both in vivo and in vitro studies have shown that only L-BVOddUMP was detected. This suggests that L-BVOddUMP may be the active drug metabolite.

BVDU was the first bromovinyl analog to show anti-VZV activity (De Clercq et al., 1979, 1980). The good affinity for mitochondrial deoxypyrimidine kinase and the degradation by pyrimidine phosphorylase limits its usage in clinical treatment (Cheng et al., 1981b; Desgranges et al., 1983). The most potent BVDU derivative is BV-araU, which is more than 1000 times more potent against VZV than ACV in vitro (Machida and Sakata, 1984). It also has a favorable toxicity profile and is already in clinical trial in the United States (Gnann et al., 1998). The major side effect of this drug is that the BV-araU can be cleaved by intestinal mucosa flora to produce bromovinyl uracil (BVU; Nakayama et al., 1997). BVU is a strong inhibitor of dihydropyrimidine dehyrogenase (DPD). DPD is an enzyme involved in the degradation of 5-fluorouracil (5-FU), a commonly used anticancer drug. Therefore, if taken together with 5-FU during anticancer chemotherapy, BV-araU can cause toxic accumulation of 5-FU and result in death (Diasio, 1998). Because most enzymes are stereospecific with respect to their substrates, with the non-natural L-configuration, L-BVOddU may have a toxicity profile different from D-configuration analogs. We examined the metabolites in vitro and did not detect any cleavage of L-BVOddU into BVU by mammalian DPD (data not shown). This may give L-BVOddU great clinical potential and a special advantage when used in combination with cancer chemotherapy agents like 5-FU. It will be of interest to examine the metabolism of L-BVOddU in vivo.

In summary, we have discovered an L-configuration nucleoside analog, L-BVOddU, that shows potent inhibitory activity against VZV growth in vitro. The biological activity of this compound requires VZV TK activity. The active metabolite may be the L-BVOddU monophosphate.

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