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ACCELERATED COMMUNICATION

Specific Recognition of the Bicyclic Pyrimidine Nucleoside Analogs, a New Class of Highly Potent and Selective Inhibitors of Varicella-Zoster Virus (VZV), by the VZV-Encoded Thymidine Kinase

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ABSTRACT

Recently, an entirely new class of bicyclic nucleoside analogs (BCNAs) was found to display exquisite potency and selectivity as inhibitors of varicella-zoster virus (VZV) replication in cell culture. A striking difference in their ability to convert the BCNAs to their phosphorylated derivatives was observed between the VZV-encoded thymidine kinase (TK) and the very closely related herpes simplex virus type 1 (HSV-1) TK. Whereas VZV TK efficiently phosphorylated the BCNAs, HSV-1 TK was unable to do so. In addition, the thymidylate (dTMP) kinase activity of VZV TK further converted BCNA-5′-MP to BCNA-5′-DP. The BCNAs (or their phosphorylated derivatives) were not a substrate for cytosolic TK, mitochondrial TK, or cytosolic dTMP kinase. Human erythrocyte nucleoside diphosphate (NDP) kinase was unable to phosphorylate the BCNA 5′-diphosphates to BCNA 5′-triphosphates. Under the same experimental conditions, the anti-herpetic (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) derivative was efficiently converted to BVDU-MP and BVDU-DP by both VZV TK and HSV-1 TK and further, into BVDU-TP, by NDP kinase. Our observations may account for the unprecedented specificity of BCNAs as anti-VZV agents.

The activity of many known antiherpetic purine nucleoside analogs such as 9-(2-hydroxyethoxymethyl)guanine (acyclovir, ACV) (Elion et al., 1977) depends on specific phosphorylation by thymidine kinase (TK) encoded by herpes simplex virus type 1 (HSV-1), HSV-2, and/or varicella-zoster virus (VZV). Cellular kinases convert the resulting monophosphate to the corresponding di- and triphosphates. The nucleoside triphosphate derivatives are responsible for the antiherpetic activity by interacting with the viral DNA polymerase as alternative substrates and/or as competitive inhibitors of the natural substrates for the virus-encoded DNA polymerase. Thus, the selectivity of antiherpetic purine nucleoside analogs can be attributed at least in part to their preferential phosphorylation by herpes virus-encoded pyrimidine deoxynucleoside kinases (Cheng, 1977; Cheng et al., 1981a,b; De Clercq, 1984). It is noteworthy that the anti-VZV activity of many purine nucleoside analogs, including ACV, is suboptimal because of their relatively low affinity for VZV TK. Among the pyrimidine nucleoside analogs, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) is the best known antiherpetic agent (De Clercq et al., 1979). In contrast with the purine nucleoside analogs, BVDU also requires the thymidylate (dTMP) kinase activity of HSV-1 or VZV TK for the second phosphorylation step (conversion of BVDU-MP to BVDU-DP) (Cheng et al., 1979; De Clercq, 1997). This increases the antiviral specificity of BVDU and explains its highly potent and selective activity against HSV-1 and VZV and virtual

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ABBREVIATIONS: ACV, acyclovir; TK, thymidine kinase; VZV, varicella-zoster virus; HSV, herpes simplex virus; BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; DP, diphosphate; MP, monophosphate; NDP, nucleoside diphosphate; BCNA, bicyclic nucleoside analog; GST, glutathione-*S*-transferase; HPLC, high-performance liquid chromatography; ACN, acetonitril; BVOddU, L-β-5-bromovinyl-(2-hydroxymethyl)-(1,3-dioxolanyl)uracil; RR, ribonucleotide reductase; HEL, human erythroleukemia; TK-1, human cytosolic thymidine kinase.

inactivity against HSV-2, the TK from which is unable to carry out the second phosphorylation step.

Recently, an entirely new class of bicyclic nucleoside analogs (BCNAs) (Fig. 1) was found to display exquisite potency and selectivity as anti-VZV agents (McGuigan et al., 1999, 2000a,b). These 2′-deoxynucleoside analogs with an unusual fluorescent bicyclic base bearing a long alkyl or *p*-alkylphenyl side chain do not inhibit the closely related HSV-1 and HSV-2, or any other DNA or RNA virus in cell culture. This property gives these compounds a unique and unprecedented selectivity spectrum. Recently, side-chain modifications [i.e., terminal halogen substitution (Brancale et al., 2000), terminal unsaturation (Srinivasan et al., 2001), and pyrro and thieno substitutions (McGuigan et al., 2000c, 2001)] were conducted to clarify the role of the hydrophobic side chain and further optimize the lead compounds. Cf 1743, bearing a *p*-pentylphenyl as the side chain, has been identified as the most active anti-VZV compound (EC_{50} = 0.0003 μM) in cell culture and is at least 3000 times more active against VZV in cell culture than is the clinically used ACV (EC_{50} = 1 μM) (McGuigan et al., 2000b).

In this study, we demonstrated that the BCNAs are selectively phosphorylated to their 5′-diphosphates by the two successive enzyme activities of VZV TK (thymidine kinase and thymidylate kinase), whereas they are not recognized by the TK/dTMP kinase activity of HSV-1 under similar experimental conditions. In this respect, the BCNAs act strikingly different from BVDU, which is efficiently recognized by both VZV- and HSV-1–associated TK and dTMP kinase activity. These observations may provide an explanation for the unprecedented specificity of the BCNAs for VZV.

Materials and Methods

Compounds. BVDU was originally obtained from Searle (High Wycombe, UK), and BVDU-MP was provided by P. Herdewijn (Rega Institute for Medical Research, Leuven, Belgium).

The BCNAs were synthesized as described previously (McGuigan et al., 1999, 2000b). The synthesis of the 5′-monophosphates of Cf 1368 and Cf 1369, which were called Cf 1928 and Cf 1602, respectively (Fig. 1), was carried out using the procedure described by Yoshikawa et al. (1969). The nucleoside analogs were treated with phosphorus oxychloride using triethyl phosphate as the solvent for 5 h at 0°C. The reaction was terminated by adding a solution of 0.4 M ammonium bicarbonate, followed by the evaporation of the solvent under vacuum. After purification on a silica column and elution with propane-2-ol/water/aqueous ammonia (9:2:1), the compounds were characterized by the use of ^{31}P NMR, ^{1}H NMR, and mass spectrometry. Full synthetic details will be published elsewhere.

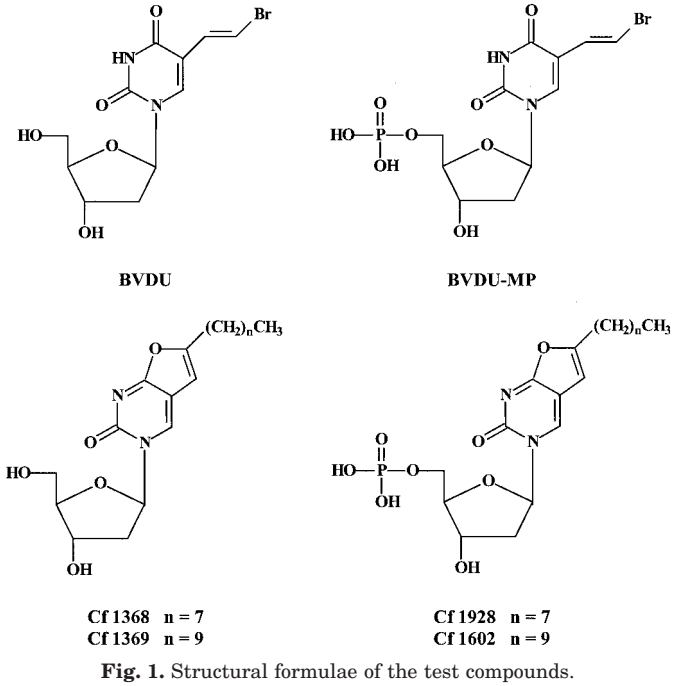

Fig. 1. Structural formulae of the test compounds.

Radiochemicals. The radiolabeled nucleoside [CH_3-^{3}H]dThd (specific radioactivity, 52 Ci/mmol) was obtained from the Radiochemical Center (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Enzymes. Human cytosolic thymidine kinase (TK-1) was obtained and purified from CEM cell cultures. Human mitochondrial TK-2 was expressed recombinantly and purified according to procedures described previously (Johansson and Karlsson, 1997). HSV-1 TK, HSV-2 TK, and VZV TK were expressed in *Escherichia coli* as glutathione-*S*-transferase (GST) fusion proteins, according to the following procedure. The HSV-1 TK coding sequence was amplified by PCR using primers 5′-GAGGAATTCATGGCTTCGTACCCCGGCCATC (a) and 5′-CTCGTCGACTC AGTTAGCCTCCCCCATCTCC (b) (Kebo Lab, Stockholm, Sweden) with the pMCTK plasmid (from D. Ayusawa, Yokohama University, Yokohama, Japan) as a template, introducing an *Eco*RI (a) and *Sal*I (b) site. The HSV-2 TK coding sequence was amplified using 5′-GAGGAATTCATGGCTTCTCACGCCGGCCAAC (a) and 5′-CTCGTCGACAG AACTCCCCCCACCTCGCGGGC (b) with the pGR18 plasmid (from D. Ayusawa) as a template. The VZV TK coding sequence was amplified using primers 5′-GAGGAATTCATGTCAACGGATAAAACCGATG (a) and 5′-CTCGTCGACA GGGAAGTGTTGTCCTGAACGGC (b) (Invitrogen, Carlsbad, CA) with the pRc/CMV/VZV TK plasmid (from J. Piette, University of Liège, Liège, Belgium) as a template. The resulting sequences were ligated between the *Eco*RI and *Sal*I sites of the pGEX-5X-1 vector (Amersham Biosciences) after subcloning in the pGEM-T vector (Promega, Madison, WI), and transformed into *E. coli* BL21 (DE3)pLysS. Bacteria were grown overnight in 2YT medium [sterile 1.6% (w/v) Bacto-tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.0] containing ampicillin (100 μg/ml) and chloramphenicol (40 μg/ml) and then diluted in fresh medium. After further growth of the bacteria at 27°C for 1 h, isopropyl-β-D-thiogalactopyranoside (Sigma, St. Louis, MO) was added to a final concentration of 0.1 mM to induce the production of the GST TK fusion proteins. After 15 h of further growth at 27°C, cells were formed into pellets (7700*g* for 10 min at 4°C) and resuspended in lysis buffer (50 mM Tris, pH 7.5, 1 mM dithiothreitol, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and 0.15 mg/ml lysozyme) (Fetzer et al., 1994). Bacterial suspensions were sonicated (on ice) and ultracentrifuged (20,000*g* for 15 min at 4°C). GST TK was purified from the supernatant using Gluthatione Sepharose 4B (Amersham Biosciences AB) according to the manufacturer's instructions. Protein content of the purified fractions was assessed using Bradford reagent (Sigma).

Enzyme Reactions. Thymidine and thymidylate kinase assays to evaluate the test compounds as a substrate for the enzyme were performed as follows: the standard reaction mixture contained 50 mM Tris HCl, pH 8, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 2.5 mM ATP, 10 mM NaF, 10 μl of Milli-Q water, test compound at various concentrations dissolved in dimethyl sulfoxide, and 5 μl of an appropriate amount (1 μg) of protein HSV-1 TK or VZV TK in a total reaction mixture of 50 μl. The reaction mixture was incubated at 37°C for 40 min, and the reaction was terminated by transferring the contents into 150 μl of ice-cold methanol, which was followed after 10 min by centrifugation at 12,000*g*. The resulting samples were injected on an HPLC (Waters, Milford, MA) to separate and quantify the 5′-mono- and 5′-diphosphates of the BCNAs. In one experiment,

1 unit of NDP kinase from human erythrocytes (Sigma; 1 unit converts 1 μmol of ADP to ATP per minute at 25°C) was added to the VZV thymidine kinase assay with Cf 1368 as the substrate to determine the formation of BCNA triphosphate.

The IC_{50} of the test compounds against phosphorylation of [CH_3-^{3}H]dThd as the natural substrate by HSV-1 TK or VZV TK was determined under the following reaction conditions: the standard reaction mixture (50 μl) contained 50 mM Tris-HCl, pH 8.0, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 2.5 mM ATP, 10 mM NaF, 1.0 mg/ml bovine serum albumin, 1 μM [CH_3-^{3}H]dThd (0.1 μCi), an appropriate amount of test compound, and 5 μl of Milli-Q water. The reaction was started by the addition of enzyme and then incubated at 37°C for 30 min, and the reaction was terminated by spotting an aliquot of 45 μl onto DE-81 discs (Whatman, Maidstone, England). After 15 min, the discs were washed 3 times for 5 min each in 1 mM $HCOONH_4$ while shaking, followed by 5 min in ethanol (70%). Finally, the filters were dried and assayed for radioactivity in a toluene-based scintillant. The IC_{50} was defined as the drug concentration required to inhibit thymidine phosphorylation by 50%.

HPLC Analysis. HPLC analysis to separate and quantify the hydrophilic (phosphorylated) reaction products was performed on a Whatman Partisphere SAX ion-exchange column (4.6 ×125 mm) using the following gradient (flow = 2 ml/min): 5 min at 5 mM $(NH_4)H_2PO_4$ (Acros Organics, Fairlawn, NJ), pH 5; 15-min linear gradient to 0.3 M $(NH_4)H_2PO_4$, pH 5; 20 min at 0.3 M $(NH_4)H_2PO_4$, pH 5; 5-min linear gradient to 5 mM $(NH_4)H_2PO_4$, pH 5; and 5-min equilibration under the same buffer conditions. Metabolites of BVDU and BVDU-MP were detected by UV spectrometry at 293 nm. HPLC analysis to separate and quantitate the lipophilic reaction products was performed on a Merck LiChroCART 125–4 RP select B (5 μm) (Merck Darmstadt, Germany) using the following gradient (flow = 1 ml/min); 2 min at 98% NaH_2PO_4 (Acros) 50 mM + heptanesulfonic acid 5 mM, pH 3.2 (buffer) (Sigma) and 2% acetonitril (ACN) (Rathburn, Walkerburn, Scotland); 6-min linear gradient to 80% buffer and 20% ACN; 2-min linear gradient to 75% buffer and 25% ACN; 2-min linear gradient to 65% buffer and 35% ACN; 8-min linear gradient to 50% buffer and 50% ACN; 10-min isocratic flow; 50-min linear gradient to 98% buffer and 2% ACN; and 5-min equilibration at the same conditions. Metabolites of the BCNAs were determined by fluorescence detection (excitation at 340 nm and emission at 415 nm).

Results

To examine the anti-VZV specificity of the BCNAs, we investigated two prototype BCNAs (designated Cf 1368 and Cf 1369) for their substrate activities against purified VZV and HSV-1 TK (Table 1). First, we incubated the TKs with 100 μM and 500 μM BCNAs for 10 and 40 min. The antiherpetic drug BVDU was included as a reference compound. VZV TK markedly converted the BCNAs to their 5′-monophosphates (86 and 93% for Cf 1368 and Cf 1369, respectively) within 10 min (Table 1). Traces (1–2%) of BCNA 5′-diphosphate formation were already observed after a 10-min incubation. After a 40-min incubation, conversion of the BCNAs to the phosphorylated derivatives was virtually complete (91–100%), and BCNA 5′-diphosphates (derived from the BCNA 5′-monophosphates) could be clearly detected in the reaction mixtures. Under similar experimental conditions, 72% of BVDU was phosphorylated after 40-min exposure to VZV TK, with half of it being in the BVDU-DP form (Table 1). At the higher drug concentrations (500 μM), the enzyme converted more than 90% of both BCNAs, indicating that VZV TK-catalyzed BCNA phosphorylation was still clearly lower than its V_{max} value. Millimolar drug concentrations could not be reproducibly evaluated because of solubility limitations of the compound in the reaction mixture. Instead, the amount of BVDU conversion to BVDU-MP leveled off considerably at 500 μM, indicating that the V_{max} for BVDU-MP formation was virtually reached at 500 μM. When a variety of BVDU concentrations were examined, the kinetic values for BVDU phosphorylation derived from Lineweaver-Burk plots were 78 μM (K_m) and 630 pmol/h (V_{max}).

When HSV-1 TK was used as the phosphorylating enzyme, no trace of BCNA phosphorylation was observed (Table 1). Under similar experimental conditions, BVDU was even more efficiently converted to its BVDU-MP derivative (K_m = 50 μM, V_{max} = 529 pmol/h) by HSV-1 TK than by VZV TK. Thus, VZV TK, but not HSV-1 TK, was able to selectively recognize the BCNAs as an efficient substrate under experimental conditions in which BVDU was markedly phosphorylated by both enzymes.

The dTMP kinase activity of VZV TK that efficiently converted BVDU-MP to BVDU-DP also converted the BCNA 5′-monophosphates to their corresponding 5′-diphosphates, although to a lesser initial extent (Fig. 2). Indeed, when various concentrations (between 100 and 2000 to 2500 μM) of the BCNA 5′-monophosphates Cf 1928 and Cf 1602 were exposed to VZV TK as a potential substrate for the thymidylate kinase activity of VZV TK, Cf 1928 and Cf 1602 were further phosphorylated in a concentration-dependent and linear manner up to 2000 to 2500 μM. Because it was tech-

TABLE 1

Percentage conversion of BCNAs and BVDU to their phosphorylated derivatives by VZV TK and HSV-1 TK

Peak identification of the monophosphate and diphosphate metabolites on the chromatograms was done by comparison with known standards.

Compound	Concentration	Incubation time	Conversion					
			VZV TK			HSV-1 TK		
			MP	DP	Total phosphorylated metabolites	MP	DP	Total phosphorylated metabolites
	μM	*min*			%			
Cf 1368	100	10	85	1	86	0	0	0
		40	79	12	91	0	0	0
	500	10	68	1	69	0	0	0
		40	87	6	93	0	0	0
Cf 1369	100	10	91	2	93	0	0	0
		40	76	24	100	0	0	0
	500	10	49	0.5	49	0	0	0
		40	77	7	84	0	0	0
BVDU	100	40	37	35	72	93	0	93
	500	40	9	11	20	14	0	14

nically difficult to evaluate higher drug concentrations and because the phosphorylation reaction still proceeded linearly at the highest compound concentrations, the V_{max} values were estimated to be much higher than 1500 and 5000 pmol/h for Cf 1928 and Cf 1602, respectively. Obviously, the K_m values that could not be calculated correctly must be in the millimolar range. In contrast, the kinetic values of BVDU-MP for VZV TK were 444 pmol/h (V_{max}) and 65 μM (K_m).

When the BCNA 5′-monophosphates were evaluated as a substrate for the dTMP kinase activity of HSV-1 TK, no trace of conversion to the 5′-diphosphates could be observed within the concentration range between 100 and 500 μM. In contrast, BVDU-MP was easily converted by HSV-1 TK to BVDU-DP with a K_m value of 150 μM and a V_{max} of 396 pmol/h. Thus, the dTMP kinase activity of VZV TK efficiently recognized the BCNA-5′-monophosphates as a substrate for further phosphorylation to the 5′-diphosphate, whereas the corresponding dTMP kinase activity of HSV-1 TK did not.

We also evaluated whether the BCNA 5′-diphosphates were further converted to their 5′-triphosphates by NDP kinase. Therefore, 1 unit of (human erythrocyte) NDP kinase was added to the reaction mixture containing 100 μM of the BCNAs Cf 1368 and VZV TK. BVDU was included as a control. Whereas 76% of BVDU-DP was further converted to BVDU-TP in the reaction mixture at which NDP kinase was added, no Cf 1368 5′-triphosphate could be detected. It was concluded, therefore, that BCNA 5′-diphosphate has very little or no substrate affinity for human NDP kinase.

To reveal whether the BCNAs may act as nonsubstrate inhibitors of HSV TK, cellular cytosolic TK-1, or mitochondrial TK-2, the BCNAs Cf 1368 and Cf 1369 were examined for their inhibitory activity against HSV-1 TK-, HSV-2 TK-, TK-1–, and TK-2–catalyzed phosphorylation of 1 μM [CH_3-^{3}H]dThd. At their highest concentrations tested (i.e., 500 μM for TK-1 and TK-2 and 5000 μM for HSV-1 TK and HSV-2 TK), the compounds did not affect [CH_3-^{3}H]dThd phosphorylation. In contrast, Cf 1368 displayed an IC_{50} of 37 $\pm$ 2.0 μM against [CH_3-^{3}H]dThd phosphorylation by VZV TK. No complete inhibition of VZV TK at the highest drug concentrations was observed because of drug insolubility. A similar observation was made for Cf 1369. Therefore, it was concluded that the BCNAs do not act as nonsubstrate inhibitors of any of the nucleoside kinases tested.

Discussion

Recently, an entirely new class of nucleoside analogs with an unusual fluorescent bicyclic pyrimidine base bearing a long alkyl or *p*-alkylphenyl side chain (McGuigan et al., 1999, 2000a, b) was found to have a unique antiviral activity spectrum. Members of this new class of compounds solely inhibit VZV replication and not any other DNA or RNA virus, including the closely related HSV-1 and HSV-2. Previous stud-

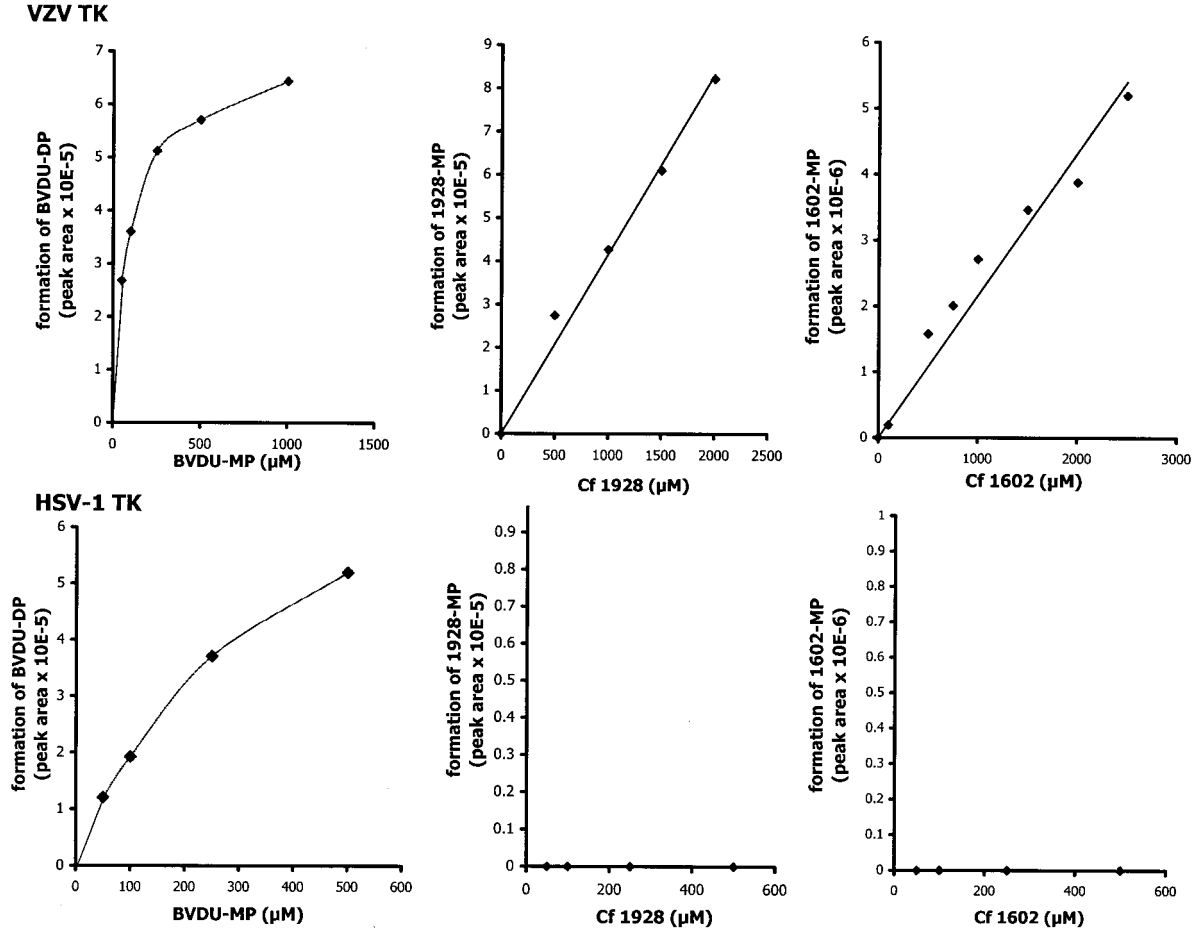

Fig. 2. Conversion of BVDU-MP, Cf 1928, and Cf 1602 by VZV TK versus HSV-1 TK

ies (McGuigan et al., 1999, 2000a) have shown that VZV strains deficient in the virus-encoded TK gene lose their sensitivity for the BCNAs, suggesting an instrumental role of this enzyme for metabolic activation of the BCNAs.

We have now demonstrated that the BCNAs are efficiently recognized by VZV TK, but not by HSV-1 TK, as a substrate for phosphorylation. Such stringent discrimination of substrate specificity between HSV-1 TK and VZV TK is unprecedented. In this respect, the BCNAs differ strikingly from BVDU, which is recognized as an efficient substrate by both VZV and HSV-1 TK enzymes to a virtually similar extent.

We also found that human cytosolic TK-1 and mitochondrial TK-2 do not recognize the BCNAs either as a substrate for phosphorylation (data not shown) or as potential nonsubstrate inhibitors of these enzymes. Thus, phosphorylation (activation) of the BCNAs by VZV TK (but not HSV-1 TK or any other cellular TK) provides a rational basis for explaining the selectivity and specificity of these compounds for VZV. A second level of selectivity may be represented by the VZV TK-associated dTMP kinase. When BCNAs were exposed to VZV TK, 5′-diphosphate formation could always be detected after prior formation of the 5′-monophosphate. Moreover, we were also able to show that two BCNA monophosphates that had been synthesized could be efficiently converted to their 5′-diphosphate by VZV TK- but not HSV-1 TK-associated dTMP kinase. Human cellular thymidylate kinase did not convert the BCNA 5′-monophosphates to their corresponding 5′-diphosphates under the experimental conditions in which dTMP was entirely converted to dTDP (B. Degrève and Y.-C. Cheng, personal communication).

Our phosphorylation data for both the TK and dTMP kinase activity of the VZV-encoded enzyme revealed that the BCNAs had a rather low affinity for VZV TK but high V_{max} (saturation of substrate conversion was not reached at 500-2000 μM). This means that the phosphorylation capacity (V_{max}/K_m) of the enzyme for the BCNAs must still be pronounced. The lack of recognition of the BCNA monophosphates by cellular dTMP kinase further adds to their selectivity as anti-VZV agents and low toxicity to normal uninfected cells.

Although we provided an explanation for the unique anti-VZV specificity of the BCNAs, how the BCNAs may exert anti-VZV activity in cell culture remains unclear. Purified human erythrocyte NDP kinase was unable to convert the BCNA diphosphates to their triphosphate derivatives under the experimental conditions in which BVDU diphosphate was readily converted to its antivirally active metabolite BVDU-TP. The inability to show conversion of BCNA 5′-diphosphate to 5′-triphosphate by purified NDP kinase does not necessarily mean that the compound cannot be converted to its 5′-triphosphate derivative in intact cells, because a variety of other (less known or less obvious) enzymes may recognize the BCNA 5′-diphosphates as a substrate. However, the inability of NDP kinase to convert the BCNA 5′-diphosphate to BCNA-5′-triphosphate is in agreement with our preliminary metabolic data that HEL cells infected with VZV could metabolize Cf 1368 to the corresponding 5′-mono- and 5′-diphosphates, whereas no traces of the 5′-triphosphate could be detected in the extracts of the BCNA-exposed VZV-infected HEL cells.

Also, Cheng and collaborators recently described a novel anti-VZV drug [designated BVOddU or L-β-5-bromovinyl-(2-hydroxymethyl)-(1,3-dioxolanyl)uracil (Bednarski et al., 1994; Li et al., 2000)] that inhibited VZV replication in HEL cells at approximately 55 nM and that was selectively phosphorylated by VZV TK and not by human cytosolic TK in cell-free assays. Unlike BVDU and BVaraU, this compound was metabolized only to its 5′-monophosphate metabolite by VZV TK, and no 5′-di- or 5′-triphosphates were detected. The authors concluded that the inhibitory mechanism of this compound may be unique and different from that of other anti-herpes virus nucleoside analogs (Li et al., 2000). It is interesting to explore whether the BCNAs and BVOddU may be endowed with a similar mechanism of antiviral action.

It may seem unlikely that the BCNAs would be targeted at the VZV DNA polymerase, because no BCNA triphosphate formation could be detected in our enzymatic assays and cell systems. If the compounds are indeed not converted to their 5′-triphosphates in intact cells, their antivirally active metabolite may well be the 5′-monophosphate [as also suggested by Li et al. (2000) for BVOddU], the 5′-diphosphate, or yet another metabolite that we have so far failed to observe.

The observed anti-VZV activity through specific inhibition of VZV-encoded ribonucleotide reductase (RR) cannot be ruled out, considering that the natural substrates of this enzyme are ribonucleoside diphosphates. Although herpes virus RR is not required for virus replication in exponentially growing cells, it has been shown that it is necessary in virus-infected nondividing cells (Duan et al., 1998) as well as for full expression of pathogenicity in animal models (Brandt et al., 1991). Although the viral and cellular RRs share many similarities, their primary amino acid sequences are markedly different. Therefore, viral RR can be considered a target for antiviral therapy. Recently, peptidomimetic inhibitors of HSV RR (i.e., BILD 1633 SE) have been described that are endowed with a more potent antiviral activity than acyclovir

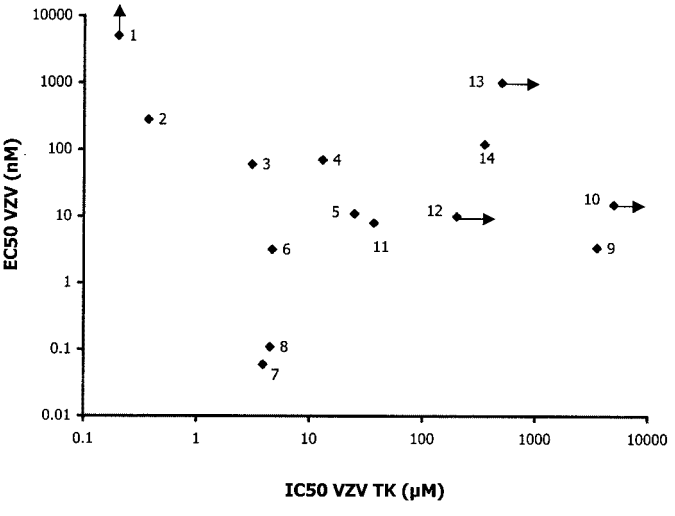

Fig. 3. Correlation of anti-VZV activity (EC_{50}) and affinity for VZV TK (IC_{50}) for a variety of BCNAs. Plot for anti-VZV activity (EC_{50}) as a function of the inhibition effects (IC_{50}) of a variety of BCNAs on VZV TK phosphorylation of thymidine (1 μM). Numbers 1 to 10 are BCNAs with a *p*-substituted (halogen or alkyl)phenyl as side chain (1, 4-F-phenyl; 2, phenyl; 3, 4-methyl-phenyl; 4, 4-ethyl-phenyl; 5, 4-propyl-phenyl; 6, 4-butyl-phenyl; 7, 4-phentyl-phenyl; 8, 4-hexyl-phenyl; 9, 4-heptyl-phenyl; and 10, 4-octyl-phenyl). Numbers 11 to 14 (see also Fig. 1), carrying an aliphatic side chain, correspond to Cf 1368 (number 11), Cf 1369 (number 12), and their corresponding 5′-monophosphate derivatives Cf 1928 (number 13) and Cf 1602 (number 14). The EC_{50} values for VZV TK were taken from Balzarini and McGuigan (2001).

and are able to suppress ACV-resistant HSV mutants (Duan et al., 1998). Also, some thiocarbonohydrazones (i.e., A11104) have been described that were found to be potent inactivators of the RR encoded by HSV-1, HSV-2, and VZV, but much weaker inactivators of human RR (Spector et al., 1989). Therefore, VZV RR is now under investigation in our laboratories as a possible target for the BCNAs.

The unmetabolized BCNA itself is unlikely to be the active form of the drug, because it was shown that TK-deficient VZV strains lose their sensitivity to the BCNAs. Also, we found no correlation between the anti-VZV activity of a variety of *p*-alkylphenyl-substituted BCNAs and their affinity for VZV TK (Fig. 3), indicating that the eventual antiviral target for the BCNAs has a different structure-affinity relationship than VZV TK. The search for the antiviral target of the BCNAs is therefore of high priority in our laboratory.

In conclusion, the anti-VZV specificity of the novel class of BCNAs was found to reside in a specific recognition of the BCNAs by the VZV TK and dTMP kinase activity, whereas the compounds were not acting as a substrate for HSV-1 TK and dTMP kinase, cytosolic TK-1, mitochondrial TK-2, or cytosolic dTMP kinase.

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