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# VARICELLA-ZOSTER VIRUS THYMIDINE KINASE

## CHARACTERIZATION AND SUBSTRATE SPECIFICITY

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**Abstract**—The varicella-zoster virus (VZV) thymidine kinase (TK) (EC 2.7.2.21) catalyzes the phosphorylation of many anti-VZV nucleosides. Purified, bacterially expressed VZV TK was characterized with regard to N-terminal amino acid sequence, pI value, pH optimum, metal ion requirement, phosphate donor and acceptor specificity, and inhibition by dTTP. Initial velocities of thymidine phosphorylation with variable MgATP concentrations fit a two-site model with apparent $K_m$ values for MgATP of 0.10 and 900 $\mu$M. dTTP was a noncompetitive inhibitor of thymidine phosphorylation but was competitive with MgATP. Phosphate donor and acceptor specificities of the bacterially expressed enzyme were indistinguishable from those of VZV TK purified from infected cells. Detailed studies of the nucleoside specificity with the bacterially expressed enzyme showed that, for a given sugar moiety, thymine nucleosides were the most efficient substrates followed by nucleosides of cytosine, uracil, adenine, and with some exceptions, guanine. For a given pyrimidine or purine (except guanine), 2′-deoxyribonucleosides were the most efficient substrates, followed by arabinosides, ribonucleosides, 2′,3′-dideoxyribonucleosides, and the acyclic moiety of acyclovir.

*Key words:* varicella-zoster virus, thymidine kinase

Varicella-zoster virus (VZV)§, a member of the herpesvirus family, encodes a thymidine kinase (TK) which is responsible for the activation of several antiviral nucleoside analogs [1–3]. The VZV TK shares significant amino acid sequence homology in the proposed substrate binding regions with the TK from herpes simplex virus (HSV) types 1 and 2 [4]. All three enzymes catalyze the phosphorylation of a broad range of nucleoside analogs [5–9]. The human TK does not share these features, and the substrate specificity differences among the human and viral kinases have been the basis for the development of selective antiviral agents [10].

While the thymidine kinases from the herpesvirus family share many structural and biochemical features, important differences exist in the substrate specificities of these enzymes. For example, 6-methoxypurine arabinoside is a good substrate for VZV TK but not for HSV TK [11]. In contrast, acyclovir is readily phosphorylated by HSV TK but poorly phosphorylated by VZV TK. Inhibition of viral replication *in vitro* by nucleoside analogs reflects these substrate specificity differences. Because of these differences, one cannot extrapolate information from substrate specificity studies with HSV TK to VZV TK. We have expressed VZV TK in *Escherichia coli*, and have purified the enzyme [9] in quantities sufficient to conduct general characterization and extensive substrate specificity studies. The bacterially expressed VZV TK was indistinguishable from viral TK from VZV-infected cells. These substrate specificity data may help explain the selective activity or toxicity of various nucleoside analogs and aid in the design of novel antiviral agents.

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§ Abbreviations: VZV, varicella-zoster virus; TK, thymidine kinase; HSV, herpes simplex virus; dThd, thymidine; $K_i'$, apparent $K_i$; $K_m'$, apparent $K_m$; rel $V_{max}$, relative maximal velocity; BVdU (*E*)-5-(2-bromovinyl)-2′-dUrd; and BVaraU, (*E*)-5-(2-bromovinyl)uracil arabinoside.

### MATERIALS AND METHODS

#### *Nucleoside analogs*

Sources of nucleoside analogs as shown in Tables 2–4 are as follows: Compound No. **5**, **14**, **22–28**, **30**, **31**, **33–37**, **42**, **43**, **46**, **50**, **52**, **53**, **55**, **56**, **58**, **60–65**, **67**, **69**, and **71–81** were synthesized at Burroughs Wellcome, Research Triangle Park, NC. Compound No. **4**, **6**, **7**, **11**, **12**, **15**, **16**, **18**, **19**, **21**, and **45** were synthesized at Wellcome Research Laboratories, Beckenham, U.K. Compound No. **38** was provided by Dr. R. T. Walker of the University of Birmingham, U.K. Compound No. **17** was provided by Dr. Kirk Field of Bristol-Myers Squibb, Princeton, NJ. Compound No. **39–41** were provided by Dr. Y. Fulmer Shealy of Southern Research Institute, Birmingham, AL. All other nucleoside analogs were from commercial sources. Nucleoside purity was assessed by reverse-phase chromatography (Pep RPC 5/5, Pharmacia, Uppsala, Sweden) with a 0–50% methanol gradient in 2 mM Tris–HCl, pH 7.5. Compounds were purified by this procedure if solutions were less than 99% homogeneous.

*Enzyme preparation and assays*

The TK gene from VZV was expressed in *E. coli* and the viral TK was purified as previously described [9]. Enzyme homogeneity, based on densitometry scans of Coomassie-stained SDS gels, was greater than 95% [9]. Native VZV TK was purified from WI-38 cells infected with Ellen strain VZV [12]. Thymidine (dThd), ATP and NaCl were removed from purified enzyme by extensive dialysis against Tris Buffer A [20 mM Tris–HCl, (pH 7.5), 1 mM dithiothreitol, 10% glycerol (v/v)] or by size exclusion chromatography (P-4, Bio-Rad, Richmond, CA). Standard assay mixtures contained 100 $\mu$M [2-$^{14}$C]-dThd (56 Ci/mol; Moravek Biochemicals, Brea, CA), 5 mM $MgCl_2$, 5 mM ATP, 100 mM Tris–HCl, (pH 7.5), 0.5 mM dithiothreitol, 5% glycerol (v/v), 0.5 mg/mL bovine serum albumin ("essentially fatty acid free"; Sigma, St. Louis, MO) and purified enzyme. Reaction mixtures in which MgATP was the variable substrate contained 260 $\mu$M free $Mg^{2+}$. Under these conditions, the concentration of uncomplexed ATP was less than 8% of the total MgATP concentration based on MgATP dissociation constants [13]. Initial velocities, based on three to five time points, were determined at 37° by the DEAE paper assay as described [9]. Rates were proportional to enzyme concentration. One microgram of enzyme catalyzed the phosphorylation of 2720 pmol dThd/min under standard conditions.

*Metal ion studies*

All reagents used in divalent metal ion studies and MgATP kinetics studies were treated with Chelex® 100 (Bio-Rad Laboratories) to remove trace metal ions. The VZV TK was dialyzed against Chelex 100 in Tris Buffer A (5 g Chelex 100 per 100 mL buffer) overnight at 4°. All stock solutions, except those of metal ions, were treated with Chelex 100 (0.05 g Chelex 100/mL stock solution) and mixed for at least 1 hr at 4°. Prior to use, aliquots of centrifuged stock solutions were transferred to vials rinsed with Chelex-treated water. Solutions of divalent metal ions were prepared with deionized water that had been passed through a Chelex 100 column (2.5 × 18 cm). All enzyme reactions were in plastic microcentrifuge tubes that had been rinsed with deionized water and Chelex-treated water.

*Phosphate acceptor specificity studies*

Relative substrate velocities for nonradioactive nucleoside analogs were determined by the phosphate transfer assay at 1 mM test compound and 1 mM Mg[$\gamma$-$^{32}$P]ATP (150 Ci/mol; New England Nuclear, Boston, MA) as described previously [11]. Initial estimates of apparent $K_i$ ($K_i'$) values were based on reactions with a single concentration of inhibitor and 0.16 $\mu$M [5-$CH_3$-$^3$H]dThd (53 Ci/mmol; Moravek Biochemicals) and were calculated from fractional inhibitions ($i = 1 - v_i/v_o$) and the equation $K_i' = ([I](1 - i))/(i(1 + [S]/K_m'))$, where $K_m'$ (apparent $K_m$) for dThd = 0.16 $\mu$M. Reported $K_i'$ values were determined by several methods: (i) $K_i'$ values were determined as described previously [9] for all 5-substituted pyrimidine deoxynucleosides and arabinosides (see Table 2) and thymine nucleosides with various sugar substitutions (see Table 3). Briefly, initial velocities of [$^3$H]dThd phosphorylation (six concentrations) were determined in the absence or presence of three concentrations of inhibitor. $K_i'$ values were calculated from weighted least-squares fits of the equation $V = (V_{max})([S])/(K_m'(1 + ([I]/K_i')) + [S])$ to the data [14], and the inhibition data were analyzed for conformity to competitive or noncompetitive models [15]. All compounds tested by this method demonstrated linear competitive inhibition. (ii) Alternatively, initial velocities at 0.16 $\mu$M [$^3$H]dThd were determined with and without five concentrations of inhibitor for compounds with relative reaction velocities greater than 5% of that with dThd or with low estimated $K_i'$ values. Data were analyzed by nonlinear least-squares fits of the equation, $V = (V_{max})([S])/(K_m'(1 + ([I]/K_i')) + [S])$ [16]. (iii) Finally, if the relative reaction velocity was less than 5% of that with dThd, and if the estimated $K_i'$ value was greater than 250 $\mu$M, the $K_i'$ estimate was used for the $K_i'$ value.

The $K_i'$ value is equal to the $K_m'$ value for alternate substrates that are linear competitive inhibitors [17, 18]. $K_i'$ values were therefore used to calculate relative maximal velocity (rel $V_{max}$) and efficiency values (rel $V_{max}/K_m'$) for those compounds that were detectably phosphorylated and were linear competitive inhibitors of dThd phosphorylation.

$K_m'$ values for [$^3$H]dThd and [2-$^{14}$C]2′-deoxycytidine (56 Ci/mol; Moravek Biochemicals) were determined as described previously [9].

*Other methods*

N-Terminal amino acid analysis of the purified, bacterially expressed VZV TK was performed by William Chestnut (Wellcome Research Laboratories) with an Applied Biosystems model 477a protein sequencer using Edman degradation chemistry with an on-line amino acid analyzer (Applied Biosystems, Foster City, CA). The pI value of the purified bacterially expressed enzyme was determined by isoelectric focusing gel electrophoresis (pH 3–9) (PhastGel, Pharmacia, Uppsala, Sweden). The MacVector sequence analysis software program (IBI, New Haven, CT) was used to predict amino acid composition from the VZV TK DNA sequence [19] and to calculate the theoretical pI value for the protein.

## RESULTS

*General characterization of the bacterially expressed VZV TK*

*Comparison of phosphate acceptor specificity between bacterially expressed and native VZV TK.* To demonstrate that the bacterially expressed enzyme was indistinguishable from native VZV TK, relative substrate velocities for 31 compounds were determined with VZV TK purified from the bacterial expression system and from VZV-infected (Ellen strain) WI-38 cells (Fig. 1). A range of substrates was selected to include high and low velocity substrates and to include purine and pyrimidine nucleosides with substitutions on the base and sugar moieties. When rates with each enzyme were normalized to the rate of 6-methoxypurine ara-

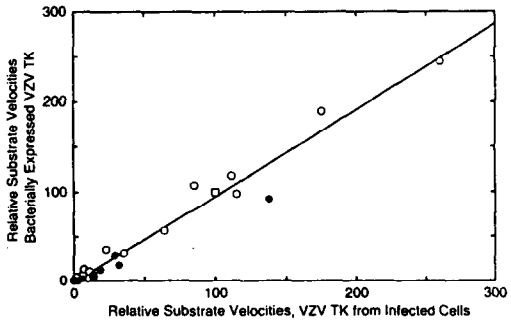



Fig. 1. Relative substrate velocities with native and bacterially expressed VZV TK. Initial phosphorylation velocities with 1 mM nucleoside and 1 mM [$\gamma$-$^{32}$P]ATP were determined by a silica gel TLC method [11]. A variety of pyrimidine (●) and purine (○) nucleosides were tested with enzyme from both sources, and the velocities were normalized to the velocity with 6-methoxypurine arabinoside (□), a selective substrate of the VZV TK.

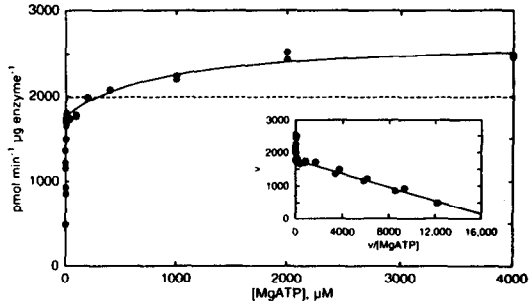



Fig. 2. dThd phosphorylation by VZV TK as a function of MgATP concentration. Reactions were conducted under standard conditions (see Materials and Methods), except the mixtures contained 10 $\mu$M [$^3$H]dThd, 0.04 $\mu$M to 4 mM MgATP, and 60 pg of purified enzyme (total volume = 60 $\mu$l). Curves represent least-squares fits of hyperbolae to the data for a two-site model (—),

$$v = \frac{V_{m1} \cdot [\text{MgATP}]}{[\text{MgATP}] + K_{m1}} + \frac{V_{m2} \cdot [\text{MgATP}]}{[\text{MgATP}] + K_{m2}}$$

or the Michaelis–Menten model (- - -),

$$v = \frac{V_m \cdot [\text{MgATP}]}{[\text{MgATP}] + K_m}$$

binoside (compound No. 77, see Table 4), a selective substrate of the VZV TK [11], relative velocities with the bacterially expressed enzyme were 0.95 ± 0.03 (SEM of the slope) times that of the corresponding relative velocities with enzyme from VZV-infected cells (correlation coefficient = 0.984, P value = 0.0001).

*N-Terminal amino acid sequence and pI determination.* The sequence of the first 15 amino acids was consistent with that predicted from the DNA sequence of the VZV TK gene [19], confirming expression had occurred in the correct reading frame and that no N-terminal blocking (formylation) or heterogeneity was evident. The enzyme focused between pH 6.7 and 7.0 on an isoelectric focusing gel, in good agreement with the predicted pI value of 6.88.*

*pH Optimum.* Thymidine phosphorylation rates at various pH values were determined under standard reaction conditions with Hepes buffer pH 5.8 to pH 7.4 or Tris buffer pH 6.7 to pH 8.8 (pH values were corrected for 37°). The pH affected reaction velocities only minimally over the range tested; maximum activity occurred at approximately pH 7.5 with a 10% decrease in activity at the extremes. These results were consistent with previously published data with extracts from infected cells [20].

*Metal ion requirements.* Partial TK activity (35% of control) remained when $MgCl_2$ was omitted from the reaction mixture. When enzyme and stock solutions were treated with Chelex 100, residual activity was reduced to 1% of control. Seventy-five percent of control activity was restored when $MgCl_2$ was added back to control levels (5 mM). These experiments indicated that there had been significant metal ion contamination, although the identity of the contaminating metal ion(s) was not determined. These results also demonstrated that a divalent metal ion was required for catalytic activity. Of the divalent metal ions tested individually at 5 mM (see Materials and Methods), $Mn^{2+}$, $Co^{2+}$, and $Fe^{2+}$ supported the TK-catalyzed reaction at 54, 49, and 45% of the rate seen with $Mg^{2+}$, respectively. $Zn^{2+}$ supported even less activity (only 29%), while $Ca^{2+}$ and $Cu^{2+}$ did not support the reaction.

*MgATP studies.* At constant MgATP concentrations (10 and 100 $\mu$M), excess $Mg^{2+}$ stimulated dThd phosphorylation slightly, whereas excess free ATP was slightly inhibitory (data not shown). Therefore, all reactions with variable MgATP contained 260 $\mu$M free $Mg^{2+}$. Under these conditions, the calculated concentration of free ATP was less than 8% of the total MgATP in the reactions.

Initial velocities of [$^3$H]dThd phosphorylation (10 $\mu$M) with variable MgATP concentrations fit a biphasic, two-site model [16, 21], in which $K_m'$ values for MgATP were 0.10 ± 0.004 (SEM) $\mu$M and 900 ± 300 $\mu$M (Fig. 2). The nonlinear plot of $v$ vs $v$/[MgATP] (Fig. 2 insert) could be explained by the presence of two nonequivalent MgATP sites, by negative cooperativity between the MgATP-binding sites, or by heterogeneous enzyme [17]. Because the VZV TK is probably a dimer [22], the MgATP sites on the two sub-units may behave differently. Enzyme heterogeneity due to contamination by bacterial enzymes was unlikely since we observed similar biphasic kinetics with the VZV TK purified from infected WI-38 cells (data not shown).

* When partially purified (Mono Q-purified, ~80% homogeneous), bacterially expressed VZV TK was applied to a chromatofocusing, Mono P HR 5/20 column (Pharmacia), activity was eluted between pH 5.8 and 5.9, below the predicted pI and the value obtained by isoelectric focusing.

Table 1. Nucleoside triphosphates as phosphate donors or inhibitors of VZV TK

| Nucleotide (2 mM) | As phosphate donor* (% of ATP reaction) | As inhibitor† |
|---|---|---|
| ATP | 100 | 100 |
| GTP | 60 | 90 |
| CTP | 81 | 97 |
| UTP | 91 | 105 |
| dATP | 89 | 90 |
| dGTP | 51 | 96 |
| dCTP | 72 | 95 |
| dTTP | 0 | 7 |

* Reactions were conducted under standard conditions with bacterially expressed enzymes, except that 5 mM MgATP was replaced by the indicated Mg-nucleoside triphosphate (2 mM).

† Mg-nucleoside triphosphate (2 mM) was added to standard reactions containing 2 mM MgATP.

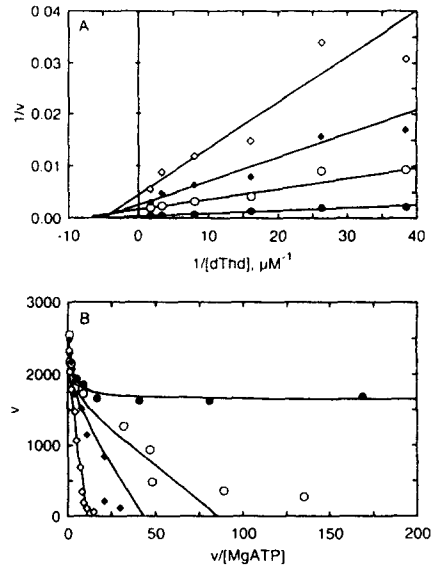



Fig. 3. Inhibition of VZV TK-catalyzed dThd phosphorylation by dTTP. Standard conditions were used (total volume = 60 μl) except as noted. Units for velocities are pmol product $min^{-1}$ μg $enzyme^{-1}$. (A) dThd as the variable substrate: Reaction mixtures contained 0.026 to 0.58 μM [$^3$H]dThd, 2 mM MgATP, 0 (●), 10 (○), 20 (◆), or 50 (◇) μM MgdTTP and 46 pg enzyme. Data were analyzed by least-squares fits of hyperbolae to the data for the noncompetitive model: $v = V_m \cdot [dThd]/(K_m \cdot (1 + [dTTP]/K_{is}) + [dThd] \cdot (1 + [dTTP]/K_{ii}))$. (B) MgATP as the variable substrate: Reaction mixtures contained 0.04 to 4,000 μM MgATP, 10 μM [$^3$H]dThd, 0 (●), 0.2 (○), 0.4 (◆), or 1.6 (◇) μM MgdTTP and 65 pg enzyme. Data were analyzed by least-squares fits of hyperbolae to the data for a two-site model in which dTTP was a competitive inhibitor at both MgATP sites;

$$v = \frac{V_{m1} \cdot [MgATP]}{[MgATP] + K_{m1} + ([dTTP] \cdot K_{m1}/K_{i1})} + \frac{V_{m2} \cdot [MgATP]}{[MgATP] + K_{m2} + ([dTTP] \cdot K_{m2}/K_{i2})}$$

*Substrate and inhibitor specificity of bacterially expressed VZV TK*

*Nucleoside triphosphates (NTP) as phosphate donors or inhibitors.* All MgNTP tested except MgdTTP were phosphate donors in the TK reaction (Table 1). In the presence of 2 mM MgATP, only MgdTTP was significantly inhibitory. These results are in agreement with those published with the VZV TK purified from infected cells [5].

Inhibition of [$^3$H]dThd phosphorylation by MgdTTP fit a noncompetitive model [14] when dThd was the variable substrate and MgATP was 2 mM ($K_{ii}$ = 3.0 μM, $K_{is}$ = 3.5 μM) (Fig. 3A). However, when dThd was maintained at 10 μM, and MgATP was varied, the data fit a two-system model [21] in which MgdTTP competitively inhibited the reaction at both MgATP sites to yield $K_i'$ values for MgdTTP of 1.0 ± 0.14 nM and 4 ± 3 μM (Fig. 3B).

*Phosphate acceptor specificity.*

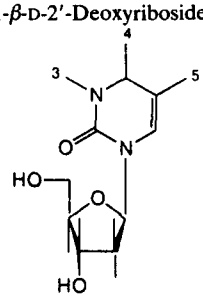
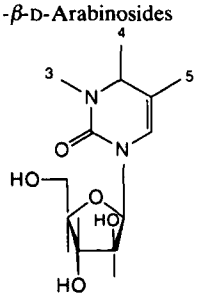
*Pyrimidine ring substitutions (Table 2).* In the 2′-deoxynucleoside series, the 5-alkyl-substituted uracil nucleosides (No. **1**, **4**, **6** and **7**) were efficient substrates of the VZV TK. Removal of the 5-alkyl group (No. **2**) resulted in an 80-fold increase in the $K_m'$ value relative to dThd (No. **1**) but no change in rel $V_{max}$. Halo (No. **3**) and halo-alkyl groups (No. **5**) at the 5-position were also well tolerated. (*E*)-5-(2-Bromovinyl)-2′-dUrd (BVdU, No. **5**) was the most efficient substrate tested. Although 5-F, -Cl, or -Br deoxyuridines have not been tested, preliminary evidence indicates that nucleoside analogs with larger halogen groups (Br and I) are better substrates than those with the smaller groups (F and Cl) (unpublished data). Methylation at the 3-position (No. **8**) resulted in a high velocity, high $K_m'$ substrate.

2′-Deoxycytidine analogs (No. **9–12**) were also high velocity, high $K_m'$ substrates. $K_m'$ values for the 5-alkyl-2′-deoxycytidines were significantly higher than the corresponding 5-alkyl-2′-deoxyuridine compounds. In contrast, the $K_m'$ value for 2′-deoxycytidine (No. **9**) was lower than the $K_m'$ for 2′-deoxyuridine (No. **2**).

Similar effects of substitutions at the 4- and 5-positions were observed with the pyrimidine arabinosides (No. **13–22**). However, for a given substitution, the rel $V_{max}$ was lower, and the $K_m'$ was higher, than that of the corresponding 2′-deoxynucleoside. Exceptions to this trend were (*E*)-5-(2-bromovinyl)uracil arabinoside (BVaraU, No. **17**), which had a very low $K_m'$ value and was the second most efficient substrate tested, and 5-(propynyl)cytosine arabinoside (No. **21**) which had a higher rel $V_{max}$ than the corresponding 2′-deoxynucleoside (No. **12**). Methylation at $N^4$ of cytosine arabinoside (No. **22**) increased the $K_m'$ value 5-fold.

*Thymine nucleoside sugar substitutions.* In the thymine series (Table 3), the 2′-deoxyribonucleoside (No. **1**) was the most efficient substrate. Modifications at the 3′-position, such as substitutions (No. **23** and **24**), extensions (No. **25** and **31**), removal of the 3′-

Table 2. Substitutions on the heterocycle of pyrimidine nucleosides: effects on VZV TK substrate phosphorylation kinetics

| | Compound No. | 3-sub | 4-sub | 5-sub | Rel $V_{max}$* | $K_m'$† ($\mu$M) | Efficiency (rel $V_m/K_m'$) |
|---|---|---|---|---|---|---|---|
| 1-$\beta$-D-2'-Deoxyribosides | 1 | H | O | $CH_3$ | 100 | 0.16 | 625 |
|  | 2 | H | O | H | 100 | 13 | 7.7 |
| | 3 | H | O | I | 120 | 0.096 | 1200 |
| | 4 | H | O | $CH=CH_2$ | 27 | 0.055 | 490 |
| | 5 | H | O | (*E*) $CH=CHBr$ | 63 | 0.043 | 1500 |
| | 6 | H | O | $C\equiv CH$ | 220 | 0.70 | 310 |
| | 7 | H | O | $C\equiv CCH_3$ | 220 | 0.52 | 420 |
| | 8 | $CH_3$ | O | $CH_3$ | 310 | 360 | 0.86 |
| | 9 | — | $NH_2$ | H | 600 | 5.3 | 110 |
| | 10 | — | $NH_2$ | $CH_3$ | 880 | 3.5 | 250 |
| | 11 | — | $NH_2$ | $C\equiv CH$ | 680 | 9.9 | 69 |
| | 12 | — | $NH_2$ | $C\equiv CCH_3$ | 180 | 2.5 | 72 |
| 1-$\beta$-D-Arabinosides | 13 | H | O | $CH_3$ | 33 | 0.55 | 60 |
|  | 14 | H | O | H | 41 | 57 | 0.72 |
| | 15 | H | O | I | 40 | 0.59 | 68 |
| | 16 | H | O | $CH=CH_2$ | 20 | 1.5 | 13 |
| | 17 | H | O | (*E*) $CH=CHBr$ | 38 | 0.030 | 1300 |
| | 18 | H | O | $C\equiv CH$ | 70 | 1.3 | 54 |
| | 19 | H | O | $C\equiv CCH_3$ | 210 | 2.2 | 95 |
| | 20 | — | $NH_2$ | H | 540 | 91 | 5.9 |
| | 21 | — | $NH_2$ | $C\equiv CCH_3$ | 460 | 140 | 3.3 |
| | 22 | — | $NHCH_3$ | H | 410 | 480 | 0.85 |

* Rel $V_{max}$ values were calculated from the relative velocities at 1 mM test compound (dThd = 100) and the $K_m'$ values (rel $V_{max}$ = rel $v(S + K_m')/S$).

† $K_m'$ values were determined directly with labeled substrate or as $K_i'$ values by [$^3$H]dThd inhibition studies (see Materials and Methods).

hydroxyl (No. **30** and **32**), or reconfiguration at the 3'-position to xylose (No. **26–28**) reduced phosphorylation efficiency. A 2'-hydroxyl was not well tolerated by the VZV TK, although the arabinoside was a better substrate than the ribonucleoside (No. **13** vs No. **29**). Thymine with a 1-(2-hydroxyethoxy)methyl acyclic sugar moiety (No. **34**) was not a substrate. Addition of another hydroxymethyl group (No. **35**), which could mimic the 3'-hydroxyl of a furanoside, resulted in a compound that was detectably phosphorylated. The $K_m'$ for this substrate, however, was the highest of the thymine nucleosides tested. The $K_i'$ for 2',5'-dideoxythymidine (No. **36**) was only slightly higher than the $K_m'$ for dThd, suggesting the 5'-hydroxyl was not important to substrate binding. Alpha-dThd (No. **37**) was not a substrate. Replacement of the sugar ring oxygen with a sulfur (No. **38**) did not affect either rel $V_{max}$ or $K_m'$ values relative to dThd. However, the carbocyclic thymine analogs (No. **39–41**, racemic mixtures) had much higher relative velocities than the corresponding furanosides (compare to No. **29**, **30** and **32**).

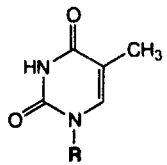
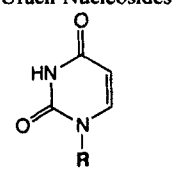
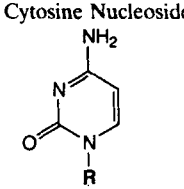
*Uracil nucleoside sugar substitutions.* Effects of sugar substitutions on phosphorylation velocities in the uracil series (No. **2**, **14**, **42–46**, Table 3) were very similar to those in the thymine series. The $K_m'$ values for the uracil nucleoside analogs, however, were more than an order of magnitude higher than the corresponding thymine analogs. Therefore, while phosphorylation efficiencies were significantly lower in the uracil series, the order of substrate efficiencies was identical to that in the thymine series [i.e. 2' - deoxyribonucleoside > arabinoside > ribonucleoside > 2',3' - dideoxyribonucleoside > (2 - hydroxyethoxy)methyl].

*Cytosine nucleoside sugar substitutions.* In the cytosine series (No. **9**, **20**, **47–50**), the rel $V_{max}$ values were much higher than with either the thymine or uracil series. Even 2',3'-dideoxy-3'-fluorocytidine was a high velocity substrate (compare No. **47** vs No. **23** and **42**). $K_m'$ values were comparable to those of the uracil nucleosides. Again, the effects of sugar substitutions on phosphorylation efficiencies were similar to those in the thymine series. Interestingly, 2',3'-dideoxycytidine was as efficient a substrate as the ribonucleoside (No. **49** vs No. **48**).

*Adenine nucleoside sugar substitutions.* Many adenine nucleosides are moderate velocity, high $K_m'$ substrates of the VZV TK. The effects of sugar substitutions in this series (Table 4, No. **51–65**) were very similar to those for thymine nucleosides. However, while carbocyclic sugar thymine nucleosides were high velocity substrates, only one of the carbocyclic adenine nucleosides (No. **61**) was detectably phosphorylated.

*Guanine nucleoside sugar substitutions.* Guanine nucleosides did not follow the trends observed in the pyrimidine and adenine series. These compounds (No. **66–74**) were generally poor substrates. One notable exception was the 3'-hydroxymethyl analog of dGuo (No. **67**). In the thymine and uracil nucleoside series, this substitution was detrimental to substrate efficiencies. In contrast, the 2'-deoxy-

Table 3. Pentosyl modifications on pyrimidine nucleosides: effects on VZV TK substrate phosphorylation kinetics

| | Compound No. | R | Rel $V_{max}$* | $K_m'$ or $K_i'$† ($\mu$M) | Efficiency (rel $V_m/K_m'$) |
|---|---|---|---|---|---|
| Thymine Nucleosides | 1 | 2-Deoxy-$\beta$-D-ribofuranosyl | 100 | 0.16 | 625 |
| | 13 | $\beta$-D-Arabinofuranosyl [araT] | 33 | 0.55 | 60 |
|  | 23 | 2,3-Dideoxy-3-fluoro-$\beta$-D-ribofuranosyl | 5 | 5.4 | 0.93 |
| | 24 | 2,3-Dideoxy-3-azido-$\beta$-D-ribofuranosyl [AZT] | [<2]‡ | 7.3 | n.c.‡ |
| | 25 | 2,3-Dideoxy-3-hydroxymethyl-$\beta$-D-ribofuranosyl | 130 | 6.9 | 19 |
| | 26 | 2-Deoxy-$\beta$-D-xylofuranosyl | [<2] | 4.4 | n.c. |
| | 27 | 2,3-Dideoxy-3-fluoro-$\beta$-D-xylofuranosyl | [<2] | 12 | n.c. |
| | 28 | 2,3-Dideoxy-3-azido-$\beta$-D-xylofuranosyl | [<2] | 49 | n.c. |
| | 29 | $\beta$-D-Ribofuranosyl | 33 | 5.3 | 6.2 |
| | 30 | 3-Deoxy-$\beta$-D-ribofuranosyl | 3 | 2.9 | 1.0 |
| | 31 | 3-Deoxy-3-hydroxymethyl-$\beta$-D-arabinofuranosyl | 15 | 45 | 0.33 |
| | 32 | 2,3-Dideoxy-$\beta$-D-ribofuranosyl [ddT] | 14 | 11 | 1.3 |
| | 33 | 2,3-Dideoxy-2,3-didehydro-$\beta$-D-ribofuranosyl [d4T] | [<3] | 28 | n.c. |
| | 34 | (2-Hydroxyethoxy)methyl | [<3] | 140 | n.c. |
| | 35 | (2-Hydroxy-1-(hydroxymethyl)ethoxy)methyl | 30 | 360 | 0.083 |
| | 36 | 2,5-Dideoxy-$\beta$-D-ribofuranosyl | [<1] | 0.31 | n.c. |
| | 37 | 2-Deoxy-$\alpha$-D-ribofuranosyl | [<2] | 15 | n.c. |
| | 38 | 2-Deoxy-4-thio-$\beta$-D-ribofuranosyl | 90 | 0.17 | 530 |
| | 39 | (±) C-Rib‖ | [170] | n.d.§ | n.c. |
| | 40 | (±) C-3-dRib‖ | [230] | n.d. | n.c. |
| | 41 | (±) C-2,3-ddRib‖ | [200] | n.d. | n.c. |
| Uracil Nucleosides | 2 | 2-Deoxy-$\beta$-D-ribofuranosyl | 100 | 13 | 7.7 |
| | 14 | $\beta$-D-Arabinofuranosyl | 41 | 57 | 0.72 |
|  | 42 | 2,3-Dideoxy-3-fluoro-$\beta$-D-ribofuranosyl | [<2] | 440 | n.c. |
| | 43 | 2,3-Dideoxy-3-hydroxymethyl-$\beta$-D-ribofuranosyl | 88 | 600 | 0.15 |
| | 44 | $\beta$-D-Ribofuranosyl | 57 | 790 | 0.072 |
| | 45 | 2,3-Dideoxy-$\beta$-D-ribofuranosyl | 19 | 730 | 0.026 |
| | 46 | (2-Hydroxyethoxy)methyl | 5 | 1700 | 0.0029 |
| Cytosine Nucleosides | 9 | 2-Deoxy-$\beta$-D-ribofuranosyl | 600 | 5.3 | 110 |
| | 20 | $\beta$-D-Arabinofuranosyl [araC] | 540 | 91 | 5.9 |
|  | 47 | 2,3-Dideoxy-3-fluoro-$\beta$-D-ribofuranosyl | 380 | 430 | 0.87 |
| | 48 | $\beta$-D-Ribofuranosyl | 840 | 1700 | 0.49 |
| | 49 | 2,3-Dideoxy-$\beta$-D-ribofuranosyl [ddC] | 240 | 450 | 0.52 |
| | 50 | 2-(Hydroxyethoxy)methyl | 23 | 1300 | 0.018 |

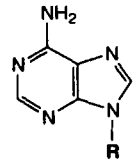
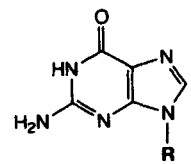
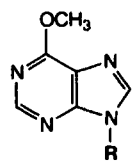
*,† See Table 2.

‡ Values in brackets are relative substrate velocities (dThd = 100) at 1 mM test compound, rather then rel $V_{max}$ values. Efficiencies were not calculated (n.c.) for compounds that were not detectably phosphorylated or for racemic mixtures (see below).

§ $K_m'$ values were not determined (n.d.) for racemic carbocyclic nucleoside analogs.

‖ Compound No. **39** is (±)-(1$\alpha$,2$\beta$,3$\beta$,4$\alpha$)-1-[2,3-dihydroxy-4-(hydroxymethyl)cyclopentyl]-5-methyl-2,4(1*H*,3*H*)-pyrimidinedione; compound No. **40** is (±)-(1$\alpha$,2$\beta$,4$\alpha$)-1-[2-hydroxy-4(hydroxymethyl)cyclopentyl]-5-methyl-2,4(1*H*,3*H*)-pyrimidinedione; compound No. **41** is (±)-cis-1-[3-(hydroxymethyl) cyclopentyl]-5-methyl-2,4(1*H*,3*H*)-pyrimidinedione.

Table 4. Pentosyl modifications on purine nucleosides: effects on VZV TK substrate phosphorylation kinetics

| | Compound No. | R | Rel $V_{max}$* | $K_m'$ or $K_i'$† ($\mu$M) | Efficiency (rel $V_m/K_m'$) |
|---|---|---|---|---|---|
| Adenine Nucleosides | 51 | 2-Deoxy-$\beta$-D-ribofuranosyl | 560 | 700 | 0.80 |
|  | 52 | 2,3-Dideoxy-3-fluoro-$\beta$-D-ribofuranosyl | [<2]‡ | 920 | n.c.‡ |
| | 53 | 2-Deoxy-$\beta$-D-xylofuranosyl | [<2] | 2200 | n.c. |
| | 54 | $\beta$-D-Ribofuranosyl | 33 | 2000 | 0.017 |
| | 55 | 2-Deoxy-2-amino-$\beta$-D-ribofuranosyl | 14 | 2500 | 0.0056 |
| | 56 | 3-Deoxy-3-hydroxymethyl-$\beta$-D-ribofuranosyl | [<1] | 3800 | n.c. |
| | 57 | $\beta$-D-Arabinofuranosyl [araA] | 500 | 1500 | 0.33 |
| | 58 | 3-Deoxy-$\beta$-D-arabinofuranosyl | 31 | 5200 | 0.0060 |
| | 59 | 2,3-Dideoxy-$\beta$-D-ribofuranosyl [ddA] | 6 | 1900 | 0.0031 |
| | 60 | (2-Hydroxyethoxy)methyl | [<3] | 10000 | n.c. |
| | 61 | (±) C-Ara‖ | [5] | n.d.§ | n.c. |
| | 62 | (±) C-Rib‖ | [<5] | n.d. | n.c. |
| | 63 | (±) C-3-dRib‖ | [<2] | n.d. | n.c. |
| | 64 | (±) C-2,3-ddRib‖ | [<5] | n.d. | n.c. |
| | 65 | (±) C-2,3-ene-ddRib‖ | [<5] | n.d. | n.c. |
| Guanine Nucleosides | 66 | 2-Deoxy-$\beta$-D-ribofuranosyl | 24 | 530 | 0.045 |
| | 67 | 2,3-Dideoxy-3-hydroxymethyl-$\beta$-D-ribofuranosyl | 1400 | 95 | 15 |
|  | 68 | $\beta$-D-Ribofuranosyl | [<3] | 420 | n.c. |
| | 69 | $\beta$-D-Arabinofuranosyl | [<5] | 660 | n.c. |
| | 70 | 2,3-Dideoxy-$\beta$-D-ribofuranosyl | [<3] | 340 | n.c. |
| | 71 | (2-Hydroxyethoxy)methyl [acyclovir] | 37 | 830 | 0.044 |
| | 72 | (2-Hydroxy-1-(hydroxymethyl)ethoxy)methyl [ganciclovir] | 16 | 1300 | 0.012 |
| | 73 | (±) C-Ara‖ | [<5] | n.d. | n.c. |
| | 74 | (−) C-2,3-ene-ddRib ((−)carbovir)‖ | 33 | 170 | 0.19 |
| 6-Methoxypurine Nucleosides | 75 | 2-Deoxy-$\beta$-D-ribofuranosyl | 980 | 170 | 5.8 |
|  | 76 | $\beta$-D-Ribofuranosyl | 120 | 300 | 0.40 |
| | 77 | $\beta$-D-Arabinofuranosyl | 680 | 200 | 3.4 |
| | 78 | 2,3-Dideoxy-$\beta$-D-ribofuranosyl | [<3] | 290 | n.c. |
| | 79 | (±) C-Ara‖ | [47] | n.d. | n.c. |
| | 80 | (±) C-2,3-ddRib‖ | [23] | n.d. | n.c. |
| | 81 | (±) C-2,3-ene-ddRib‖ | [5] | n.d. | n.c. |

*,†,‡,§ See footnotes in Tables 2 and 3.

‖ Compound No. **61** is (±)-(1$\alpha$,2$\beta$,3$\beta$,5$\beta$)-3-(6-amino-9*H*-purin-9-yl)-5-(hydroxymethyl)-1,2-cyclopentanediol; compound No. **62** is (±)-(1$\alpha$,2$\alpha$,3$\beta$,5$\beta$)-3-(6-amino-9*H*-purin-9-yl)-1,2-cyclopentanediol; compound No. **63** is (±)-(1$\alpha$,3$\alpha$,4$\beta$)-3-(6-amino-9*H*-purin-9-yl)-4-hydroxycyclopentanemethanol; compound No. **64** is (±)-cis-3-(6-amino-9*H*-purin-9-yl)cyclopentanemethanol; compound No. **65** is (±)-cis-4-(6-amino-9*H*-purin-9-yl)-2-cyclopentene-1-methanol; compound No. **73** is (±)-(1$\alpha$,2$\alpha$,3$\beta$,4$\alpha$)-2-amino-9-[2,3-dihydroxy-4-(hydroxymethyl)cyclopentyl]-1,9-dihydro-6*H*-purin-6-one; compound No. **74** is (1*R* cis)-2-amino-1,9-dihydro-9-[4-(hydroxymethyl)-2-cyclopenten-1-yl]-6*H*-purin-6-one; compound No. **79** is (±)-(1$\alpha$,2$\beta$,3$\alpha$,5$\alpha$)-3-(hydroxymethyl)-5-(6-methoxy-9*H*-purin-9-yl)-1,2-cyclopentanediol; compound No. **80** is (±)-cis-3-(6-methoxy-9*H*-purin-9-yl)cyclopentanemethanol; compound No. **81** is (±)-cis-4-(6-methoxy-9*H*-purin-9-yl)cyclopentene-1-methanol.

Table 5. Summary of VZV TK substrate efficiencies

| Sugar | Efficiency (rel $V_{max}/K_m'$) | | | | |
|---|---|---|---|---|---|
| | Thymine | Cytosine | Uracil | Adenine | Guanine |
| 2'-Deoxy-β-D-ribofuranosyl | 625 | 110 | 7.7 | 0.80 | 0.045 |
| β-D-Arabinofuranosyl | 60 | 5.9 | 0.72 | 0.33 | n.c.* |
| β-D-Ribofuranosyl | 6.2 | 0.49 | 0.072 | 0.017 | n.c. |
| 2',3'-Dideoxy-β-D-ribofuranosyl | 1.3 | 0.52 | 0.026 | 0.0031 | n.c. |
| (2-Hydroxyethoxy)methyl | n.c. | 0.018 | 0.0029 | n.c. | 0.044 |

* Efficiencies were not calculated because phosphorylation was not detected at 1 mM substrate.

3'-hydroxymethyl guanine analog was the most efficient purine nucleoside tested. The efficiency of acyclovir (No. **71**) phosphorylation was identical to that of 2'-deoxyguanosine (No. **66**). In all other series, this acyclic sugar substitution decreased substrate efficiency (No. **34**, **46**, **50**, and **60**) relative to the corresponding 2'-deoxyribonucleosides. An additional hydroxymethyl group on the acyclic moiety (ganciclovir) caused a decrease in the phosphorylation efficiency relative to acyclovir (No. **72** vs No. **71**). This was opposite to the trend seen in the thymine series (No. **35** vs. No. **34**). The (-) enantiomer of carbovir (No. **74**, natural configuration) was a substrate for the VZV TK and had a low $K_m'$ relative to other purine nucleoside analogs.

*6-Methoxypurine nucleoside sugar substitutions.* The trends with 6-methoxypurine nucleoside sugar substitutions (No. **75–81**) were very similar to those for the adenine series. Unlike most of the carbocyclic nucleosides in the adenine series, the 6-methoxypurine carbocyclic analogs (No. **79–81**) were substrates for the VZV TK.

Generalizations (with a few exceptions) can be drawn from the VZV TK substrate specificity data. For a given sugar moiety, thymine nucleosides were the most efficient substrates for the VZV TK, followed by cytosine, uracil and adenine nucleosides. And for a given base moiety, 2'-deoxyribonucleosides were the most efficient substrates, followed by arabinosides, ribonucleosides, 2',3'-dideoxyribonucleosides and the acyclic moiety of acyclovir. These trends, summarized in Table 5, did not always apply to the guanine nucleosides.

## DISCUSSION

The VZV TK plays a critical role in the activation of many anti-VZV nucleosides. Detailed studies of this enzyme have been hampered by the difficulty of purifying sufficient quantities from infected cells. We have expressed the VZV TK in *E. coli* and purified the enzyme [9] to a specific activity 10-fold higher than that previously reported [5]. Bacterially expressed and native VZV TK behave similarly during purification, have the same molecular mass, and are both precipitated by antibodies raised against the bacterially expressed enzyme [9]. Biochemical characterizations presented in this paper further demonstrated that the bacterially expressed VZV TK was indistinguishable from enzyme purified from VZV-infected cells. Optimum pH range, phosphate donor specificity, and dTTP inhibition of dThd phosphorylation with the bacterially expressed VZV TK were similar to published results with enzyme from infected cells [5, 20]. We observed biphasic, non-Michaelis–Menten kinetics for MgATP both with bacterially expressed enzyme and with enzyme from VZV-infected cells. Finally, a strong correlation was observed between relative substrate velocities for a wide variety of compounds with the enzyme from the two sources.

Comparisons of our $K_m'$ and $K_i'$ values with those in the literature are more difficult because reaction conditions vary. While we determined a $K_m'$ of 0.16 μM for dThd, others [5, 23, 24] have reported $K_m'$ and $K_i'$ values 2- to 4-fold higher. $K_m'$ and $K_i'$ values for thymine arabinoside [5], BVdU [3, 6, 24], BVaraU [3], and 5-iodo-2'-deoxyuridine [5] are also 2- to 6-fold higher than the values we observed. Literature $K_m'$ values for 2'-deoxycytidine range from 1 to 240 μM [1, 2, 5, 23] whereas we observed a $K_m'$ of 5.3 μM. Interestingly, there is very good agreement among the reported $K_i'$ values for acyclovir (830 vs. 820–860 μM) [3, 24]. A single $K_m'$ value for MgATP (16 μM) has been reported for the native enzyme [5] in contrast to our observations of biphasic kinetics. We cannot account for the difference because experimental details were not given [5].

Several generalizations about the substrate specificity of VZV TK can be drawn from our data. For pyrimidine nucleosides, several substitutions at the 5-position were well tolerated. 4-Amino and 3-methyl substitutions increased velocities but also increased $K_m'$ values, decreasing substrate efficiency overall. The effects of base substitutions on the efficiency of purine arabinoside phosphorylation were reported previously [11, 25]. Purine arabinosides are high-velocity, high-$K_m'$ substrates for the VZV TK. Small alkoxy and alkyl amines at the 6-position improve phosphorylation kinetics, while 2-substitutions are not well tolerated. Interestingly, while methylation at $N^6$ of adenosine arabinoside significantly improves phosphorylation efficiency [25] relative to the parent compound, the same substitution at $N^4$ of cytosine arabinoside (No. **22**) increased the $K_m'$ value, thereby decreasing substrate efficiency.

Effects of alterations in the sugar moiety of

pyrimidine nucleosides can be generalized as follows: The VZV TK required a 3′-hydroxyl in the sugar moiety for efficient phosphorylation. 3′-Substitutions, extension or reconfiguration to xylose all reduced or eliminated phosphorylation activity. A hydroxyl at the 2′-position was not well tolerated, though arabinosides were better substrates than ribonucleosides. Substituting a carbon for the furanosyl ring oxygen increased the phosphorylation velocity.

Most of these generalizations also applied to adenine and 6-methoxypurine nucleosides. One notable exception was that the carbocyclic analogs were poor substrates. Also, guanine nucleoside phosphorylation kinetics did not follow the trends observed in the other series. Acyclovir (No. **71**) was as efficient as 2′-deoxyguanosine (No. **66**), and (−)carbovir (No. **74**) was an even better substrate. Unexpectedly, 2′,3′-dideoxy-3′-hydroxymethyl guanosine (No. **67**) was the most efficient purine nucleoside tested.

The substrate specificity of the VZV TK can provide a biochemical explanation for antiviral activity. Within a series of *active* compounds, there is a qualitative correlation between *in vitro* $IC_{50}$ values from the literature and our phosphorylation efficiency data. BVdU (No. **5**) and BVaraU (No. **17**), two of the most active nucleosides against VZV *in vitro* [26, 27], were the most efficient substrates we tested. Thymine arabinoside, 5-vinyluracil arabinoside and cytosine arabinoside, are less active against VZV and, in parallel, were less efficient substrates. A correlation between antiviral activity and VZV TK phosphorylation efficiencies for purine arabinosides has also been demonstrated [11, 25]. These correlations presumably represent a rate-limiting role for the viral TK in formation of the antiviral triphosphates.

VZV TK phosphorylation data are not necessarily *predictive* of antiviral activity. 5-Iodo-2′-deoxyuridine and BVaraU had very similar phosphorylation efficiencies (compare No. **3** and **17**), yet the $IC_{50}$ for 5-iodo-2′-deoxyuridine is three orders of magnitude higher than that for BVaraU [27]. 2-Chloro-6-methoxypurine arabinoside, a compound with no anti-VZV activity, is a better substrate for the VZV TK than a number of closely related compounds which do inhibit VZV *in vitro* [11]. Although activation by the viral kinase is usually a requisite step for *in vitro* antiviral activity, it is not sufficient to ensure such activity. Nucleoside analogs must first cross the cell membrane. Subsequent to phosphorylation by the VZV TK, compounds must be phosphorylated by mono- and diphosphokinases, and the triphosphate must then inhibit the viral DNA polymerase. Catabolic enzymes including nucleoside deaminases and phosphorylases can degrade nucleoside analogs, and cellular toxicity due to nonselective phosphorylation by host cell nucleoside kinases and inhibition of host DNA polymerase can mask antiviral activity. Any one of these steps can affect the antiviral activity of a VZV TK substrate.

The substrate specificity of VZV TK can be compared to mammalian nucleoside kinases. Such comparisons serve to explain the antiviral activity and cellular toxicity of a nucleoside analog. The 5-halodeoxyuridine analogs have anti-VZV activity *in vitro* but are also cytotoxic [26, 27]. These compounds are substrates for the VZV TK and for human cytosolic and mitochondrial thymidine kinases [28]. Phosphorylation by these human enzymes probably contributes to the cellular toxicity. BVdU and thymine arabinoside are poor substrates for cytosolic TK yet are readily phosphorylated by the VZV TK. These compounds exhibit selective antiviral activity with little host cell toxicity. However, the mitochondrial TK can also phosphorylate these compounds [28]. Again, this activity may be responsible for some of the limited cytotoxicities observed with these compounds [29].

Deoxycytidine kinase and adenosine kinase have overlapping substrate specificities with VZV TK. Like the VZV TK, deoxycytidine kinase can phosphorylate pyrimidine and purine deoxynucleosides and arabinosides as well as cytosine nucleosides with various sugar substitutions [28, 30]. Adenine arabinoside, which is a substrate for both of these enzymes and for adenosine kinase, has antiviral and cytotoxic activities. The $N^6$-monomethyl- and dimethyl-adenine arabinoside analogs are better substrates than the parent compound for the VZV TK, yet are not detectably phosphorylated by calf thymus deoxycytidine kinase or rabbit liver adenosine kinase [25]. In a corresponding manner, these compounds have better *in vitro* anti-VZV activity and are less cytotoxic than adenine arabinoside.

Although there is considerable substrate specificity overlap between the VZV TK and various cellular kinases, the "permissive" nature of the VZV TK has allowed development of compounds that are phosphorylated by the viral enzyme but not by human kinases. Using the bacterially expressed VZV TK, we have expanded our knowledge of this enzyme's substrate specificity. These studies, in turn, will provide insight as to why some antiviral nucleosides are active and will help guide the discovery of new analogs with selective antiviral activity.

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