The A167Y Mutation Converts the Herpes Simplex Virus Type 1 Thymidine Kinase into a Guanosine Analogue Kinase†

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ABSTRACT: The thymidine (dThd) kinase (TK) encoded by herpes simplex virus type 1 (HSV-1) is not only endowed with dThd kinase, but also with thymidylate (dTMP) kinase and 2′-deoxycytidine (dCyd) kinase (dCK) activity. HSV-1 TK also recognizes a variety of antiherpetic guanine nucleoside analogues such as acyclovir (ACV), ganciclovir (GCV), lobucavir (LBV), penciclovir (PCV), and others (i.e., A5021). Site-directed mutagenesis of the highly conserved Ala-167 to Tyr in HSV-1 TK completely abolished TK, dTMP-K, and dCK activity, but maintained ACV-, GCV-, LBV-, PCV-, and A5021-phosphorylating capacity. A variety of 5-substituted pyrimidine nucleoside substrates, but also a number of selective HSV-1 TK inhibitors structurally related to thymine lost significant binding affinity for the mutant enzyme and did not markedly compete with GCV phosphorylation by the mutant enzyme. These findings could be explained by computer-assisted modeling data that revealed steric hindrance of the pyrimidine ring in the HSV-1 TK active site by the large 4-hydroxybenzyl ring of 167-Tyr, while the positioning of the purine ring of guanine-based HIV-1 TK substrates in the active site was kept virtually unaltered. Surprisingly, the efficiency of conversion the antiherpetic 2′-deoxyguanosine analogues ACV, GCV, LBV, PCV, and A5021 to their phosphorylated forms by the A167Y mutant HSV-1 TK was far more pronounced than for the wild-type enzyme. Therefore, the single A167Y mutation converts the wild-type HSV-1 TK from a predominantly pyrimidine nucleos(t)ide kinase into a virtually exclusive purine (guanine) nucleoside analogue kinase.

Herpes simplex virus type 1 (HSV-1)[1] encoded thymidine kinase (TK) differs from mammalian nucleoside kinases in terms of substrate specificity. It recognizes besides 2′-deoxythymidine (dThd) and 2′-deoxyuridine (dUrd) also 2′-deoxycytidine (dCyd) and a variety of dUrd and dCyd analogues that are not, or only poorly, recognized by mammalian cytosolic TK-1 (*1*−*5*). One of the most striking differences in recognition of nucleoside analogues by HSV-1 and cytosolic TK is exemplified by the potent antiherpetic (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (5-BV-dUrd) (Figure 1) (*6*), which is an excellent substrate for HSV-1 TK, while being not recognized as a substrate by cytosolic TK-1 (*7*, *8*). Moreover, HSV-1 TK is also endowed with thymidylate kinase activity (*9*), converting dTMP but also the 5′-monophosphate of 5-BV-dUrd to their corresponding 5′-diphosphate derivatives (*3*). The selective and potent anti-HSV-1 activity of 5-BV-dUrd is due to its efficient and selective phosphorylation to 5-BV-dUMP and 5-BV-dUDP

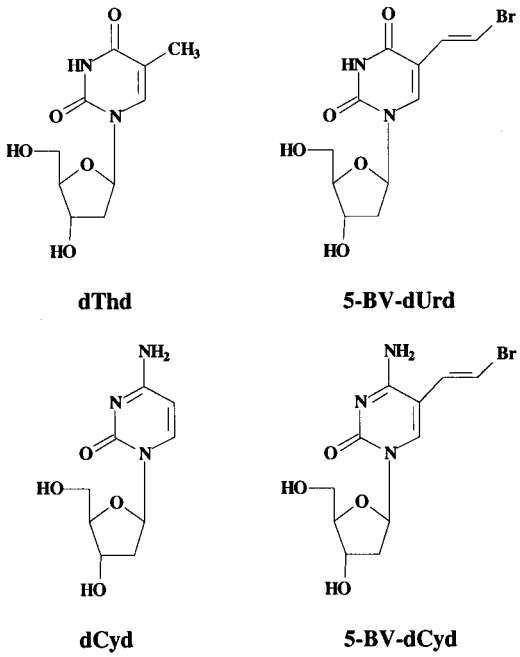

FIGURE 1: Structural formulas of natural and antiherpetic pyrimidine nucleoside analogues.

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[1] Abbreviations: dThd, thymidine; TK, dThd kinase; HSV-1, herpes simplex virus type 1; dTMP, thymidylate; dCyd, 2′-deoxycytidine; dCK, dCyd kinase; ACV, acyclovir; GCV, ganciclovir; LBV, lobucavir; PCV, penciclovir; 5-BV-dUrd, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; 5-BV-dCyd, (*E*)-5-(2-bromovinyl)-2′-deoxycytidine; IPTG, isopropyl-β-D-thiogalactopyranoside; GST, glutathiontransferase; DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride; d4T, stavudine; dUrd, 2′-deoxyuridine; dGuo, 2′-deoxyguanosine; 5-F-dUrd, 5-fluoro-2′-deoxyuridine; 5-Me-dCyd, 5-methyl-2′-deoxycytidine; dUMP, 2′-deoxyuridylate; AZT, azidothymidine; AZT-MP, azidothymidine 5′-monophosphate.

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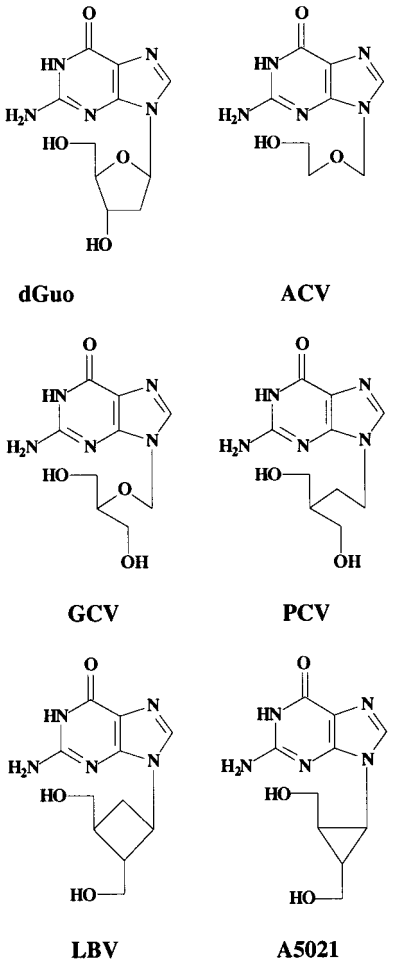

FIGURE 2: Structural formulas of natural and antiherpetic guanosine analogues.

by the two successive catalytic activities of HSV-1 TK, after which 5-BV-dUDP is further converted to the triphosphate derivative by a cellular nucleoside diphosphate kinase.

The HSV-1 TK has also another interesting property: it recognizes a variety of guanine-based (antiherpetic) nucleoside analogues such as acyclovir (ACV), ganciclovir (GCV), lobucavir (LBV), penciclovir (PCV), and A-5021 (Figure 2), albeit at much lower efficiency than pyrimidine nucleosides (*10*). The crystal structures of HSV-1 TK complexed with either the natural substrate dThd and antiherpetic drugs such as GCV have been resolved and revealed that both pyrimidine and purine nucleosides bind to the same substrate binding site of the enzyme (*11*, *12*). The pyrimidine and purine bases of the nucleoside substrates are kept in place by the aromatic ring of tyrosine Y-172 on one side of the base ring and the methyl group of methionine M-128 on the other side of the base ring. Interestingly, the carbonyl and amide groups of glutamine Q-125 form hydrogen bonds with the N^3-amido and O^4-carbonyl oxygen of thymine, but also with the N^1-imino and O^6-carbonyl oxygen of the guanine of ganciclovir after switching the glutamine carbonyl and amide entities of Q125 by virtually 180 °C (Figure 3). As a result, the thymine base does not entirely overlap with the guanine base but partially occupies a space in the active site of the enzyme that is different from that of guanine.

On the basis of the distinct characteristics of dThd versus GCV binding to the enzyme, and on our computer-assisted modeling studies, we decided to introduce a tyrosine instead of the fairly conserved alanine at position 167 of HSV-1 TK (Figure 3) in an attempt to abolish the pyrimidine nucleoside phosphorylation site in the enzyme while preserving its purine nucleoside phosphorylation capacity. Detailed kinetic characterization of this novel mutant enzyme was performed. As an enzyme source, a highly purified recombinant GST-TK fusion protein was used. We found that the A167Y mutant HSV-1 TK completely lost its pyrimidine nucleos(t)ide phosphorylating capacity, but acquired a markedly greater ability to phosphorylate guanosine analogues such as the antiviral nucleosides acyclovir, ganciclovir, lobucavir, and penciclovir. Hence, this single mutation of the fairly conserved amino acid 167 of the HSV-1 TK

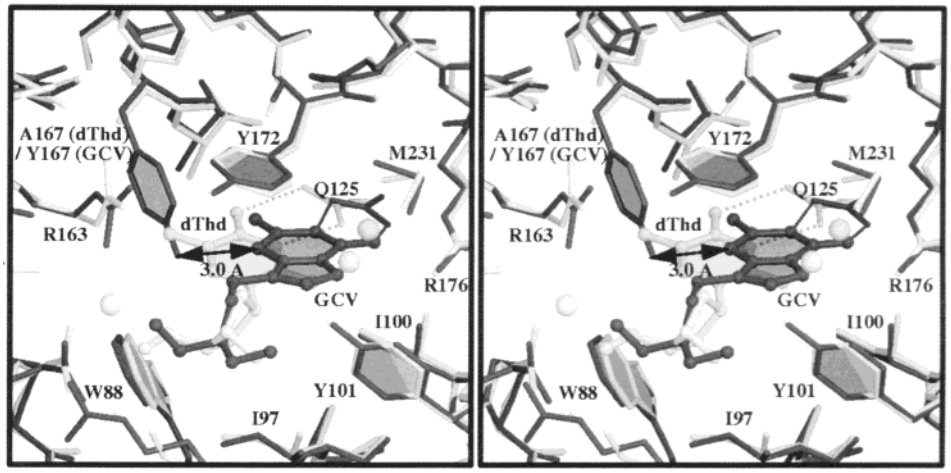

FIGURE 3: Stereodiagram of a model attempting to explain the different specificities of wild-type and A167Y-mutant HSV-1 TK. The crystal structure of wild-type TK in complex with dThd (PDB code 2VTK) (*12*) is shown in pale gray with water molecules shown by small spheres. The position of dThd is defined largely by interactions with Q125. The crystal structure of wild-type TK in complex with ganciclovir (GCV) (PDB code 1KI2) *(11)* was modified to bear the A167Y mutation and then the optimal position of the tyrosine within a static binding pocket was determined using RotSearch (*13*). This model is shown in dark gray. The closest contact between 167Y and GCV is 3.0A suggesting that minor rearrangements would allow A167Y-mutated TK to accommodate GCV. However, the same procedure with dThd as a substrate gave a severe clash (minimum distance less than 0.5A) between 167Y and dThd. Figure drawn using BobScript *(14)* and rendered with Raster3D.

converted the pyrimidine deoxynucleoside kinase into a purine (guanine) nucleoside analogue kinase.

MATERIALS AND METHODS

Expression and Purification of Wild-Type and A167Y Mutant HSV-1 TK. The plasmid vector, containing the TK mutant, has been constructed as described (*15*) and transfected into *Escherichia coli* BL21(DE3)pLysS. Bacteria were grown overnight at 37 °C in 2YT medium containing ampicillin (100 μg/mL) and chloramphenicol (40 μg/mL), and then diluted 1:10 in fresh medium. After further growth of the bacteria at 27 °C (for 1 h), isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) was added to a final concentration of 0.1 mM to induce the production of the GST-TK fusion protein. After 15 h of further growth at 27 °C, cells were pelleted (6000*g* for 10 min at 4 °C) and resuspended in lysis buffer [50 mM Tris, pH 7.5, 1 mM DTT, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.15 mg/mL lysosyme]. Bacterial suspensions were homogenized and lysed by means of a "French Pressure cell press", and ultracentrifuged (20000*g* for 15 min at 4 °C). GST-TK was purified from the supernatant using Glutathione Sepharose 4B (Amersham Pharmacia Biotech) as described by the Supplier. Briefly, a 50% slurry of glutatione Sepharose was added to the bacterial supernatant (1.5 mL/1.5 L of broth), incubated for 30 min at 4 °C, and then washed 3 times with 10 bed volumes (7.5 mL) of lysis buffer without lysosyme and PMSF. Bound proteins were eluted in 50 mM Tris (pH 8.0) containing 0.1% Triton X-100 and 10 mM glutathione. Protein content of the purified fractions was assessed using Bradford reagent (Sigma Chemical Co.).

Compounds. 5-BV-dUrd, 5-BV-dCyd, and 5-BV-dUMP were kindly provided by Dr. P. Herdewijn and Dr. A. Van Aerschot of the Rega Institute for Medical Research (Katholieke Universiteit Leuven, Leuven, Belgium). GCV was from Roche (Brussels, Belgium) and lobucavir (LBV) and stavudine (d4T) from Bristol-Myers Squibb (Wallingford, CT). Penciclovir (PCV) was obtained from Dr. I. Winkler (Hoechst, Frankfurt, Germany) and A5021 was from Ajinomoto Co (Kawasaki, Japan). dThd, dUrd, dCyd, dGuo, 5-F-dUrd, 5-Me-dCyd, dTMP, dUMP, AZT, and AZT-MP were from Sigma (St. Louis, MO). The thymine-based inhibitor [N-2,4-dichlorophenoxy-2-propionamide]-5′-amino-5′-deoxy-5-ethyl-2′-deoxyuridine (Ro316197) was from Roche (Hertfordshire, U. K.), and (*E*)-5-(2-bromovinyl)-5′-*O*-triphenylmethoxy-2′-deoxyuridine (KIN-5) was obtained from Dr. M. -J. Pérez-Pérez (CSIC, Madrid, Spain). The guanine-based inhibitors N^2-(m-trifluoromethylphenyl)guanine (m-CF_3pG) and N^2-phenyl-2′-deoxyguanosine (PhdG) were kindly provided by Dr. G. E. Wright, Washington, MA.

Radiolabeled Compounds. [^{3}H]ganciclovir (12.4 Ci/mmol), [^{3}H]lobucavir (26 Ci/mmol), and [^{3}H]penciclovir (16 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA).

TK/dTMP Kinase Assays. The ability of the purified GST-HSV-1 TK (WT) and GST-HSV-1 A167Y TK preparations to phosphorylate the test compounds listed in Table 1 was determined as follows. The standard reaction mixture contained 2.5 mM $MgCl_2$, 10 mM dithiotreitol, 1 mg/mL BSA, 2.5 mM ATP, 10 mM NaF, 100 μM test compound, and 5.65 μg of protein for WT HSV-1 TK and 8.0 μg protein for mutant HSV-1 A167Y TK preparation in a total reaction mixture of 50 μL of 50 mM Tris-HCl, pH 8.0. The reaction mixture was incubated at 37 °C for 90 min. The reaction mixtures were subjected to HPLC analysis using a Partisphere-SAX column. A linear gradient of 5 mM $(NH_4)H_2PO_4$, pH 5.0 (buffer A) to 0.3 M $(NH_4)H_2PO_4$, pH 5.0 (buffer B) was used to separate the metabolites as follows: 5 min of 100% buffer A, 15 min of a linear gradient to 100% buffer B, 20 min of 100% buffer B; 5 min of a linear gradient to 100% buffer A, and 5 min of equilibration with buffer A. The flow rate was 2 mL/min.

Table 1: Conversion of (100 μM) Nucleoside Analogues to Their Monophosphates by Wild-Type HSV-1 TK and Mutant HSV-1 A167Y TK after 90 min of the Enzyme Reaction

phosphorylation of	AUC[a] HSV-1 TK[b] WT	AUC[a] HSV-1 TK[b] A167Y	retention time of phosphorylated derivative (min)	λ_{max}[c] (nm)
dThd	508 362	ND[d]	5.7	266
d4T	ND	ND	6.8	266
AZT	ND	ND	6.7	266
dUrd	763 701	ND	6.3	262
5-F-dUrd	196 449	ND	5.9	262
5-BV-dUrd	890 000	ND	6.4	294
dCyd	448 806	ND	4.7	274
5-Me-dCyd	670 380	ND	4.0	274
5-BV-dCyd	199 520	ND	6.1	294
araC	ND	ND		274
dTMP	629 070	ND	12.4	266
d4TMP				266
AZTMP	ND	ND	12.7	266
dUMP	9 052	ND	12.6	262
5-BV-dUMP	132 582	ND	13.2	294
dCMP	ND	ND	13.6	274
dGuo	ND	62 089	9.6	253
ACV	167 526	348 865	9.5	253
GCV	603 927	817 885	8.4	253
LBV	22 150	920 834	8.5	253
PCV	110 500	1 358 184	8.6	253
A5021	61 560	960 740	9.1	253

[a] Area under the curve. [b] Appearance of the phosphorylated derivatives of the nucleos(t)ide analogues measured as area under the curve (AUC). The phosphorylated nucleos(t)ide derivatives had an identical u.v. absorption spectrum as their parent compounds. [c] The optimal (or near-optimal) wavelength at which the appearance of the phosphorylated nucleos(t)ide analogue was examined. ϵ_{max} of dTMP: 9.5×10^{-3}; ϵ_{max} of dGMP: 13.7×10^{-3}; ϵ_{max} of dCMP: 9.0×10^{-3}. [d] ND: not detectable (detection limit: AUC: 5000).

The influence of dThd on the phosphorylation of [8-^{3}H]-GCV was determined by incubating 50 μL reaction mixtures at 37 °C for 60 min in the presence of 1.6 μM [8-^{3}H]GCV (1 μCi), a variety of dThd concentrations (i.e., 1, 5, 25, 100, 500, and 2000 μM) and 0.71 μg of WT HSV-1 TK or 0.83 μg of mutant HSV-1 A167Y TK. The reactions were terminated by spotting an aliquot of 45 μL onto DE-81 disks (Whatman, Maidstone, UK) that were instantly immersed and thoroughly washed three times in 1 mM $HCOONH_4$ and one time in ethanol (70%). Finally, the disks were dried and assayed for radioactivity in a toluene-based scintillant.

The ability of test compounds, listed in Table 2, to inhibit phosphorylation of 1 μM [^{3}H]GCV (0.3 μCi) by wild-type HSV-1 TK and A167Y mutant HSV-1 TK was essentially examined as described above, but in the presence of 0.23 μg of protein of wild-type HSV-1 TK and 0.40 μg of protein

Table 2: Inhibition of HSV-1 TK-Catalyzed [^{3}H]GCV Phosphorylation by Nucleos(t)ide Analogues

compound	50% inhibitory concentration[a] (μM) wild-type HSV-1 TK	A167Y mutant HSV-1 TK
pyrimidine nucleoside HSV-1 TK substrates		
dThd	1.8	500
dUrd	16	>500
dCyd	1000	>1000
pyrimidine nucleotide HSV-1 TK substrate		
dTMP	145	>2000
thymine-based HSV-1 TK inhibitors		
Ro316197	0.12	73
KIN-5	1.4	162
guanine nucleoside HSV-1 TK substrates		
dGuo	364	115
LBV	152	90
PCV	17	23
A5021	12	17
guanine-based HSV-1 TK inhibitors		
mCF_3PG	1.7	80
PhdG	0.85	>500

[a] In the presence of 1 μM [^{3}H]GCV Ro316197, [*N*-2,4-dichlorophenoxy-2-propionamide]-5′-amino-5′-deoxy-5-ethyl-2′-deoxyuridine KIN-5, (*E*)-5-(2-bromovinyl)-5′-*O*-triphenylmethoxy-2′-deoxyuridine mCF_3PG, N^2-(m-trifluoromethylphenyl)guanine PhdG, N^2-phenyl-2′-deoxyguanosine.

of A167Y mutant HSV-1 TK. The reaction mixture was incubated for 30 min at 37 °C.

The phosphorylation of different concentrations of [^{3}H]-GCV, [^{3}H]LBV, [^{3}H]PCV, and [^{3}H]ACV (i.e., 400, 200, 150, 125, 100, 67, 40, 20, 15, 12.5, 10, 6.7, and 4 μM) in the presence of 0.71 μg of protein of wild-type HSV-1 TK and 0.83 μg of protein of A167Y mutant HSV-1 TK was carried out as described above. The reaction mixtures were incubated for 30 min at 37 °C.

RESULTS

Phosphorylation of the Natural Pyrimidine Nucleosides by Wild-Type and A167Y Mutant HSV-1 TK. Hundred micromolar of the natural substrates of HSV-1 TK (e.g., dThd, dCyd, and dTMP) were exposed to equal amounts of wild-type HSV-1 TK and A167Y mutant HSV-1 TK, and conversion to the phosphorylated nucleoside derivative was measured after 90 min by HPLC analysis (Table 1). Whereas dThd, dCyd, and dTMP were markedly converted to their phosphorylated derivatives dTMP, dCMP, and dTDP, respectively, by wild-type HSV-1 TK, the A167Y mutant HSV-1 TK enzyme was unable to measurably convert any of the natural substrates to their phosphorylated derivatives within the evaluated time period. Taken into account the limit of detection, it could be inferred that the pyrimidine nucleoside (dThd, dCyd) and pyrimidine nucleotide (dTMP) kinase activity of the A167Y mutant HSV-1 TK enzyme was definitely much less than 1% of wild-type enzyme.

Conversion of Antiviral and Anticancer Pyrimidine and Purine Nucleoside and Nucleotide Analogues by Wild-Type and A167Y Mutant HSV-1 TK. A number of dThd, dCyd, and dTMP analogues were included in our study to determine their substrate activity by the wild-type and A167Y mutant HSV-1 TK enzymes. As observed for the natural substrates dThd and dUrd, also the antiviral 5-BV-dUrd and anticancer 5-F-dUrd derivatives were efficiently converted to their monophosphate derivatives by wild-type enzyme, but not by the A167Y mutant HSV-1 TK (Table 1). Measurable phosphorylation of d4T and AZT by either wild-type or by A167Y mutant HSV-1 TK did not occur under our experimental conditions.

Among the dCyd analogues tested, 5-BV-dCyd and 5-methyl-dCyd were markedly converted to their 5′-monophosphates by wild-type enzyme, but again, no trace of phosphorylated derivative could be detected in the reaction mixture to which A167Y mutant HSV-1 TK was added. For the anticancer drug araC, no activity was recorded with both wild-type and mutant HSV-1 TK enzyme.

The potential substrate properties of the natural nucleotide dTMP and related analogues (including 5-BV-dUMP) were also investigated (Table 1). Besides the natural dTMP substrate, also 5-BV-dUMP and, to a much lesser extent, dUMP acted as substrate for wild-type HSV-1 TK to be converted to their 5′-diphosphate derivatives, whereas no traces of conversion of these monophosphate derivatives to their 5′-diphosphates could be recorded in the presence of A167Y mutant HSV-1 TK. Also we found no conversion of d4T-MP and AZT-MP to their 5′-diphosphates by the mutant enzyme. 2′-dCMP was not a substrate for either enzyme (Table 1).

In contrast, a variety of antiherpetic guanosine analogues including ACV, GCV, LBV, PCV, and A5021 were to a marked extent converted to their monophosphate derivatives by both wild-type HSV-1 TK and A167Y mutant HSV-1 TK (Table 1). Interestingly, 2′-deoxyguanosine was only measurably converted to dGMP by the mutant enzyme.

Inhibitory Effect of Nucleoside Analogues on [3H]GCV Phosphorylation by Wild-Type HSV-1 and A167Y Mutant HSV-1 TK. Although it was found that none of the pyrimidine nucleos(t)ides shown in Table 1 were measurably phosphorylated by A167Y mutant HSV-1 TK, it could not be excluded that the pyrimidine nucleosides could still be recognized by the mutant enzyme and bound in its active site, but were unable to undergo catalytic conversion to their phosphorylated derivatives. Therefore, in a preliminary experiment, thymidine was added at several concentrations (i.e., 1, 5, 25, 100, 500, and 2000 μM) to the enzymes in the presence of 7 μM [^{3}H]GCV. Whereas the lower dThd concentrations ($\leq$5 μM) did not measurably affect conversion of 7 μM GCV to its phosphorylated derivative by wild-type HSV-1 TK, the higher dThd concentrations (e.g., 100, 500, and 2000 μM) completely prevented [^{3}H]GCV phosphorylation by wild-type HSV-1 TK. In contrast, addition of these higher dThd concentrations had only a minor effect on phosphorylation of 7 μM [^{3}H]GCV by A167Y mutant HSV-1 TK, indicating that the A167Y mutant HSV-1 TK enzyme was unable to phosphorylate dThd to dTMP due to a marked loss of overall affinity for the natural substrate. This was also confirmed for other pyrimidine nucleos(t)ides (Table 2). Indeed, whereas dThd, dUrd, and dTMP showed a marked measurable inhibitory effect on [^{3}H]GCV phosphorylation by wild-type HSV-1 TK (expressed as their IC_{50} values required to inhibit phosphorylation of 1 μM [^{3}H]GCV by 50%), A167Y mutant HSV-1 TK-catalyzed [^{3}H]GCV phosphorylation was not significantly affected by markedly higher

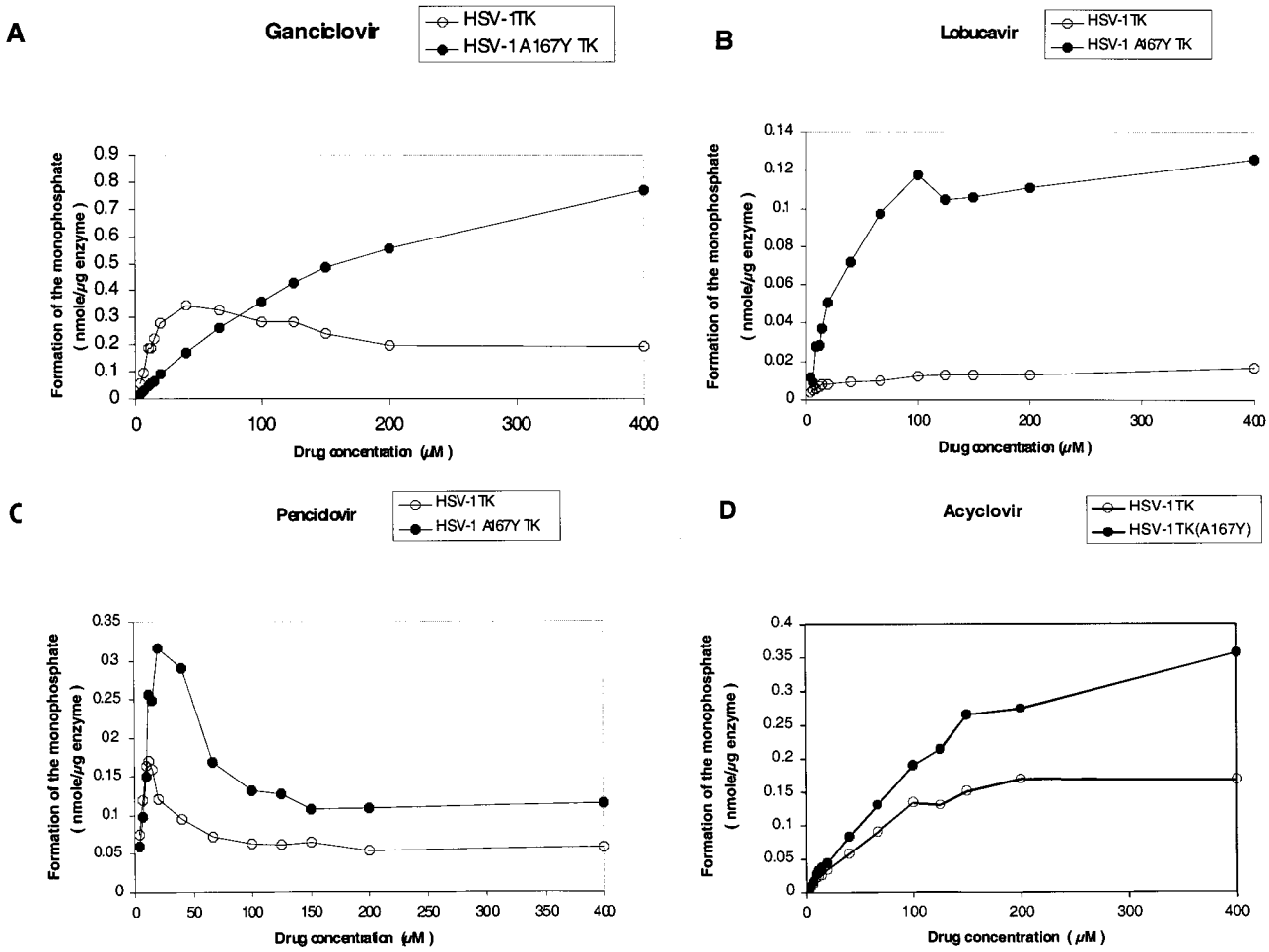

FIGURE 4: Phosphorylation pattern of [^{3}H]ganciclovir (panel A), [^{3}H]penciclovir (panel B), [^{3}H]lobucavir (panel C), and [^{3}H]acyclovir (panel D) by comparable amounts of wild-type (-○-) and mutant HSV-1 A167Y TK (-●-) enzymes at a variety of substrate concentrations.

(>15- to >300-fold) pyrimidine nucleoside concentrations than required for inhibition of wild-type HSV-1 TK (Table 2).

In contrast, dGuo and the antiherpetic guanosine analogues LBV, PCV, and A5021 had equal inhibitory (competitive) effects on [^{3}H]GCV phosphorylation by both wild-type and A167Y mutant HSV-1 TK, PCV and A5021 being more potent in their inhibitory action on [^{3}H]GCV phosphorylation by both enzymes than LBV and dGuo (Table 2).

Interestingly, Ro316197 and KIN-5 that represent two highly specific HSV-1 TK inhibitors (lacking any substrate activity) and that consist of a thymine base in their molecular structure, substantially prevented [^{3}H]GCV phosphorylation by wild-type HSV-1 TK, but required at least 70- to 600-fold higher concentrations to inhibit mutant HSV-1 A167Y TK-catalyzed [^{3}H]GCV conversion to its monophosphate derivative. Surprisingly, mCF_3PG and PhdG, two guanine-based specific inhibitors of HSV-1 TK (lacking any substrate activity), also lost substantial inhibitory potential when evaluated against the A167Y mutant HSV-1 TK (Table 2).

Kinetic Analysis of Wild-Type HSV-1 TK and A167Y Mutant HSV-1 TK. The conversion of [^{3}H]dThd by wild-type HSV-1 TK was measured at a variety of dThd concentrations that ranged between 0.1 and 50 μM. Whereas the conversion of [3H]dThd to its 5′-monophosphate followed typical Michaelis−Menten kinetics at the lower dThd concentrations (0.1 to 5.0 μM), higher dThd concentrations resulted in lower enzyme activity than the optimal TK activity recorded at 5 μM (data not shown). A similar kinetic enzyme activity curve was also observed for the phosphorylation of [^{3}H]GCV by wild-type HSV-1 TK (Figure 4A). At concentrations between 1 and 10 μM, the conversion of GCV to GCV-MP occurred proportionally with increasing substrate concentrations, but at GCV concentrations higher than 10 μM, the increasing enzymatic activity slowed and reached an optimal conversion rate at ~40 μM GCV. At higher GCV drug concentrations, enzymatic activity (GCV phosphorylating capacity) again decreased to levels that were only ~40% of the optimal GCV substrate conversion levels (Figure 4A). In sharp contrast, GCV phosphorylation by A167Y mutant HSV-1 TK followed typical Michaelis−Menten kinetics at concentrations up to 400 μM. At this GCV concentration, the V_{max} was not reached yet. Interestingly, whereas GCV phosphorylation by the mutant enzyme was clearly inferior to wild-type enzyme at concentrations lower than 70 μM, phosphorylation of GCV was far more pronounced in the presence of the mutant enzyme at 400 μM GCV, exceeding the phosphorylation levels afforded by wild-type HSV-1 TK enzyme by ~5-fold (Figure 4A). A similar phosphorylation curve by A167Y mutant HSV-1 TK was also found for ACV (data not shown).

Comparative phosphorylation of three other antiherpetic guanine nucleoside analogues (i.e., PCV, LBV, and ACV) by wild-type and mutant HSV-1 TK has also been carried

Table 3: Kinetic Properties of Guanine Nucleoside Analogues against Wild-Type and A167Y HSV-1 Thymidine Kinase

	wild-type TK			mutant A167Y TK		
compound	V_{max}[a]	K_m[b]	V_{max}/K_m	V_{max}[a]	K_m[b]	V_{max}/K_m
ganciclovir	0.36 ± 0.04	13 ± 2.3	0.0276	0.47 ± 0.09	120 ± 11	0.0039
acyclovir	0.13 ± 0.03	69 ± 8.5	0.0019	0.25 ± 0.04	141 ± 28	0.0018
penciclovir	0.39 ± 0.16	15 ± 7.0	0.026	0.56 ± 0.08	23 ± 11	0.024
lobucavir	0.009 ± 0.004	14 ± 2.5	0.0006	0.072 ± 0.035	37 ± 1.3	0.0019

[a] Expressed in nmol/μg of enzyme/30 min. [b] Expressed in μM.

out. In all cases, the phosphorylating capacity of A167Y mutant HSV-1 TK for the guanine derivatives was markedly superior to that of wild-type HSV-1 TK, as demonstrated for LBV (Figure 4B), PCV (Figure 4C), and ACV (Figure 4D). The phosphorylation pattern of penciclovir showed a virtual identical profile for both wild-type and mutant enzymes at low substrate concentrations, but whereas PCV phosphorylation by wild-type HSV-1 TK decreased again at concentrations that were higher than 10 μM, the mutant enzyme gave at least 4-fold higher PCV-MP levels at these higher drug concentrations (Figure 4C). More strikingly, lobucavir was subject of a 20-fold (or higher) degree of phosphorylation by mutant enzyme than wild-type HSV-1 TK at all tested substrate concentrations (Figure 4B). Acyclovir was 2-fold more efficiently converted to its monophosphate derivative by the A167Y mutant HSV-1 TK than wild-type enzyme (Figure 4D). The V_{max} values of the guanine nucleoside analogues for the wild-type and mutant A167Y HSV-1 TK were calculated from the Lineweaver−Burk plots of the kinetic data, and presented in Table 3. They were found to be increased for the mutant enzymes. The K_m values for the guanine nucleoside analogues were also increased in most cases (Table 3).

DISCUSSION

Before any X-ray crystallographic studies were performed, Darby and colleagues had already investigated the active center of the HSV-1 TK and characterized mutations that altered the affinity of the enzyme for nucleoside substrates (*16*). They found that HSV-1 TK, containing a A168T mutation, kept dThd phosphorylating activity but had seriously compromised 5-BV-dUrd affinity. It was concluded that residue 168 presumably did not have a direct role in dThd binding, but may have been positioned in close proximity to the nucleoside binding site so that when a bulkier side-chain was substituted the binding of substrate analogues (such as 5-BV-dUrd) carrying large substituents at the C-5 position of the pyrimidine ring was hindered. Among a wide variety of herpesviral TKs, only alanine or serine is present at the amino acid position 168 (Table 4) (*17*). For the neigboring amino acid position 167, a similar situation, allowing solely alanine or serine, seems to occur (Table 4), and this is in agreement with our observations that a more bulky functional group at position 167 destroys the pyrimidine kinase activity of the enzyme. Since 9 out of 12 different herpesviruses all contain an alanine at position 167 in their TK, this amino acid position can be considered as being fairly conserved.

Our findings that the A167Y mutant HSV-1 GST-fusion TK enzyme was deprived of any measurable pyrimidine (dThd) recombinant phosphorylating activity is also in line with the findings of Dube et al. (*10*), who created a series of artificial HSV-1 TK mutants by the insertion of random oligonucleotides into the putative nucleoside binding site. When the amino acid mutations were introduced at a variety of codons followed by screening for active enzyme mutants by complementation of TK-deficient *E. coli* bacteria, these investigators found that the alanine residue at position 167 could at best be replaced by an uncharged functional serine, threonine, or valine group: no active mutant TK was found that contained a tyrosine or phenylalanine residue at amino acid position 167. Munir et al. (*18*) obtained similar findings when performing mutagenesis studies for 11 codons from positions 165 to 175 in the HSV-1 TK gene. The functional group of the A167 amino acid obviously must be kept small and may be meant to optimally interact with the 5-methyl group and/or C5−C6 part of the pyrimidine ring (Figure 3). Therefore, replacing this small functional group by the bulky 4-hydroxybenzyl group of tyrosine may result in considerable steric hindrance with the pyrimidine ring (Figure 3). This steric hindrance may prevent the enzyme to optimally fit thymidine (and dUrd and dCyd) in its active site, resulting in poor, if any, measurable affinity of the enzyme for its natural pyrimidine nucleoside substrates and leading to undetectable levels of pyrimidine nucleoside (i.e., dThd, BVDU) phosphorylation.

Table 4: Sequence Alignment of the Region of HSV-1 TK Containing Ala-167 with the Homologous Regions from Nucleoside Kinases of Other Origin

herpes simplex virus type 1 TK	161-FDRHPI**A**ALLCYPA-174
herpes simplex virus type 2 TK	161-FDRHPI**A**SLLCYPA-175
Varicella-zoster virus TK	128-SDRHPI**A**STICFPL-141
Epstein−Barr virus TK	391-HDRHLL**S**ASVVFPL-404
equine herpesvirus type 1 TK	137-FDRHPV**A**SAVCFPA-150
equine herpesvirus type 4 TK	137-FDRHPV**A**STVCFPA-150
bovine herpesvirus type 1 TK	132-FDRHPV**A**ACLCYPF-145
marmoset herpesvirus TK	129-VDRHAV**A**SMVCYPL-142
herpesvirus saimiri TK	314-FDRHPL**S**ATVVFPY-327
turkey herpesvirus TK	120-LDRHPV**A**AILCFPI-133
pseudorabies virus TK	107-FDRHPV**A**ATVCFPL-120
Marek's disease virus TK	129-VDRHPV**S**ATVCFPI-142

Our competition experiments of GCV with a variety of pyrimidine nucleoside substrates (dThd, BVDU) and pyrimidine nucleoside inhibitors (Ro316197, KIN-5) revealed that the thymidine analogues not only lost their potential to act as an efficient substrate or inhibitor of the mutant enzyme, but also that their binding to, and thus recognition by the mutant enzyme was seriously compromised. In striking contrast, the activity site for guanosine analogue phosphorylation that partially overlaps with the substrate-binding site occupied by thymidine, was obviously not affected by the A167Y mutation. Instead, the mutant enzyme had a markedly increased phosphorylating capacity for the antiherpetic guanosine analogues. Indeed, all guanine-based nucleoside substrates having different modifications in the sugar ring

(i.e., an aliphatic 1,3-dihydroxy-2-methoxypropyl in GCV, a 2-hydroxyethoxymethyl in ACV, a 2,3-dihydroxymethyl cyclobutyl in LBV, a 2,3-dihydroxymethyl cyclopropyl in A5021 or an aliphatic 3-hydroxymethyl-4-hydroxybutyl in PCV) (Figure 2) were markedly better converted to their monophosphorylated derivatives by the mutant enzyme compared with wild-type enzyme at identical enzyme protein concentrations. Thus, by mutating alanine at amino acid position 167 to tyrosine, HSV-1 TK predominantly phosphorylating pyrimidine nucleosides such as dThd and 5-BV-dUrd could be converted into an enzyme that virtually completely lacked pyrimidine nucleoside phosphorylating capacity, but efficiently phosphorylated guanine nucleosides such as the antiherpetic ACV, GCV, PCV, LBV, and A-5021 derivatives. The substrate inhibition of the wild-type enzyme, noted for GCV, had completely disappeared for the mutant enzyme (Figure 4A). It is puzzling to explain the inhibition of wild-type enzyme in the presence of excess of substrate, but this observation may be related to a change of the oligomeric state of the enzyme in the presence of high substrate concentrations. However, this has not been determined.

Cellular dGuo kinase, a mitochondrial 2′-deoxynucleoside kinase preferentially recognizes the natural substrate dGuo, and also the anticancer nucleoside analogues 2-chloro-dAdo and araG (*19*), but does not show any measurable affinity for dThd or for the antiherpetic acyclic guanosine analogues ACV and GCV. In contrast, HSV-1 TK efficiently recognizes dThd (and dCyd) lacks any measurable dGuo kinase activity but also shows significant affinity (and substrate activity) for the antiherpetic (acyclic) guanosine nucleosides. In fact, there is very little sequence similarity between the narrower substrate-specific dGuo kinase and the broader substrate-specific HSV-1 TK (*20*). In this respect, mitochondrial dGuo kinase and herpes dThd kinase have entirely opposite nucleoside (analogue) kinase activities. However, the mutant herpetic enzyme reported herein has knocked-out pyrimidine nucleoside kinase activity and gained a markedly more pronounced guanosine analogue (e.g., ACV, GCV, PCV, LBV, A5021) kinase activity in terms of increased V_{max} values (Table 3). The mutant enzyme also acquired a limited but measurable dGuo phosphorylating ability. In addition, both the anticancer guanosine derivatives araG and 2′,2′-difluoro-2′-deoxyguanosine that did not measurably act as a substrate for wild-type HSV-1 TK were found to be converted to a minor extent to their 5′-monophosphate derivatives by the A167Y mutant HSV-1 TK (data not shown). Thus, the single A167Y mutant HSV-1 TK mimics much more the substrate specifications of dGuo kinase than wild-type HSV-1 TK, but different from dGuo kinase can efficiently convert the antiherpetic guanosine analogues to their monophosphorylated forms. In this respect, the A167Y mutant HSV-1 TK is clearly superior (with regard to V_{max}) to wild-type HSV-1 TK in phosphorylating the antiherpetic guanosine analogues (Table 3).

From a structural viewpoint, the substrate-binding pocket in HSV-1 TK is very large and can obviously accommodate a wide variety of substrates. It may therefore bind a substrate both in the active position but also in an inactive (and hence competing inhibitory) position. The A167Y mutant HSV-1 TK enzyme has a much larger side chain protruding in the substrate binding pocket and filling it partially. This will not only result in a collision with the thymine ring of dThd (resulting in an annihilation of the dThd substrate activity), but will also restrict the number of available binding modes of GCV and related guanine nucleoside analogues. In this respect, the higher V_{max} values for the guanosine analogues may be explained by lesser possibilities for the mutant enzyme to have inactive binding modes (autoinhibition) of the substrates due to binding restrictions afforded by the 4-hydroxybenzyl ring at amino acid position 167. Such reduced amount of autoinhibition would, indeed, apparently increase the V_{max}. However, further research is needed to prove this hypothesis to explain the consistently and significantly higher V_{max} values of the mutant enzyme for the antiherpetic guanine nucleoside substrate analogues.

Several mutations in the HSV-1 TK have been described to increase the substrate affinity of GCV for the mutated HSV-1 TK enzyme (*10*, *18*, *21*−*23*), while maintaining dThd phosphorylating capacity. It would be of particular interest to introduce these amino acid changes in the A167Y mutated genetic background of the enzyme to reveal whether the affinity for GCV and other antiherpetic guanine nucleoside analogues can be further improved. Our molecular modeling and experimental findings provide a rational molecular basis that enable us to create a HSV-1 nucleoside kinase enzyme with high efficiency and shifted substrate selectivity toward guanosine derivatives. Such mutated enzyme may prove useful in improving the HSV-1 TK based suicide gene therapy with compounds such as GCV by increasing the substrate phosphorylating capacity for antiherpetic guanosine analogues on one hand and by diminishing or annihilating the competition of natural pyrimidine nucleosides (such as dThd) with the phosphorylation of the antiherpetic drugs by the mutant HSV-1 TK.

NOTE ADDED AFTER ASAP POSTING

This article appeared on ASAP on 4/26/02 with an error. Paragraph 2 of the introduction incorrectly referred to M-112 rather than M-128. This correct version was posted 5/14/02.

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