

# Selective Abolishment of Pyrimidine Nucleoside Kinase Activity of Herpes Simplex Virus Type 1 Thymidine Kinase by Mutation of Alanine-167 to Tyrosine

BART DEGRÈVE, ROBERT ESNOUF, ERIK DE CLERCQ, and JAN BALZARINI

*Rega Institute for Medical Research, Laboratory of Virology and Chemotherapy, Leuven, Belgium (B.D., E.D.C., J.B.); and Division of Structural Biology, Wellcome Trust Centre for Human Genetics, Oxford, United Kingdom (R.E.)*

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## ABSTRACT

Herpes simplex virus type 1 (HSV-1) encodes a thymidine kinase (TK) that markedly differs from mammalian nucleoside kinases in terms of substrate specificity. It recognizes both pyrimidine 2'-deoxynucleosides and a variety of purine nucleoside analogs. Based on a computer modeling study and in an attempt to modify this specificity, an HSV-1 TK mutant enzyme containing an alanine-to-tyrosine mutation at amino acid position 167 was constructed. Compared with wild-type HSV-1 TK, the purified mutant HSV-1 TK(A167Y) enzyme was heavily compromised in phosphorylating pyrimidine nucleosides such as (E)-5-(2-bromovinyl)-2'-deoxyuridine and the natural substrate dThd, whereas its ability to phosphorylate the purine nucleoside analogs ganciclovir (GCV) and lobucavir was only reduced ~2-fold. Moreover, a markedly decreased competition of nat-

ural pyrimidine nucleosides (i.e., thymidine) with purine nucleoside analogs for phosphorylation by HSV-1 TK(A167Y) was observed. Human osteosarcoma cells transduced with the wild-type HSV-1 TK gene were extremely sensitive to the cytostatic effects of antiherpetic pyrimidine [i.e., (E)-5-(2-bromovinyl)-2'-deoxyuridine] and purine (i.e., GCV) nucleoside analogs. Transduction with the HSV-1 TK(A167Y) gene sensitized the osteosarcoma cells to a variety of purine nucleoside analogs, whereas there was no measurable cytostatic activity of pyrimidine nucleoside analogs. The unique properties of the A167Y mutant HSV-1 TK may give this enzyme a therapeutic advantage in an in vivo setting due to the markedly reduced dThd competition with GCV for phosphorylation by the HSV-1 TK.

Advances in gene technology offer new possibilities for the treatment of malignancies, including the artificial creation of biochemical differences between neoplastic and non-neoplastic cells. By the insertion in tumor cells of genes encoding for specific enzymes not normally present in mammalian cells, the tumor cells are rendered selectively sensitive to chemotherapeutic agents. These susceptibility genes, often of viral or prokaryotic origin, are designated "suicide genes" because they trigger the transduced cell to commit metabolic suicide by converting the chemotherapeutic agent into a highly toxic metabolite that is able to kill the transduced cell (Blaese et al., 1994; Freeman et al., 1996).

The thymidine kinase (TK) gene encoded by herpes sim-

plex virus type 1 (HSV-1) is by far the most intensively studied suicide gene. HSV-1 TK is able to phosphorylate the naturally occurring nucleosides dThd, dUrd, and dCyd, and is also endowed with thymidylate kinase activity (Fyfe, 1982). In addition, HSV-1 TK is able to phosphorylate a wide range of pyrimidine and purine nucleoside analogs, which forms the basis for the selective therapy for herpesviral disease (De Clercq, 2000) and now, also, cancer gene therapy. The first in vivo demonstration of HSV-1 TK as a cancer suicide gene was about a decade ago, when Culver et al. (1992) showed complete regression of established brain tumors in rats after in situ transduction with the HSV-1 TK gene and subsequent treatment with the acyclic guanosine derivative ganciclovir (GCV; Cymevene; Hoffman-La Roche, Nutley, NJ). After preferential phosphorylation of this acyclic guanosine nucleoside analog by HSV-1 TK gene-transduced cells, the resulting monophosphate derivative is further me-

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**ABBREVIATIONS:** TK, thymidine kinase; HSV-1, herpes simplex virus type 1; GCV, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (ganciclovir); BVDU, (E)-5-(2-bromovinyl)-2'-deoxyuridine; BVDU-MP, 5'-monophosphate of BVDU; BVaraU, (E)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil; S-BVDU, (E)-5-(2-bromovinyl)-2'-deoxy-4'-thiouridine; araT, 1-β-D-arabinofuranosylthymine; LBV, (R)-9-[2,3-bis(hydroxymethyl)cyclobutyl]guanine (lobucavir); ACV, 9-(2-hydroxyethoxymethyl)guanine (acyclovir); PCV, 9-[4-hydroxy-3-(hydroxymethyl)but-1-yl]guanine (penciclovir); BCV, (R)-9-(3,4-dihydroxybutyl)guanine (buciclovir); VZV, varicella-zoster virus; WT, wild-type; GFP, green fluorescent protein; GST, glutathione S-transferase; FACS, fluorescence-activated cell-sorting analysis.

tabolized by cellular enzymes (i.e., guanylate kinase and nucleoside diphosphate kinase) to the triphosphate form. GCV-TP inhibits DNA synthesis by acting as a competitive inhibitor of cellular DNA polymerase (with respect to the natural substrate dGTP) and/or as an alternative substrate for incorporation into the growing DNA chain (Reid et al., 1988).

So far, the few reports of early (predominantly phase I) clinical trials have been encouraging (Izquierdo et al., 1997; Ram et al., 1997; Klatzmann et al., 1998a,b; Sterman et al., 1998; Shand et al., 1999). Possible improvements of the HSV-1 TK/GCV system that have been explored in animal experiments, but that have not yet progressed to clinical trials, include the use of thymidine kinases from other herpesviruses such as herpes simplex virus type 2 (Shimizu et al., 1986; Balzarini et al., 1987), varicella-zoster virus (Huber et al., 1991; Degreuve et al., 1999) or equine herpesvirus type 4 (Loubiere et al., 1999), the use of more efficient and less toxic prodrugs (Balzarini et al., 1993; Shewach et al., 1994), the use of replication-competent vectors (Boviatsis et al., 1994; Wildner et al., 1999), the development of novel routes of GCV administration (Engelmann et al., 1999), and combinations with other anticancer approaches.

Modifications of the wild-type HSV-1 TK may provide another route to improvement. Molecular analysis of the crystal structures of dThd (protein data bank code 2VTK; Wild et al., 1997) and GCV (protein data bank code 1KI2; Champness et al., 1998) complexed with HSV-1 TK revealed that the side chain of the alanine residue at position 167 is very close to the 5-methyl group of thymidine, but that there is a cavity between alanine-167 and the purine ring of GCV. Models for 167-mutated TKs [produced by MidasPlus (Ferrin et al., 1988) and optimized using RotSearch (Esnouf et al., 1997)] showed that the tyrosine mutation might be particularly favorable for discriminating between dThd and GCV substrates (Fig. 1). No acceptable model for dThd bound to A167Y could be constructed, whereas GCV could be accommodated with only minor adjustments. Furthermore, in our model the hydroxyl group of the mutant side chain occupies approximately the same position as a water molecule in the wild-type TK-GCV crystal structure, suggesting that it is a favorable position. Therefore, a specific mutation was introduced in HSV-1 TK (i.e., the replacement of the alanine residue at position 167 by a tyrosine residue) in an attempt to delete the pyrimidine nucleoside kinase activity while preserving the purine nucleoside kinase activity. It was demonstrated that the A167Y mutation resulted in the specific

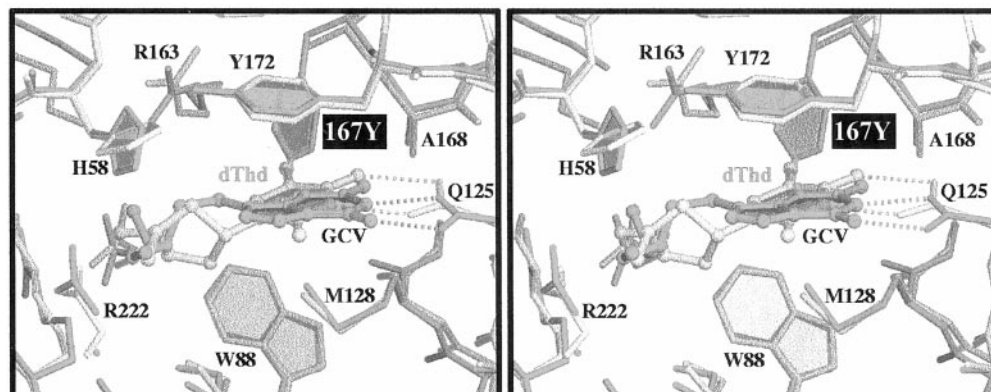
phosphorylation of purine nucleoside analogs but not pyrimidine nucleoside analogs, including the abundant natural substrate dThd. Lack of significant endogenous competition of purine nucleoside analogs with natural nucleosides for phosphorylation may be an attractive approach to improve the phosphorylation of purine nucleoside analogs such as GCV in an in vivo setting, resulting in an increased cytostatic potential of these compounds.

## Materials and Methods

**Compounds.** BVDU and BVDU-MP were synthesized by Prof. P. Herdewijn at the Rega Institute for Medical Research (Katholieke Universiteit Leuven, Leuven, Belgium). BVaraU was a kind gift of Prof. H. Machida (Yamasa Shoyu Co., Choshi, Japan). The late Prof. R. T. Walker (University of Birmingham, UK) provided S-BVDU. AraT was a kind gift from M. Sandvold and F. Myhren (Norsk Hydro, Porsgrunn, Norway). GCV was from Roche (Brussels, Belgium) and lobucavir (LBV) from Bristol-Myers Squibb (Wallingford, CT). Acyclovir (ACV) was obtained from the former Wellcome Research Laboratories (Research Triangle Park, NC). Penciclovir (PCV) was obtained from Dr. I. Winkler (Hoechst, Frankfurt, Germany) and buciclovir (BCV) from Astra Läkemedel (Södertälje, Sweden). dThd and dTMP were from Sigma (St. Louis, MO).

**Cells.** Adherent human osteosarcoma 143B cells deficient in cytosol TK (designated OstTK<sup>-</sup>) were kindly provided by Prof. M. Izquierdo (Universidad Autónoma de Madrid, Madrid, Spain). OstTK<sup>-</sup>, OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup>, and OstTK<sup>-</sup>/HSV-1 TK(A167Y)<sup>+</sup> cells were maintained at 37°C in a humidified CO<sub>2</sub>-controlled atmosphere in Eagle's minimal essential medium (Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (Integro, Zaandam, The Netherlands), 2 mM L-glutamine (Life Technologies), 0.075% (w/v) NaHCO<sub>3</sub> (Life Technologies), 0.5 µl/ml geomycine (Gentamycin, 40 mg/ml; Schering-Plough, Madison, NJ), and 0.5 µl/ml Amphotericin.

**Construction, Expression, and Purification of Wild-Type and A167Y Mutant HSV-1 TK.** HSV-1 TK(WT) and HSV-1 TK(A167Y) were expressed in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins as follows. The HSV-1 TK coding sequence was amplified by polymerase chain reaction using primers 5'-GAGGAATTCATGGCTTCGTACCCCGGCCATC-3' and 5'-CTCGTCGACTCAGTTAGCCTCCCCCATCTCC-3' (Kebo Lab, Stockholm, Sweden) with the pMCTK plasmid (kindly provided by Dr. D. Ayusawa, Yokohama University, Yokohama, Japan) as a template, and ligated between the *Eco*RI and *Sal*I sites of the pGEX-5X-1 vector (Amersham Pharmacia Biotech, Uppsala, Sweden). From this plasmid, the pGEX-5X-1-HSV-1 TK(A167Y) vector was constructed according to the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) protocol. The A167Y mutant primers (5'-GCACAGGAGGGCGTAGATGGGATGCGGTCT-3' and complementary antisense primer) were from Life



**Fig. 1.** A stereodiagram showing the superimposed substrate binding sites for the crystal structure of HSV-1 TK complexed with dThd (gray sticks for the protein with dThd as a gray ball-and-stick model) and for a model for A167Y-mutated HSV-1 TK complexed with GCV (black sticks for the protein with GCV as a black ball-and-stick model). Figure produced with Bobscript (Esnouf, 1997) and rendered with Raster 3D (Merritt and Murphy, 1994).

Technologies. After linear amplification of the primers using *Pfu* DNA polymerase (Stratagene) and pGEX-5X-1-HSV-1 TK(WT) vector as a template in a temperature cycler program (30 min at 95°C; 20 cycles 30 min at 95°C, 1 min at 55°C, 12 min at 68°C), wild-type (methylated) plasmid was digested with *DpnI* restriction enzyme (Stratagene) and the mutant (unmethylated) DNA was transformed into competent *E. coli* DH5 $\alpha$ . Plasmid preparations from ampicillin-resistant colonies were checked by automated fluorescence sequencing (ALFexpress; Amersham Pharmacia Biotech) and transfected in *E. coli* BL21(DE3)pLysS. Transfected bacteria were grown overnight in 2YT medium containing ampicillin (100  $\mu$ g/ml) and chloramphenicol (40  $\mu$ g/ml) and then diluted in fresh medium. After further growth of the bacteria at 27°C (for 1 h), isopropyl- $\beta$ -D-thiogalactopyranoside (Sigma) 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 pelleted (7700g 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 (20,000g 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. The protein content of the purified fractions was assessed using Bradford reagent (Sigma).

**Construction of Wild-Type and Mutant HSV-1 TK Mammalian Expression Vectors.** The HSV-1 TK(WT) and HSV-1 TK(A167Y) genes were ligated into the pEGFP-N1 N-terminal protein fusion vector (Clontech, Palo Alto, CA). The construction of the mammalian HSV-1 TK(WT)-GFP expression vector was described previously (Degrève et al., 1999). The HSV-1 TK(A167Y) gene was also ligated into the pEGFP-N1 N-terminal protein fusion vector (Clontech) after amplification from the pGEX-5X-1-HSV-1 TK(A167Y) vector. The TK sequences were subsequently checked using automated fluorescence sequencing (ALFexpress).

**Transduction of Tumor Cells and FACS Analysis.** The HSV-1 TK(WT)-GFP and HSV-1 TK(A167Y)-GFP fusion gene constructs were introduced into OstTK<sup>-</sup> cells via membrane fusion-mediated transfer using plasmid/liposome complexes (Lipofectamine reagent; Life Technologies), as described by the supplier. The OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup> cell line was established as described previously (Degrève et al., 1999). OstTK<sup>-</sup>/HSV-1 TK(A167Y)<sup>+</sup> cells were isolated after selection in the presence of 0.5 mg/ml Geneticin (Duchefa, Haarlem, The Netherlands) and cloned by limited dilution. Nontransduced and TK gene-transduced cell lines were prepared for FACS analysis by trypsinization, two wash steps with PBS, and fixation with 1% paraformaldehyde in PBS (10<sup>6</sup> cells/ml). The fluorescence of the cells was measured on a FACScan flow cytometer equipped with CellQuest software (Beckton Dickinson, Grenoble, France).

**Inhibition of Tumor Cell Proliferation by Antiherpetic Compounds.** The cytostatic activity of antiviral nucleoside analogs against wild-type and TK gene-transduced osteosarcoma cells was evaluated as described previously (Degrève et al., 1999). Briefly, 10<sup>4</sup> OstTK<sup>-</sup>, OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup>, or OstTK<sup>-</sup>/HSV-1 TK(A167Y)<sup>+</sup> cells/well were plated in 96-well microtiter plates and subsequently incubated in the presence of 5-fold dilutions (in normal growth medium) of the compounds. After 3 days, the number of cells was evaluated in a Coulter counter (Coulter Electronics Ltd., Harpenden, UK). IC<sub>50</sub> was defined as the drug concentration required to inhibit tumor cell proliferation by 50%.

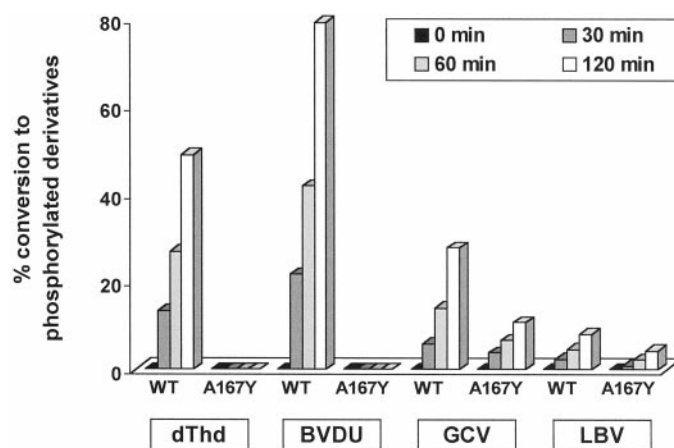
**TK/dTMP Kinase Assays.** The ability of the purified GST-HSV-1 TK(WT) and GST-HSV-1 TK(A167Y) preparations to phosphorylate dThd, dTMP, BVDU, BVDU-MP, GCV, and LBV was determined as follows. The standard reaction mixture contained 2.5 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 1 mg/ml BSA, 2.5 mM ATP, 10 mM NaF, 100  $\mu$ M substrate, and 0.066  $\mu$ g TK preparation in a total reaction mixture of 50  $\mu$ l of 50 mM Tris-HCl, pH 8.0. The reaction mixture was incubated at 37°C for 30, 60, and/or 120 min. The reaction mixtures were subjected to HPLC analysis using a Partisphere-SAX column. A

linear gradient of 5 mM (NH<sub>4</sub>)H<sub>2</sub>PO<sub>4</sub>, pH 5.0 (buffer A) to 0.3 M (NH<sub>4</sub>)H<sub>2</sub>PO<sub>4</sub>, 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.

The influence of dThd on the phosphorylation of [8-<sup>3</sup>H]GCV was determined by incubating 50- $\mu$ l reaction mixtures at 37°C for 2 h in the presence of 6.8  $\mu$ M [8-<sup>3</sup>H]GCV (5  $\mu$ Ci) and a variety of dThd concentrations (i.e., 1, 5, 25, and 100  $\mu$ M). The reactions were terminated by spotting an aliquot of 30  $\mu$ l onto DE-81 discs (Whatman, Maidstone, UK) that were instantly immersed and thoroughly washed in ethanol (70%). Finally, the discs were dried and assayed for radioactivity in a toluene-based scintillant.

## Results

**Pyrimidine and Purine Nucleoside Kinase Activity of HSV-1 TK(WT) and HSV-1 TK(A167Y).** The purified HSV-1 TK(WT) and HSV-1 TK(A167Y) enzymes were compared with regard to their abilities to phosphorylate dThd, BVDU, GCV, LBV, dTMP, and BVDU-MP. As shown previously, wild-type HSV-1 TK converted dThd and BVDU efficiently to the corresponding monophosphate forms (Fig. 2). In addition, a fraction of the monophosphorylated dThd and BVDU was further converted to the corresponding diphosphate derivatives. Up to 50% of 100  $\mu$ M dThd and even up to 80% of 100  $\mu$ M BVDU were phosphorylated by HSV-1 TK(WT) after a 120-min incubation period (Fig. 2). In sharp contrast, purified HSV-1 TK(A167Y) did not measurably recognize dThd or BVDU as a substrate because no phosphorylated derivatives were detectable after 120 min of incubation (the detection limit was approximately 0.5% of wild-type enzyme activity). Wild-type HSV-1 TK was able to phosphorylate 6, 14, and 28% of 100  $\mu$ M GCV after 30-, 60-, and 120-min incubation, respectively, whereas mutant HSV-1 TK(A167Y) was only 1.5- to 2.5-fold less efficient, phosphorylating 4, 7, and 11% of the available GCV at these time points (Fig. 2). Also, up to 8.0% of 100  $\mu$ M LBV was phosphorylated by wild-type HSV-1 TK after a 2-h incubation



**Fig. 2.** Purine and pyrimidine nucleoside kinase activity of purified HSV-1 TK(WT) and HSV-1 TK(A167Y). The ability of purified GST-HSV-1 TK(WT) and GST-HSV-1 TK(A167Y) preparations to phosphorylate dThd, BVDU, GCV, and LBV was determined. Kinase reaction mixtures were subjected to HPLC analysis using a Partisphere-SAX column. The relative conversion of the substrates was calculated from the chromatogram peak areas. The columns indicating the relative conversion of dThd and BVDU in A and B represent the sum of the conversions to the mono- and diphosphate derivatives.



period, whereas mutant HSV-1 TK(A167Y) phosphorylated up to 4.3% of 100  $\mu$ M LBV under the same experimental conditions (Fig. 2). Wild-type HSV-1 TK recognized dTMP or BVDU-MP as substrates, phosphorylating 88% of dTMP and 35% of BVDU-MP to the corresponding diphosphate forms after 2 h of incubation. No diphosphorylated metabolites were detectable with the HSV-1 TK(A167Y) mutant when either monophosphorylated dThd or BVDU was added to the reaction mixture (data not shown).

We next determined to what extent dThd is able to interfere with the phosphorylation of GCV by wild-type and A167Y mutant HSV-1 TK. As shown in Fig. 3, the phosphorylation of [8-<sup>3</sup>H]GCV by HSV-1 TK(WT) was virtually completely inhibited in the presence of dThd concentrations of 25 and 100  $\mu$ M. In contrast, the phosphorylation of [8-<sup>3</sup>H]GCV by HSV-1 TK(A167Y) was only moderately inhibited under these experimental conditions (Fig. 3).

**Transduction of Osteosarcoma Cells with Wild-Type and Mutant HSV-1 TK Genes.** The wild-type and A167Y-mutant HSV-1 TK genes were transduced into TK-deficient human osteosarcoma cells (designated OstTK<sup>-</sup>) as fusion genes with the coding sequence of GFP from the jellyfish *Aequorea victoria* (Chalfie et al., 1994; Rizzuto et al., 1995). The expression of the wild-type HSV-1 TK-GFP and the A167Y-mutant HSV-1 TK-GFP fusion proteins in the OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup> and OstTK<sup>-</sup>/HSV-1 TK(A167Y)<sup>+</sup> cell lines, respectively, was evaluated by FACS analysis. Nontransduced OstTK<sup>-</sup> cells were included as a control (Fig. 4A). The levels of fluorescence, and hence the levels of TK-GFP fusion gene expression, in the OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup>

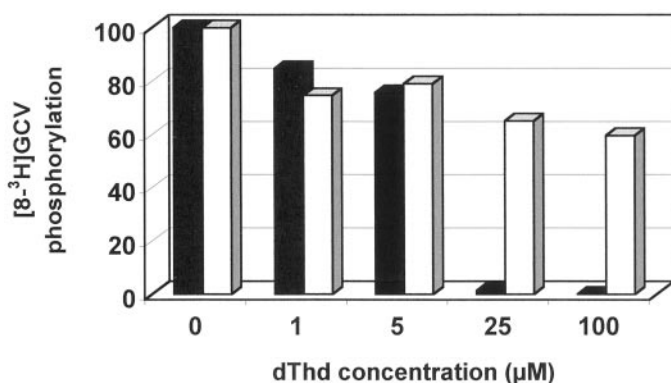
(Fig. 4B) and OstTK<sup>-</sup>/HSV-1 TK(A167Y)<sup>+</sup> (Fig. 4C) cell lines were very pronounced and highly comparable.

**Cytostatic Effect of Antiherpetic Compounds against HSV-1 TK(WT) and HSV-1 TK(A167Y) Gene-Transduced Osteosarcoma Cells.** OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup> cells were compared with OstTK<sup>-</sup>/HSV-1 TK(A167Y)<sup>+</sup> cells with regard to their sensitivity to the cytostatic action of a selection of antiherpetic pyrimidine and purine nucleoside analogs (Fig. 5). The results for OstTK<sup>-</sup> and OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup> cells have been published (Degrève et al., 1999) but are included in Table 1 to allow proper comparison. The proliferation of OstTK<sup>-</sup> cells was not markedly inhibited by any of the evaluated compounds except for LBV and GCV, which inhibited OstTK<sup>-</sup> cell growth by 50% at concentrations of 18 and 44  $\mu$ M, respectively. All purine nucleoside analogs showed potent inhibition of the proliferation of OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup> cells, displaying 50% inhibitory concentrations ranging from 0.006  $\mu$ M (LBV) to 0.059  $\mu$ M (ACV). ACV and GCV were 10- to 30-fold less inhibitory to OstTK<sup>-</sup>/HSV-1 TK(A167Y)<sup>+</sup> cells compared with OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup> cells. The cytostatic activities of the other purine nucleoside analogs (i.e., LBV, PCV, and BCV) against HSV-1 TK gene-transduced tumor cells were not markedly influenced by the A167Y mutation (Table 1).

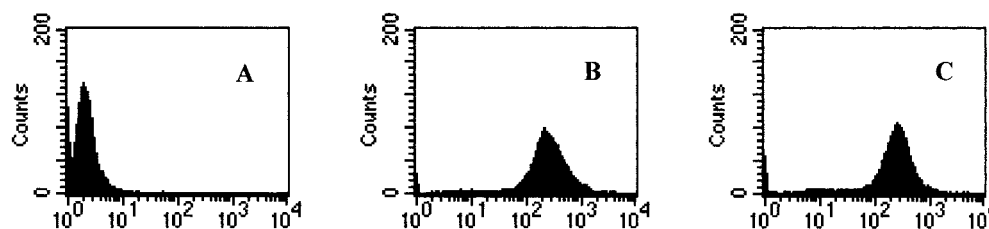
All the pyrimidine nucleoside analogs that were evaluated (i.e., BVDU, BVaraU, S-BVDU, and araT) showed potent inhibitory effects on the growth of OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup> cells. In striking contrast, the pyrimidine nucleoside analogs were devoid of any measurable cytostatic activity against HSV-1 TK(A167Y) gene-transduced tumor cells at concentrations as high as 250  $\mu$ M. Thus, the pyrimidine nucleoside analogs were at least 7,000- to 62,000-fold less inhibitory to OstTK<sup>-</sup>/HSV-1 TK(A167Y)<sup>+</sup> cells compared with OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup> cells (Table 1).

## Discussion

Because purine nucleoside analogs such as GCV have a relatively poor affinity for HSV-1 TK, endogenous dThd may seriously hamper the efficiency of HSV-1 TK-mediated GCV phosphorylation. Therefore, if HSV-1 TK can be engineered to a more selective enzyme that exclusively phosphorylates purine but not pyrimidine nucleosides (including dThd), lack of significant endogenous competition with natural substrates may favor GCV phosphorylation. With this in mind, the Ala-to-Tyr mutation at position 167 of HSV-1 TK was introduced in an attempt to increase the relative affinity of HSV-1 TK for purine over pyrimidine nucleosides. Although the predicted increased selectivity of the mutant enzyme for phosphorylating the guanine nucleoside derivatives was observed, superior cytostatic activity of GCV and other purine



**Fig. 3.** Influence of dThd on the phosphorylation of [8-<sup>3</sup>H]GCV by purified HSV-1 TK(WT) (■) and HSV-1 TK(A167Y) (□). The ability of purified GST-HSV-1 TK(WT) and GST-HSV-1 TK(A167Y) preparations to phosphorylate 6.8  $\mu$ M [8-<sup>3</sup>H]GCV was determined in the presence of different concentrations of dThd. Nucleoside kinase reactions mixtures were spotted on DE-81 discs that were subsequently washed and assayed for radioactivity.

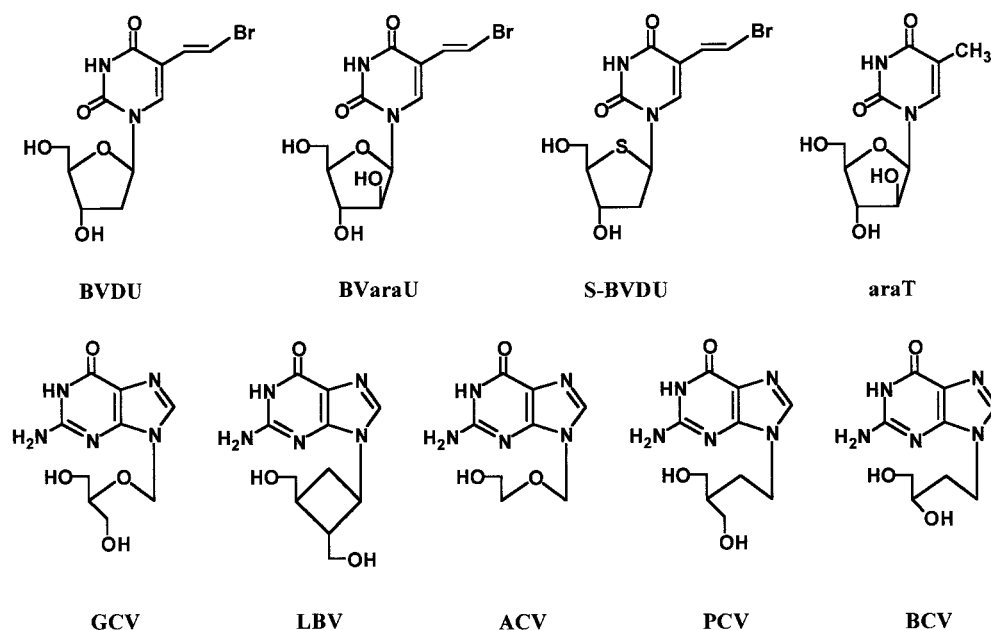


**Fig. 4.** Flow cytometric assessment of TK-GFP fusion gene expression in HSV-1 TK(WT) and HSV-1 TK(Q125N) fusion gene-transduced OstTK<sup>-</sup> cells. HSV-1 TK-GFP fusion gene-transduced cells were analyzed on a FACSscan flow cytometer to assess the level of fusion gene expression, as described under *Materials and Methods*. Nontransduced OstTK<sup>-</sup> cells served as control. A, OstTK<sup>-</sup>; B, OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup>; C, OstTK<sup>-</sup>/HSV-1 TK(A167Y)<sup>+</sup>.

nucleoside analogs in the A167Y-mutant HSV-1 TK gene-transduced cells was not observed. However, it may be assumed that endogenous dThd pools are relatively limited in tumor cell cultures but larger in living subjects due to the uptake of dThd from food. Therefore, *in vivo* studies are underway to assess the value of this novel mutant enzyme in the combined gene/chemotherapy of cancer.

Our data corroborate nicely the experiments performed by Dube et al. (1991), who generated a series of HSV-1 TK mutants by inserting random nucleotide sequences at codons 166 and 167 and then screening for active mutants by complementation of TK-deficient *E. coli* bacteria. They concluded that the alanine residue at position 167 can best be replaced by hydrophobic amino acids (alanine, tryptophan, or valine) or by polar amino acids with an uncharged functional group (serine and threonine). Interestingly, no active TK mutants were found that contained a tyrosine or phenylalanine residue at position 167. A similar procedure was later used to select for active HSV-1 TK mutants after randomization of 11 codons in the HSV-1 TK gene (coding for amino acids 165–175). Again, no active mutants were isolated that contained a tyrosine or phenylalanine residue at position 167, whereas replacement of the alanine-167 residue by

other amino acids (e.g., serine, threonine, and glycine) was tolerated (Munir et al., 1992). These mutagenesis data are in agreement with alignments of primary amino acid sequences of herpesvirus thymidine kinases (Table 2), where the alanine at position 167 is highly conserved and is only replaced by a serine residue in the TKs of a few members of the herpesvirus family (Balasubramaniam et al., 1990). Alanine-167 lies between two highly conserved sites in herpesviral TKs [i.e., between the -DRH- motif and the -C(Y/F)P motif (Balasubramaniam et al., 1990)]. Also, human 2'-deoxycytidine kinase and human 2'-deoxyguanosine kinase contain an alanine at the corresponding amino acid position, whereas human TK2 and the recently cloned multifunctional insect deoxynucleoside kinase contain a valine residue at the equivalent amino acid position (Johansson et al., 1999) (Table 2). Data in Table 2 also indicate that an alanine residue at position 167 is not the only determining factor for purine/pyrimidine nucleoside kinase specificity because, for example, varicella-zoster virus (VZV) TK has a preference for pyrimidine nucleosides yet does contain an alanine residue at position 167, whereas 2'-deoxyguanosine kinase, which has a high specificity for certain purine nucleosides, also contains an alanine residue at position 167.



**Fig. 5.** Structural formulae of antiherpetic pyrimidine and purine nucleoside analogs.

**TABLE 1**

Cytostatic activity of antiherpetic nucleoside analogs against OstTK<sup>-</sup>, OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup>, and OstTK<sup>-</sup>/HSV-1 TK(A167Y)<sup>+</sup> cells

Compound	IC <sub>50</sub> (μM) <sup>a</sup>			IC <sub>50</sub> ratio <sup>b</sup>
	OstTK <sup>-b</sup>	OstTK <sup>-</sup> /HSV-1 TK(WT) <sup>+</sup> c	OstTK <sup>-</sup> /HSV-1 TK(A167Y) <sup>+</sup>	
BVDU	862 ± 192	0.035 ± 0.006	>250	>7,000
BVaraU	942 ± 47	0.004 ± 0.001	>250	>62,000
S-BVDU	911 ± 105	0.008 ± 0.004	>250	>31,000
araT	231 ± 27	0.004 ± 0.001	>250	>62,000
GCV	44 ± 22	0.0011 ± 0.0005	0.035 ± 0.008	32
LBV	18 ± 1	0.008 ± 0.001	0.011 ± 0.001	1.3
ACV	73 ± 29	0.059 ± 0.015	0.51 ± 0.19	8.6
PCV	231 ± 13	0.013 ± 0.002	0.037 ± 0.007	2.8
BCV	173 ± 67	0.006 ± 0.001	0.012 ± 0.002	2.0

<sup>a</sup> The 50% inhibitory concentration, or the compound concentration required to inhibit tumor-cell proliferation by 50%. Data are the mean ± S.D. of at least three independent experiments.

<sup>b</sup> Ratios of the IC<sub>50</sub> values for OstTK<sup>-</sup>/HSV-1 TK(A167Y)<sup>+</sup> cells versus the IC<sub>50</sub> values for OstTK<sup>-</sup>/HSV-1 TK(WT)<sup>+</sup> cells.

Previous experiments performed in our laboratory demonstrated that combinations of nucleoside analogs (such as GCV and BVDU) did not exert superior cytostatic activities against HSV-1 TK gene-transduced tumor cells compared with the individual compounds. This was most likely due to mutual competition for phosphorylation by HSV-1 TK. The availability of VZV TK, which has a selective preference for pyrimidine but not purine nucleosides (Degrève et al., 1999), and the purine nucleoside-selective A167Y mutant of HSV-1 TK now allows us rationally to combine TKs with different substrate specificities to minimize the competition of purine and pyrimidine nucleoside analogs for phosphorylation. Indeed, combined transduction of tumor cells with the genes encoding for VZV TK (displaying high affinity for pyrimidine but not purine nucleoside analogs) and HSV-1 TK(A167Y) (displaying high affinity for purine but not pyrimidine nucleoside analogs) could give rise to concomitant high intracellular levels of both pyrimidine and purine nucleoside triphosphates. We recently constructed a mutant HSV-1 TK enzyme that lacked the thymidylate kinase activity but preserved the thymidine kinase activity (our unpublished data). The combination of this HSV-1 TK mutant and the HSV-1 TK(A167Y) mutant is even more promising because the thymidylate kinase-deficient mutant will yield high intracellular levels of pyrimidine nucleoside monophosphates (inhibiting cellular thymidylate synthase), whereas the A167Y mutant should give rise to high levels of purine nucleoside triphosphates (inhibiting cellular DNA polymerase and/or incorporating into cellular DNA). In this way, two different cytostatic targets can be simultaneously envisioned in one tumor cell population.

In conclusion, we have rationally designed and constructed a mutant of HSV-1 TK with an altered substrate specificity for potential use in TK suicide gene therapy of cancer. The A167Y mutant of HSV-1 TK proved incapable of measurably phosphorylating pyrimidine nucleoside analogs, including the natural substrate dThd, which could enhance the phosphorylation of purine nucleoside analogs such as GCV and LBV in an in vivo setting.

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TABLE 2

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-FDRHP1AALLCYPA-174
Herpes simplex virus type 2 TK	162-FDRHP1ASLLCYPA-175
Varicella-zoster virus TK	128-SDRHP1ASTICFPL-141
Epstein-Barr virus TK	391-HDRHLLSASVFP-404
Equine herpes virus type 1 TK	137-FDRHPVAVSACFPA-150
Equine herpes virus type 4 TK	137-FDRHPVASTVCFPA-150
Bovine herpesvirus type 1 TK	132-FDRHPVAAALCYPF-145
Marmoset herpesvirus TK	129-VDRHAVASMCYPL-142
Herpesvirus saimiri TK	314-FDRHPLSATVFPY-327
Turkey herpesvirus TK	120-LDRHPVAAILCFPI-133
Pseudorabies virus TK	107-FDRHPVAATVCFPL-120
Marek's disease virus TK	129-VDRHPVSATVCFPI-142
Infectious laryngotracheitis virus TK	146-VDRHPLAALCFVPY-159
Canine herpes virus TK	114-FDRHP1ASIVCFPL-127
Human 2'-deoxyguanosine kinase	146-SDRYIFAKNL-FEN-158
Human 2'-deoxycytidine kinase	132-SDRYIFAKNL-YES-144
Human TK2	107-SARYIFVENL-YRS-119
<i>Drosophila melanogaster</i> 2'-deoxynucleoside kinase	109-SARYCFVENM-RRN-121

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**Send reprint requests to:** Jan Balzarini, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium. E-mail: jan.balzarini@rega.kuleuven.ac.be

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