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Mutation of Gln125 to Asn Selectively Abolishes the Thymidylate Kinase Activity of Herpes Simplex Virus Type 1 Thymidine Kinase

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ABSTRACT

The broad substrate specificity of herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) has provided the basis for selective antiherpetic therapy and, more recently, suicide gene therapy for the treatment of cancer. We have now constructed an HSV-1 TK mutant enzyme, in which an asparagine (N) residue is substituted for glutamine (Q) at position 125, and have evaluated the effect of this amino acid change on enzymatic activity. In marked contrast with wild-type HSV-1 TK, which displays both thymidine kinase and thymidylate kinase activities, the HSV-1 TK(Q125N) mutant was unable to phosphorylate pyrimidine nucleoside monophosphates but retained significant phosphorylation activity for thymidine and a series of antiherpetic pyrimidine and purine nucleoside analogs. The abrogation of HSV-1 TK-associated thymidylate kinase activity resulted in a 100-fold accumulation of the monophosphate form of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) in osteosarcoma cells transfected with the HSV-1 TK(Q125N) gene compared with osteosarcoma cells expressing wild-type HSV-1 TK. BVDU monophosphate accumulation gave rise to a much greater inhibition of cellular thymidylate synthase in HSV-1 TK(Q125N) gene-transfected cells than wild-type HSV-1 TK gene-transfected osteosarcoma tumor cells without significantly changing the cytostatic potency of BVDU for the HSV-1 TK gene-transfected tumor cells. Accordingly, the presence of the Q125N mutation in HSV-1 TK gene-transfected tumor cells was found to result in a multilog decrease in the cytostatic activity of those pyrimidine nucleoside analogs that in their monophosphate form do not have marked affinity for thymidylate synthase [i.e., 1-β-D-arabinofuranosylthymine and (*E*)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil].

Targeted transduction of tumor cells with a gene whose product can convert a relatively nontoxic prodrug to a toxic metabolite, thereby creating artificial metabolic differences between normal and malignant cells, constitutes the basic principle underlying metabolic suicide gene therapy. The most intensively studied suicide gene/prodrug system is represented by the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) gene in combination with the antiherpetic nucleoside analog ganciclovir (GCV; Cymevene) (Balzarini et al., 1985, 1993, 1994; Culver et al., 1992). After preferential phosphorylation of this acyclic guanosine nucleoside analog by HSV-1 TK, the resulting monophosphate derivative is further metabolized by cellular enzymes 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 an alternative substrate for incorporation into the growing DNA chain (Reid et al., 1988).

Culver et al. (1992) designed the first in situ suicide gene transfer experiment to treat cerebral gliomas. Rats carrying a previously implanted 9L gliosarcoma received a stereotaxic intratumoral injection of murine fibroblasts that had been engineered to release murine replication-defective retroviruses containing the HSV-1 TK gene. Subsequent GCV treatment was capable of mediating complete tumor destruction in 80% of the rats with several long-term survivors (Culver et al., 1992), although the efficiency of tumor transduction was shown to be suboptimal (Short et al., 1990; Ram et al., 1993). This phenomenon was designated the 'bystander effect' and

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ABBREVIATIONS: HSV, herpes simplex virus; TK, thymidine kinase; GCV, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (ganciclovir); ACV, 9-(2-hydroxyethoxymethyl)guanine (acyclovir); BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; TS, thymidylate synthase; 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); BCV, (*R*)-9-(3,4-dihydroxybutyl)guanine (buciclovir); PCV, 9-[4-hydroxy-3-(hydroxymethyl)but-1-yl]guanine (penciclovir); GST, glutathione-*S*-transferase; WT, wild-type; HPLC, high-performance liquid chromatography; GFP, green fluorescent protein.

was shown to be mediated by: 1) transfer of phosphorylated GCV metabolites through intercellular gap junctions (Bi et al., 1993; Degrève et al., 1999); 2) phagocytosis by nontransfected cells of apoptotic vesicles, containing GCV metabolites, from dying HSV-1 TK gene-transfected cells (Freeman et al., 1993); 3) induction of an antitumor immune response (Vile et al., 1994); and/or 4) GCV-mediated destruction of the tumor vasculature upon occasional transfection of endothelial cells with the HSV-1 TK gene (Ram et al., 1994).

Fifty of the 400 gene marker/gene therapy protocols that have been submitted for approval by the Recombinant DNA Advisory Committee, altogether enrolling one sixth of all gene therapy patients, involve the transfer of suicide genes, almost exclusively evaluating the combination of the HSV-1 TK gene with GCV (Freeman et al., 1996; Rosenberg et al., 1999). These early (predominantly phase I) clinical trials have indicated the potential efficacy of the HSV-1 TK/GCV system, but have also emphasized the limitations of this combination treatment such as the dose-limiting toxicity of systemic GCV treatment (Markham and Faulds, 1994) and the incompetence of current gene therapy vectors to introduce the TK gene into all the cells of a particular tumor (Ram et al., 1997). Possible improvements of the HSV-1 TK/GCV system include the development of more efficient and less toxic prodrugs such as (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) (Balzarini et al., 1985; Balzarini et al., 1993, 1994; Shewach et al., 1994), the use of thymidine kinases from other herpesviruses, such as HSV-2 (Shimizu et al., 1986; Balzarini et al., 1987), varicella-zoster virus (Huber et al., 1991; Degrève et al., 1997), or Equine herpesvirus type 4 (Loubiere et al., 1999), or the use of nucleoside kinases from other sources such as the recently cloned multifunctional insect deoxynucleoside kinase (Johansson et al., 1999).

Modifications of the wild-type HSV-1 TK may provide another route to improvement. Black et al. (1996) have performed random sequence mutagenesis in the putative nucleoside binding site of HSV-1 TK and identified mutants that, upon transfection in mammalian cells, displayed enhanced sensitivity to GCV and ACV. One of these mutants, containing six amino acid substitutions, was later shown to mediate markedly enhanced tumor cell killing in vitro and in vivo compared with wild-type HSV-1 TK (Kokoris et al., 1999). The enhanced sensitization apparently originated from the increased preference of the mutant enzyme for phosphorylating GCV and acyclovir (ACV) over the competing natural substrate, thymidine (Black et al., 1996; Kokoris et al., 1999).

We have now constructed a mutant HSV-1 TK with a Gln-to-Asn substitution at position 125, and expressed the wild-type and Q125N mutant HSV-1 TK in *Escherichia coli* and human osteosarcoma cells. In vitro analysis of purified HSV-1 TK(Q125N) revealed a complete lack of thymidylate kinase activity (which is associated with wild-type HSV-1 TK), while keeping pronounced thymidine kinase activity. Treatment of HSV-1 TK(Q125N) gene-transfected tumor cells with BVDU resulted in a marked accumulation of BVDU monophosphate and, hence, strong inhibition of thymidylate synthase. Conversely, pyrimidine nucleoside analogs for which the monophosphate derivatives do not have affinity for thymidylate synthase (TS), were 100- to 10,000-fold less inhibitory to the growth of cells transfected by the wild-type compared with those transfected with the Q125N HSV-1 TK mutant. The Q125N mutation fully sustained the potent cytostatic and bystander effect of a series of purine nucleoside analogs, including GCV.

Materials and Methods

Compounds. BVDU and BVDU-MP were synthesized by P. Herdewijn at the Rega Institute for Medical Research (Katholieke Universiteit Leuven, Belgium). (*E*)-5-(2-Bromovinyl)-1-β-D-arabinofuranosyluracil (BVaraU) was a kind gift of H. Machida (Yamasa Shoyu Co., Choshi, Japan). (*E*)-5-(2-Bromovinyl)-2′-deoxy-4′-thiouridine (*S*-BVDU) was provided by the late R. T. Walker (University of Birmingham, UK). 1-β-D-Arabinofuranosylthymine (araT) was a kind gift from M. Sandvold and F. Myhren (Norsk Hydro, Porsgrunn, Norway). GCV was from Roche (Brussels, Belgium) and lobucavir (LBV) was from Bristol-Myers Squibb (Wallingford, CT). ACV was obtained from the former Wellcome Research Laboratories (Research Triangle Park, NC). Penciclovir (PCV) was obtained from I. Winkler (Hoechst, Frankfurt, Germany) and buciclovir (BCV) from Astra Läkemedel (Sodertälje, Sweden). dThd and dTMP were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. Adherent human osteosarcoma 143B cells deficient in cytosol TK (designated OstTK$^-$) were kindly provided by Prof. M. Izquierdo (Universidad Autónoma de Madrid, Spain). OstTK$^-$, OstTK$^-$/HSV-1 TK(WT)$^+$ and OstTK$^-$/HSV-1 TK(Q125N)$^+$ cells were maintained at 37°C in a humidified CO_2-controlled atmosphere in Eagle's minimal essential culture 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) $NaHCO_3$ (Life Technologies), 0.5 μl/ml geomycine (40 mg/ml gentamycin; Schering-Plough, Madison, NJ) and 0.5 μl/ml Amphotericin B (5 mg/ml Fungizone, Bristol-Myers Squibb, Brussels, Belgium).

Construction, Expression, and Purification of Wild-Type and Q125N Mutant HSV-1 TK. HSV-1 TK(WT) and HSV-1 TK(Q125N) were expressed in *E. coli* as glutathione-*S*-transferase (GST) fusion proteins as follows. The HSV-1 TK coding sequence was amplified by PCR using primers 5′-GAGGAATTCATGGCTTCGTACCCCGGCCATC and 5′-CTCGTCGACTCAGTTAGCCTCCCCCATCTCC (Kebo Lab, Stockholm, Sweden) with the pMCTK plasmid (kindly provided by Dr. D. Ayusawa, Yokohama University, 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(Q125N) vector was constructed according to the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) protocol. The Q125N mutant primers (5′-GGTAATGACAAGCGCCAACATAACAATGGGCATGC and complementary antisense primer) were from Life 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 s at 95°C; 20 cycles of 30 s at 95°C, 1 min at 55°C, and 12 min at 68°C), wild-type (methylated) plasmid was digested with *Dpn*I restriction enzyme (Stratagene) and the mutant (unmethylated) DNA was transformed into competent *E. coli* DH5α. Plasmid preparations from ampicillin-resistant colonies were checked by automated fluorescence sequencing (ALFexpress; Amersham Pharmacia Biotech).

The pGEX-5X-1-HSV-1 TK(WT) and pGEX-5X-1-HSV-1 TK(Q125N) vectors were transfected in *E. coli* BL21(DE3)pLysS. Transfected bacteria were grown overnight in medium containing 16 g/l tryptone, 10 g/l yeast extract, and 5 g/l NaCl, with ampicillin (100 μg/ml) and chloramphenicol (40 μg/ml) and then diluted in fresh medium. After further growth of the bacteria at 27°C (for 1 h), IPTG 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 (7700*g* for 10 min at 4°C) and resuspended in lysis buffer (50 mM Tris, pH 7.5, 1 mM dithiothreitol, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and 0.15 mg/ml lysozyme) (Fetzer et al., 1994). Bacteria suspensions

were sonicated (on ice) and ultracentrifuged (20,000*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. Protein content of the purified fractions was assessed using Bradford reagent (Sigma Chemical Co.).

Construction of Wild-Type and Q125N Mutant HSV-1 TK Mammalian Expression Vectors. The HSV-1 TK(WT) and HSV-1 TK(Q125N) genes were ligated into the pEGFP-N1 N-Terminal Protein Fusion Vector (CLONTECH, Palo Alto, CA). The construction of the HSV-1 TK(WT)-GFP expression vector has been described previously (Degrève et al., 1998). The HSV-1 TK(Q125N)-GFP vector was constructed by amplification of the Q125N mutant HSV-1 TK gene from the pGEX-5X-1-HSV-1 TK(Q125N) vector and ligation into the pEGFP-N1 vector. The TK sequences were checked using automated fluorescence sequencing (ALFexpress).

Stable and Transient Transfection of Tumor Cells. The HSV-1 TK(WT)-GFP and HSV-1 TK(Q125N)-GFP fusion gene constructs were introduced into OstTK$^-$ cells via membrane fusion-mediated transfer using plasmid/liposome complexes (LipofectAMINE Reagent, Life Technologies), as described by the supplier. The OstTK$^-$/HSV-1 TK(WT)$^+$ cell line was established as described previously (Degrève et al., 1998). OstTK$^-$/HSV-1 TK(Q125N)$^+$ cells were isolated after selection in the presence of 0.5 mg/ml Geneticin (Duchefa; Haarlem, The Netherlands) and cloned by limited dilution. Nontransfected and TK gene-transfected cell lines were prepared for fluorescence-activated cell sorting analysis by trypsinization, two wash steps (PBS), and fixation with 1% paraformaldehyde in PBS (10^6 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-transfected osteosarcoma cells was evaluated as described previously (Degrève et al., 1999). Briefly, 10^4 OstTK$^-$, OstTK$^-$/HSV-1 TK(WT)$^+$ or OstTK$^-$/HSV-1 TK(Q125N)$^+$ 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_{50} 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(Q125N) preparations to phosphorylate the four substrates dThd, dTMP, BVDU, and BVDU-MP was determined as follows. The standard reaction mixture contained 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 1 mg/ml bovine serum albumin, 2.5 mM ATP, 10 mM NaF, 100 μM substrate, and 0.1 μg 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 30, 60, and 120 min. The reaction mixtures were subjected to high performance liquid chromatography (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.

Intracellular Metabolism of Radiolabeled BVDU and GCV. [2′-^{3}H]BVDU (specific radioactivity, 1 Ci/mmol) and [8-^{3}H]GCV (specific radioactivity, 14.6 Ci/mmol) were from Moravek Biochemicals (Brea, CA). OstTK$^-$/HSV-1 TK(WT)$^+$ and OstTK$^-$/HSV-1 TK(Q125N)$^+$ cells were seeded in 5-ml culture bottles (10^6 cells/flask) and incubated with 0.06 μM [8-^{3}H]GCV or 1 μM [2′-^{3}H]BVDU (5 μCi/bottle). Parallel cultures were incubated with nonradiolabeled compound for the evaluation of cell proliferation by cell counting using a Coulter Counter. After 24 h, cells were trypsinized, centrifuged at 200*g*, washed twice with cold medium, and precipitated with cold (66%) methanol. After centrifugation at 10,000*g* for 10 min, the supernatants were subjected to HPLC analysis as described above. The different fractions of the eluates were assayed for radioactivity.

Determination of Tritium Release from [5-^{3}H]dUrd in Intact Cells. Activity of TS in intact OstTK$^-$/HSV-1 TK(WT)$^+$ and OstTK$^-$/HSV-1 TK(Q125N)$^+$ cells was measured by evaluation of tritium release from [5-^{3}H]dUMP (formed in the cells from [5-^{3}H]dUrd) in the reaction catalyzed by TS. This method has been described previously (Balzarini and De Clercq, 1984) but was modified as follows. Cells were seeded in 24-well plates at a density of 200,000 cells/well. After 24 h, medium was aspirated and 400 μl of growth medium containing an appropriate amount of BVDU was added to each well for 3 h. 1 μCi [5-^{3}H]dUrd (Amersham Pharmacia Biotech, specific radioactivity: 12.6 Ci/mmol) was then added to each well (in 100 μl growth medium) and the cells were further incubated at 37°C. At 0, 1 and 2 h, 400 μl of a cold suspension (0.1 g/ml) of carbon black (UCB, Leuven, Belgium) in 5% TCA was added to each well and the resulting mixtures were centrifuged at 1,100*g* for 10 min, after which supernatants (800 μl) were analyzed for radioactivity. The IC_{50} was defined as the drug concentration required to inhibit the release of tritium from [5-^{3}H]dUrd by 50%.

Bystander Effect. The procedure used to evaluate the bystander effect of the compounds was as recently described (Degrève et al., 1999). Briefly, OstTK$^-$ cells were mixed with OstTK$^-$/HSV-1 TK(WT)$^+$ and OstTK$^-$/HSV-1 TK(Q125N)$^+$ cells in percentages ranging from 0 to 100% TK gene-transfected cells, and subsequently incubated in the presence of GCV or BVDU at a concentration of 10 or 50 μM (in 2% FCS-containing medium). After 3 days, cell viability was determined using the Cell Titer 96 Aqueous Nonradioactive 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Cell Proliferation Assay (Promega, Madison, WI). Untreated cell cultures served as control cultures.

Results

Thymidine Kinase and Thymidylate Kinase Activity of Purified HSV-1 TK(WT) and HSV-1 TK(Q125N). The wild-type and Q125N mutant HSV-1 TK enzymes were expressed as fusion proteins with GST in *E. coli* and purified from bacterial cell extracts using Glutathione Sepharose 4B. The purified enzymes were evaluated for their abilities to phosphorylate dThd, dTMP, BVDU, and BVDU-MP. Wild-type HSV-1 TK was very efficient at phosphorylating the natural substrate dThd, phosphorylating 24, 47, and 81% of 100 μM dThd in the reaction mixture after 30, 60, and 120 min of incubation, respectively, at 37°C (Fig. 1A). BVDU was also efficiently phosphorylated by wild-type HSV-1 TK, with 75% of the available BVDU phosphorylated after a 120 min-incubation period (Fig. 1B). The HSV-1 TK(Q125N) mutant enzyme was at least as efficient as the wild-type HSV-1 TK enzyme with regard to dThd (Fig. 1C) and BVDU (Fig. 1D) kinase activity. From Lineweaver-Burk plots, it was calculated that the K_m values of wild-type HSV-1 TK and mutant HSV-1 TK (Q125N) were 0.56 μM and 21 μM, respectively, whereas their V_{max} values were 4.9 and 18 nmol/μg/h, respectively, for dThd as the natural substrate. Wild-type HSV-1 TK was able to phosphorylate up to 50% of the available dTMP in the reaction mixture to dTDP (Fig. 1A) and up to 94% of the available BVDU-MP to BVDU-DP (Fig. 1B). However, in marked contrast, no measurable amounts of diphosphorylated dThd or BVDU were formed from the corresponding nucleosides or nucleoside monophosphates by the Q125N mutant HSV-1 TK enzyme (Fig. 1, C and D).

Transfection of Osteosarcoma Cells with Wild-Type and Mutant HSV-1 TK Genes. Adherent human osteosarcoma 143B cells deficient in cytosol TK (designated OstTK$^-$)

were transfected with mammalian expression vectors containing the wild-type or Q125N mutant HSV-1 TK genes fused to the coding sequence of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Chalfie et al., 1994; Rizzuto et al., 1995). The two resulting cell lines, designated OstTK$^-$/HSV-1 TK(WT)$^+$ and OstTK$^-$/HSV-1 TK(Q125N)$^+$, respectively, were evaluated by fluorescence-activated cell sorting analysis to compare the level of TK-GFP fusion gene expression. Nontransfected OstTK$^-$ cells served as control (Fig. 2A). The level of fluorescence, and hence the level of TK-GFP fusion gene expression, was comparable between the OstTK$^-$/HSV-1 TK(WT)$^+$ (Fig. 2B) and OstTK$^-$/HSV-1 TK(Q125N)$^+$ (Fig. 2C) cell lines.

Cytostatic Activity of Antiherpetic Compounds against Wild-Type and Mutant HSV-1 TK Gene-Transfected Osteosarcoma Cells. To evaluate the efficiency of the Q125N mutant HSV-1 TK gene in the killing of tumor cells by antiherpetic pyrimidine and purine nucleoside analogs, selected compounds (Fig. 3) were evaluated for their cytostatic effects against human osteosarcoma cells stably expressing either the wild-type or the Q125N mutant HSV-1 TK gene. The majority of the 50% inhibitory concentrations (IC_{50} values) of the compounds for the proliferation of OstTK$^-$ and OstTK$^-$/HSV-1 TK(WT)$^+$ cells have been published before, but are included in Table 1 for comparative reasons. All evaluated antiherpetic compounds showed pronounced cytostatic activities against OstTK$^-$/HSV-1 TK(WT)$^+$ cells, with 50% inhibitory concentrations that proved 1,250-fold (ACV) to 240,000-fold (BVaraU) lower than the concentrations required to inhibit the proliferation of the corresponding nontransfected OstTK$^-$ cells (data not shown). The cytostatic effect of BVDU and its 4′-thio analog *S*-BVDU was not markedly affected by the Q125N mutation in the HSV-1 TK gene, with IC_{50} values in the low nanomolar range for both TK gene-transfected tumor cell lines. However, BVaraU and araT, both of which displayed IC_{50} values of 0.004 μM for OstTK$^-$/HSV-1 TK(WT)$^+$ cells, demonstrated a 100-fold (araT) to 10,000-fold (BVaraU) increase in IC_{50} for OstTK$^-$/HSV-1 TK(Q125N)$^+$ cells (IC_{50} values of 0.53 μM and 50 μM, respectively). The cytostatic activity of a variety of purine nucleoside analogs, including the prototype compound ganciclovir, proved independent of the nature of the HSV-1 TK gene (i.e., wild-type or Q125N mutant HSV-1 TK) (Table 1). Also, the cytostatic effects of the oral prodrugs of penciclovir (famciclovir) and acyclovir (valacyclovir) were not significant different between OST TK$^-$/HSV-1 TK$^+$ (WT) and OST TK$^-$/HSV-1 TK(Q125N)$^+$ cell lines (IC_{50} values: 255 and 180 μg/ml for famciclovir, and 0.35 and 0.19 μg/ml for valacyclovir). The markedly higher IC_{50} values for famciclovir, compared with penciclovir, are probably caused by the inability of the osteosarcoma cells to convert famciclovir to its free guanine derivative penciclovir.

The bystander effect of BVDU and GCV (i.e., the killing of nontransfected tumor cells by nearby TK gene-transfected cells upon treatment with these nucleoside analogs) was evaluated in cell cultures containing mixtures of OstTK$^-$ cells and either OstTK$^-$/HSV-1 TK(WT)$^+$ or OstTK$^-$/HSV-1 TK(Q125N)$^+$ cells. We recently reported that purine nucleoside analogs such as GCV are endowed with a marked bystander effect, whereas pyrimidine nucleosides (e.g., BVDU) are virtually incapable of bystander killing under similar experimental conditions (Degrève et al., 1999). The dashed lines in Fig. 4 depict the theoretically calculated values for cell survival if no interaction between nontransfected cells and TK gene-transfected cells occurs (i.e., when no bystander effect is exerted). For example, a mixture of 75% TK gene-transfected cells and 25% nontransfected cells should give 25% cell survival after 3 days of incubation in the presence of nucleoside analogs compared with control cell cultures without compound. As shown in Fig. 4, BVDU was inefficient in the killing of nontransfected cells in cocultures of OstTK$^-$ cells with either wild-type or Q125N mutant HSV-1 TK gene-transfected cells. A weak bystander effect was detected only in mixed cell cultures containing at least 25% TK gene-transfected tumor cells. Thus, the Q125N mutation in the HSV-1 TK gene did not improve the bystander effect of

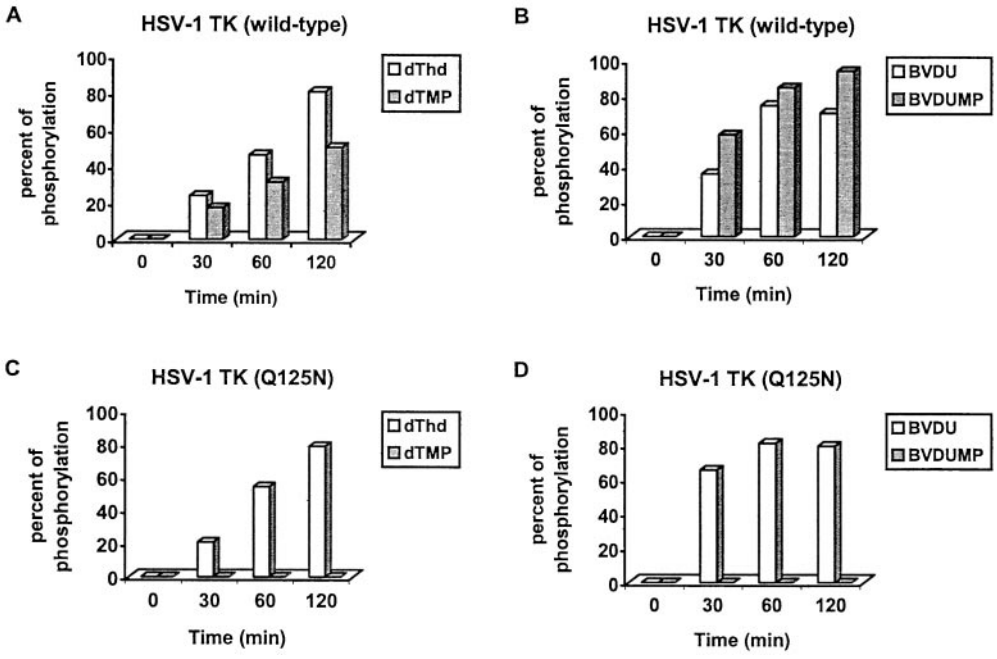

Fig. 1. Thymidine kinase and thymidylate kinase activities of purified HSV-1 TK(WT) and mutant HSV-1 TK(Q125N). The abilities of purified GST-HSV-1 TK(WT) (A and B) and GST-HSV-1 TK(Q125N) (C and D) preparations to phosphorylate dThd and dTMP (A and C) and BVDU and BVDU-MP (B and D) were determined as described under *Materials and Methods*. The relative conversion of the substrates was calculated from the HPLC chromatogram peak areas. The bars indicating the relative conversion of dThd and BVDU in A and B represent the sum of the conversions to the mono- and diphosphate forms.

BVDU. GCV, on the other hand, demonstrated a pronounced dose-dependent bystander effect, killing as much as 75% of the cells in a tumor cell population that contained only 10% TK gene-transfected cells. The Q125N mutation in the HSV-1 TK gene did not affect the pronounced bystander effect of GCV in mixed cultures of nontransfected and TK gene-transfected cells (Fig. 4).

Metabolism and Antimetabolite Studies. The metabolism of [8-^{3}H]GCV and [2′-^{3}H]BVDU in the OstTK$^-$/HSV-1 TK(WT)$^+$ and OstTK$^-$/HSV-1 TK(Q125N)$^+$ cell lines was investigated by performing HPLC analysis on extracts of cell cultures that had been incubated with these radiolabeled compounds for 24 h. Results are summarized in Table 2. [8-^{3}H]GCV was converted to its mono-, di-, and triphosphate derivatives to a comparable extent in the HSV-1 TK(WT) and Q125N mutant HSV-1 TK gene-transfected tumor cell lines. The major metabolites found were [8-^{3}H]GCV-MP and [8-^{3}H]GCV-TP. In contrast, the metabolism of [2′-^{3}H]BVDU showed an impressive 20-fold accumulation of [2′-^{3}H]BVDU-MP in the OstTK$^-$/HSV-1 TK(Q125N)$^+$ cell line over the di- and triphosphate forms, as opposed to comparable levels for mono-, di-, and triphosphorylated [2′-^{3}H]BVDU in the osteosarcoma cell line expressing wild-type HSV-1 TK. Moreover, the level of [2′-^{3}H]BVDU-MP in OstTK$^-$/HSV-1 TK(Q125N)$^+$ cells was more than 100-fold higher than in OstTK$^-$/HSV-1 TK(WT)$^+$ cells (Table 2).

Tritium release from [5-^{3}H]dUrd in intact tumor cells has been considered as a valuable parameter for measuring TS activity in situ (Balzarini and De Clercq, 1984). Indeed, after [5-^{3}H]dUrd has been converted to [5-^{3}H]dUMP, the C-5 tritium atom on the pyrimidine base is released during the TS reaction. The ability of BVDU to inhibit tritium release from [5-^{3}H]dUrd was compared in HSV-1 TK(WT) and HSV-1 TK(Q125N) gene-transfected cells. No inhibition of TS was noted in OstTK$^-$/HSV-1 TK(WT)$^+$ cells grown in the presence of BVDU for 3 h at concentrations up to 100 μM. In sharp contrast, BVDU mediated pronounced TS inhibition, displaying a 50% inhibitory concentration in the lower micromolar range for the inhibition of tritium release from [5-^{3}H]dUrd (Table 3).

Discussion

Mutants of HSV-1 TK have been studied for several reasons. First, herpesvirus strains have emerged (both in vitro and in patients) that are resistant to TK-dependent antiherpetic nucleoside analogs because of mutations or deletions in the TK gene (Larder et al., 1983; Nugier et al., 1991). Second, site-directed mutagenesis has been performed on specific amino acids of HSV-1 TK to reveal their function in substrate or inhibitor binding and to reveal other enzyme characteristics (Liu and Summers, 1988; Black and Loeb, 1993; Michael et al., 1995). Third, mutations have been introduced in HSV-1 TK to identify enzyme mutants with enhanced ability to phosphorylate antiherpetic nucleoside analogs. This could be helpful in the HSV-1 TK-mediated killing of tumor cells by such antiherpetic compounds (Black et al., 1996; Kokoris et al., 1999).

In this report, we describe an HSV-1 TK mutant, which contains an Asn residue at position 125 instead of the (wild-type) Gln residue. In crystallographic structures of HSV-1 TK complexed with thymidine, the carboxamide moiety of the Gln-125 was shown to form hydrogen bonds with the N3 and O4α-atoms of thymidine (Fig. 5). Interestingly, similar hydrogen bonds were formed with the N1 and O6α-atoms of guanine in GCV-complexed HSV-1 TK, but with the carboxamide moiety rotated by 180° (Brown et al., 1995). Alignment of primary amino acid sequences have shown that Gln-125 is strictly conserved among herpesvirus thymidine kinases (Balasubramaniam et al., 1990; Gentry, 1992), mitochondrial TK2 and the multifunctional *Drosophila melanogaster* deoxynucleoside kinase (Johansson et al., 1999), indicating that this residue may be of crucial importance in the catalytic activity of TKs. In addition to its ability to monophosphorylate antiherpetic such nucleoside analogs as GCV and BVDU, HSV-1 TK exhibits an associated thymidylate kinase activity that can further metabolize BVDU-MP to its diphosphate form (Fyfe, 1982). GCV-MP is further metabolized by cellular guanylate kinase to the corresponding diphosphate form. BVDU-DP and GCV-DP are then converted by cellular nucleoside diphosphate kinases to their triphosphate derivatives, which are able to interfere with DNA synthesis. We have now demonstrated that the Q125N mutation selectively ablates the thymidylate kinase function of HSV-1 TK, while keeping the thymidine kinase activity intact. Indeed, whereas purified HSV-1 TK(Q125N) was at least as efficient as wild-type HSV-1 TK at monophosphorylating 100 μM thymidine and BVDU, no diphosphate metabolites were detectable in our TK (Q125N) enzymatic assays (Fig. 1).

Despite the range of available TK structures, modeling studies on the effects of residue 125 are hampered because relatively little data are available relating to the HSV-1 TK thymidylate kinase activity. Rechtin et al. (1996) used photo cross-linking techniques to show that the pyrimidine base was bound at the same site during both dThd and dTMP kinase activities. All HSV-1 TK crystal structures with nucleosides show the bases sandwiched between residues Tyr172 and Met128 (Fig. 5). This particularly favorable ar-

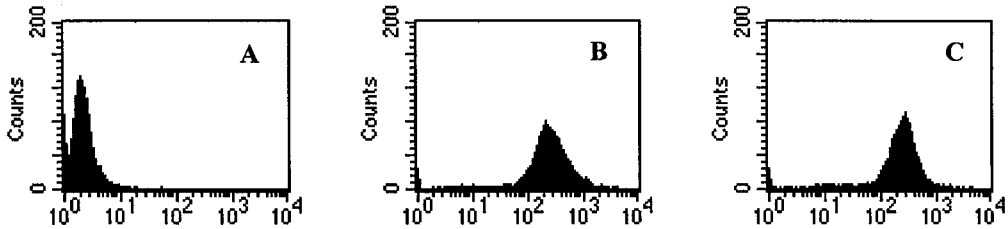

Fig. 2. Flow cytometric assessment of TK-GFP fusion gene expression in HSV-1 TK(WT) and HSV-1 TK(Q125N) fusion gene-transfected OstTK$^-$ cells. HSV-1 TK-GFP fusion gene-transfected cells were analyzed on a FACScan flow cytometer to assess the level of fusion gene expression, as described under *Materials and Methods*. Nontransfected OstTK$^-$ cells served as control. A, OstTK$^-$; B, OstTK$^-$/HSV-1 TK(WT)$^+$; C, OstTK$^-$/HSV-1 TK(Q125N)$^+$.

rangement seems likely to be conserved for both dThd and dTMP kinase functions, pinning the base in place and allowing only a restricted amount of rotation. The ability of HSV-1 TK to activate BVDU, and other nucleoside analogs with large 5-position substituents, to their diphosphates would also seem to rule out dramatically different conformations of dThd and dTMP in their catalytic complexes with TK. Thus, the mechanism whereby the Q125N mutation can abolish dTMP kinase activity but have only a relatively small effect on dThd kinase activity is still unclear. Interestingly, one other mutant, A168T, has also been observed to specifically compromise dTMP kinase activity (Wilber and Docherty, 1994). This residue lies adjacent to residue 125 in the binding pocket, suggesting a common mode of action. One possibility is a change in hydrogen bonding pattern: the Q125N mutant may form a strong hydrogen bond with the carbonyl oxygen of residue 168. The A168T mutant presents an alternative hydrogen bonding option for the carboxamide group of residue 125. In either case the interaction between residue 125 and dTMP may be compromised.

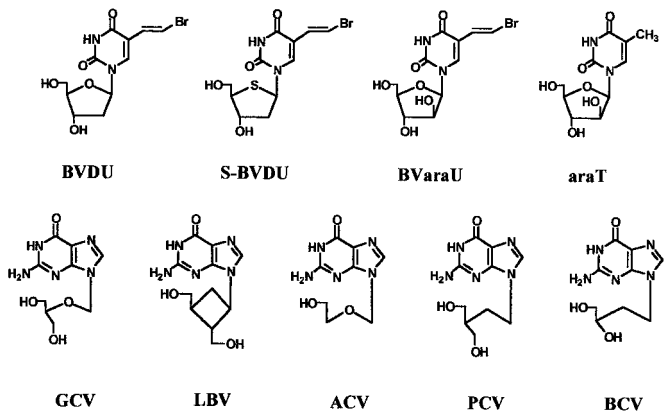

Fig. 3. Structural formulae of antiherpetic pyrimidine and purine nucleoside analogs.

TABLE 1

Cytostatic activity of antiherpetic nucleoside analogs against HSV-1 TK(WT) gene- and HSV-1 TK(Q125N) gene-transfected Ost TK$^-$ cells

Data are the mean ± S.D. of at least three independent experiments.

Compound	IC_{50}		IC_{50} ratio[b]
	Ost TK$^-$/HSV-1 TK(WT)$^+$[a]	Ost TK$^-$/HSV-1 TK(Q125N)$^+$	
	μM		
BVDU	0.035 ± 0.006	0.015 ± 0.007	0.4
BVaraU	0.004 ± 0.001	50 ± 35	12,500
S-BVDU	0.008 ± 0.004	0.024 ± 0.006	3.0
araT	0.004 ± 0.001	0.53 ± 0.11	133
GCV	0.0011 ± 0.0005	0.0006 ± 0.0003	0.5
LBV	0.008 ± 0.001	0.006 ± 0.003	0.8
ACV	0.059 ± 0.015	0.079 ± 0.019	1.3
PCV	0.013 ± 0.002	0.020 ± 0.002	1.5
BCV	0.006 ± 0.000	0.005 ± 0.001	0.8

[a] Data taken from Degreve et al. (1999).
[b] Ratios of IC_{50} values for Ost TK$^-$/HSV-1 TK(Q125N)$^+$ cells versus the IC_{50} values for Ost TK$^-$/HSV-1 TK(WT)$^+$ cells.

A very recent report describes three conservative mutations of residue 125 to Asp, Asn, and Glu (Hinds et al., 2000). In line with the results presented here (K_m values for wild-type TK and the Asn TK mutant were 0.56 and 21 μM, respectively), the measured K_m values with dThd as the substrate were 0.3, 20, 6000, and 3 μM for the wild-type and the Asn, Asp, and Glu mutants, respectively. With GCV as the substrate the equivalent kinetic constants were 69, 50, undetermined, and 473 μM, respectively. Hinds et al. (2000) also noted compromised thymidylate kinase activity from all these mutants. Kussmann-Gerber et al. (1998) have previously performed a site-directed mutagenesis study on the Gln-125 residue of HSV-1 TK, to clarify the relevance of this residue to the binding of thymidine and ACV. These investigators found that the Q125N mutant accepts thymidine and ACV as substrates with apparent K_m values of 10 μM and 555 μM, respectively, compared with apparent K_m values for wild-type HSV-1 TK of 0.2 μM and 170 μM, respectively.

Drake et al. (1999) expressed the HSV-1 TK(Q125N) mutant in mammalian cells and found that the mutant enzyme was not able to metabolize pyrimidine nucleosides, as HSV-1 TK(Q125N) gene-transfected cells did not metabolize radiolabeled thymidine and 2′-deoxycytidine any better than the corresponding nontransfected cells. These findings are in disagreement with the data of Kussmann-Gerber et al. (1998) and our own findings that the HSV-1 TK(Q125N) mutant is able to phosphorylate thymidine as efficiently as wild-type HSV-1 TK.

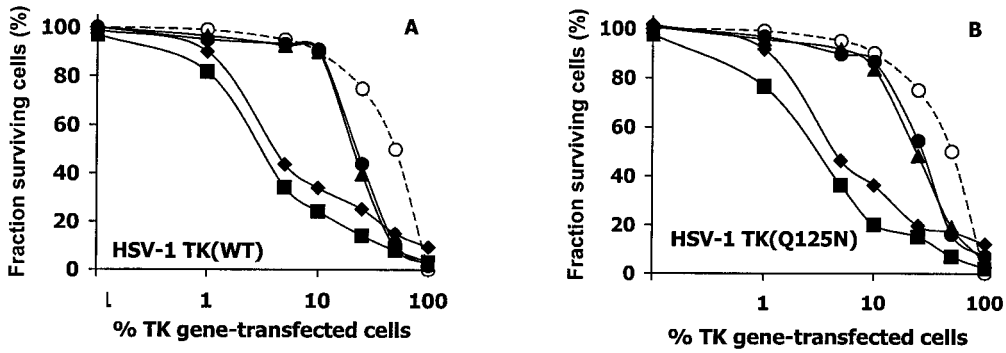

Fig. 4. Bystander effect of GCV and BVDU in mixed cell cultures containing mixtures of OstTK$^-$ and either HSV-1 TK(WT) or HSV-1 TK(Q125N) gene-transfected OstTK$^-$ cells. OstTK$^-$ cells were mixed in various ratios with OstTK$^-$/HSV-1 TK(WT)$^+$ or OstTK$^-$/HSV-1 TK(Q125N)$^+$ cells, incubated in the presence of GCV or BVDU and evaluated for cell viability using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, as described under *Materials and Methods*. The plotted data are the mean of at least three independent experiments and represent the percentages of the absorbance in the drug-treated mixed cell cultures to the absorbance of the same mixed cell culture in the absence of compound. The dashed line (with open symbols) in each graph depicts the theoretically calculated value for cell survival if no interaction between nontransfected and HSV-1 TK-GFP gene-transfected cells occurred (i.e., when no bystander effect was exerted). ■, 50 μM GCV; ♦, 10 μM GCV; ▲, 50 μM BVDU; ●, 10 μM BVDU.

In a previous study, we investigated the potent cytostatic activity of BVDU and closely related analogs [including (*E*)-5-(2-iodovinyl)-2′-deoxyuridine, (*E*)-5-(2-bromovinyl)-2′-deoxycytidine and (*E*)-5-(2-bromovinyl)-2′-deoxy-4′-thiouridine] against murine mammary carcinoma FM3A cells transfected with the wild-type HSV-1 TK and HSV-2 TK genes (designated FM3ATK$^-$/HSV-1 TK$^+$ and FM3ATK$^-$/HSV-2 TK$^+$, respectively). Intensive biochemical studies revealed that inhibition of TS, not the incorporation into cellular DNA, was the predominant mechanism of action of these pyrimidine nucleoside analogs (Balzarini et al., 1987; Balzarini et al., 1993, 1994). TS catalyzes the reductive methylation of dUMP to dTMP and is a crucial enzyme in the de novo pathway of dTMP synthesis in the cell. It is considered the principal target enzyme for the anticancer activity of compounds such as 5-fluorouracil. Surprisingly, we found that BVDU was unable to inhibit TS in human OstTK$^-$/HSV-1 TK(WT)$^+$ cells, as opposed to the murine FM3ATK$^-$/HSV-1 TK$^+$ and FM3ATK$^-$/HSV-2 TK$^+$ cells. The molecular basis for the different mechanism of action of BVDU in HSV-1 gene-transfected murine and human cells is currently unclear.

Transfection of the OstTK$^-$ cells with the mutant HSV-1 TK(Q125N) gene resulted in a marked accumulation of BVDU-MP and associated inhibition of TS in the transfected tumor cells. A change in the mechanism of cytostatic action of BVDU against TK gene-transfected tumor cells was thus achieved by mutation of a single residue in HSV-1 TK (i.e., Gln-125). These data were corroborated by our findings that the cytostatic activity of the antiherpetic arabinosyl derivatives of thymidine and BVDU (i.e., araT and BvaraU), was severely diminished by the Q125N mutation, because arabinofuranosyl nucleoside monophosphates have been previously shown to have poor affinity for TS (Santi, 1980). Presumably, araT and BVaraU need to be phosphorylated to their 5′-diphosphate and 5′-triphosphate derivatives to exert their cytostatic activity. When the viral thymidylate kinase activity in the HSV-1 TK(Q125N) gene-transfected tumor cells is deleted, araTMP and BVaraUMP can no longer be converted to their 5′-di- and 5′-triphosphate derivatives;

TABLE 2

Metabolism of [8-^{3}H]GCV and [2′-^{3}H]BVDU in OST TK$^-$ cells transfected with the HSV-1 TK(WT) and HSV-1 TK(Q125N) gene

Cell cultures were incubated with [8-^{3}H]GCV or [2′-^{3}H]BVDU, after which cell extracts were subjected to HPLC analysis and the different fractions of the eluates assayed for radioactivity.

Metabolite	Elution Time	Ost TK$^-$/HSV-1 TK(WT)$^+$	Os TK$^-$/HSV-1 TK(Q125N)$^+$
	min	*pmol / 10^6 cells*	
GCV-MP	8–9	7.6	10
GCV-DP	13–14	2.1	1.4
GCV-TP	25–26	5.4	5.0
BVDU-MP	8	0.28	32
BVDU-DP	14	0.23	0.56
BVDU-TP	23	0.27	0.91

TABLE 3

Inhibition of thymidylate synthase by BVDU in intact Ost TK$^-$ cells transfected with the HSV-1 TK(WT) and HSV-1 TK(Q125N) gene

The ability of BVDU to inhibit thymidylate synthase in Ost TK$^-$/HSV-1 TK(WT)$^+$ and Ost TK$^-$/HSV-1 TK(Q125N)$^+$ cells was measured by the evaluation of tritium release from [5-^{3}H]dUrd after 1 and 2 h.

[5-^{3}H]dUrd Incubation time	Ost TK$^-$/ HSV-1 TK(WT)$^+$	Ost TK$^-$/ HSV-1 TK(Q125N)$^+$
1 h	>100 μM	9.8 ± 5.1μM
2 h	>100 μM	3.3 ± 2.2μM

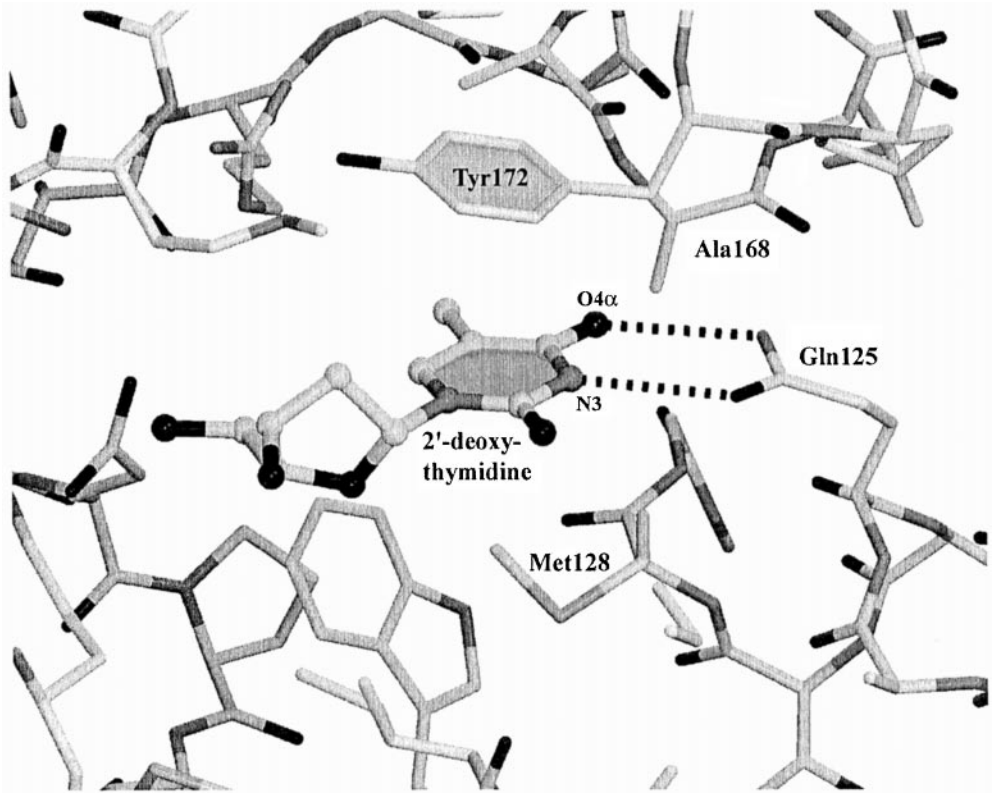

Fig. 5. 2′-deoxythymidine bound in the HSV-1 TK active site showing the importance of residue 125.

thus, their cytostatic potential may become heavily compromised.

We have previously reported the differential in vitro bystander effect of purine and pyrimidine nucleoside analogs in combination with HSV-1 TK gene transfection (Degrève et al., 1999). All purine nucleoside analogs that were evaluated, including GCV, ACV, PCV, BCV, and LBV, demonstrated pronounced killing of nontransfected osteosarcoma cells when cocultured with HSV-1 TK gene-transfected cells. In contrast, the pyrimidine nucleoside analogs (including BVDU, *S*-BVDU, BVaraU, and araT) proved markedly less capable of bystander cell killing. These findings were corroborated by autoradiographic analysis that showed that [2′-^{3}H]BVDU metabolites formed in the herpes TK gene-transfected osteosarcoma cells were much less efficiently incorporated in the DNA of bystander cells than [8-^{3}H]GCV. Transfection of tumor cells with the Gln125Asn TK mutant that changed the intracellular metabolic fate of BVDU, did not change the (lack of appreciable) bystander effect of BVDU.

Recently, we constructed an HSV-1 Ala167Tyr TK mutant enzyme that virtually completely lost its pyrimidine nucleoside (i.e., dThd, BVDU) kinase activity, but markedly kept its purine nucleoside (i.e., GCV, LBV) kinase activity (Degrève et al., 2000). Consequently, concomitant transfection of tumor cells with the mutant Ala167Tyr and Gln125Asn TK gene constructs may allow us to treat these tumor cells with a combination of BVDU and ganciclovir. Indeed, there should not be a marked competition between BVDU and GCV for phosphorylation to their monophosphates in such double-transfected tumor cells because of the differential substrate affinities for both TK enzyme constructs. Moreover, both drugs may exert their cytostatic activity through two different mechanisms of action [i.e., thymidylate synthase on the one hand (for BVDU when activated by the Glu125Asn TK mutant) and DNA polymerase/DNA incorporation (for GCV when activated by the Ala167Tyr TK mutant) on the other]. The construction of tumor cells containing both mutated TK genes is currently ongoing in our laboratory.

In conclusion, substitution in HSV-1 TK of Asn at position 125 for Gln resulted in the complete ablation of the thymidylate kinase activity associated with wild-type HSV-1 TK. Introduction of the mutant HSV-1 TK(Q125N) gene in human osteosarcoma cells brought about a marked accumulation (upon BVDU-treatment) of BVDU-MP, which in turn resulted in the inhibition of thymidylate synthase, an effect that was not observed in osteosarcoma cells transfected with wild-type HSV-1 TK.

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