Mutation of herpesvirus thymidine kinase to generate ganciclovir-specific kinases for use in cancer gene therapies

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Understanding the functional and mechanistic properties of the multi-substrate herpes simplex virus type-1 thymidine kinase (HSV-1 TK) remains critical to defining its role as a major pharmacological target in herpesvirus and gene therapies for cancer. An inherent limitation of the activity of HSV-TK is the >70-fold difference in the \(K_m\) for phosphorylation of thymidine over the pro-drug ganciclovir (GCV). To engineer an HSV-1 TK isomert that is specific for GCV as the preferred substrate, 16 site-specific mutants were generated. The mutations were concentrated at conserved residues involved in nucleoside base binding, Gln125 and near sites 3 and 4 involved in catalysis and substrate binding. The substrate preferences of each mutant enzyme were compared with wild-type HSV-1 TK. One mutant, termed Q7530 TK, had a lower \(K_m\) for GCV than thymidine. Expression of the Q7530 TK in tumor cells indicated comparable metabolism to and improved sensitivity to GCV over wild-type HSV-1 TK, with minimal thymidine phosphorylation activity. A molecular modeling simulation of the different HSV-1 TK active-sites was done for GCV and thymidine binding. It was concluded that mutations at Gln125 and near site 4, especially at Ala168, were responsible for loss of deoxypyrimidine substrate binding.

**Keywords:** ganciclovir/gene therapy/thymidine/thymidine kinase/thymidylate kinase

Introduction

The thymidine kinase of herpes simplex virus type-1 (HSV-1 TK) is a multifunctional enzyme that phosphorylates a broad range of pyrimidine nucleosides/nucleotides and purine nucleosides, including thymidine, thymidylate, deoxycytidine, acyclovir and ganciclovir (GCV) (Chen et al., 1979; Machida, 1986). Each of these substrates has been shown to bind in the same active-site pocket of the enzyme and HSV-1 TK is unique in its ability to phosphorylate both thymidine and thymidylate in the same site (Chen and Prusoff, 1978; Chen et al., 1979; Brown et al., 1995; Rechtin et al., 1995; Champness et al., 1998). Analogous to its role in anti-herpesvirus treatments, this substrate diversity of HSV-1 TK has allowed for the selective killing of tumor cells in vivo, an advantage that has proved useful in the clinical effectiveness of HSV-1 TK/GCV suicide gene therapies for cancer (Freeman et al., 1996; Klatzmann et al., 1998; Sterman et al., 1998). Tumor cells expressing HSV-1 TK specifically phosphorylate GCV to its monophosphate, GCV-MP, which is further metabolized by cellular enzymes to its triphosphate, GCV-TP. Incorporation of GCV-TP into the nascent DNA leads to chromosome breaks and sister chromatid exchanges (Thust et al., 2000a,b), resulting in an S- and/or G2-phase cell cycle arrest (Boucher et al., 1998; Halloran and Fenton, 1998; Rubsam et al., 1998; Wei et al., 1998; Drake et al., 1999). Predominantly by an apoptotic mechanism (Boucher et al., 1998; Rubsam et al., 1998; Drake et al., 1999; Thust et al., 2000b), a multi-log cell killing in tumor cells expressing HSV-1 TK is observed, an effect not seen in cytotoxic studies using other chemotherapeutic substrates (Rubsam et al., 1998).

Because of its long history as the chemotherapeutic target for antiviral agents, the structure/activity of HSV-1 TK has been extensively studied via characterization of drug-resistant viral strains (Larder et al., 1983; Darby et al., 1986), site-directed or random sequence mutagenesis (Liu and Summers, 1988; Munir et al., 1992; Michael et al., 1994; Black and Loeb, 1996), photoaffinity labeling (Rechtin et al., 1995, 1996) and X-ray crystallography (Brown et al., 1995; Champness et al., 1998). As a result of these studies, mutant HSV-1 TKs have been generated that display selective preferences for different HSV-1 TK substrates. For example, we had previously described mutations at Gln125 to Asn that resulted in poor utilization of thymidine as a substrate, but retained the ability to phosphorylate GCV (Drake et al., 1999; Hinds et al., 2000). Another approach has been the use of random oligonucleotide mutagenesis to produce drug-specific HSV-1 TK mutant enzymes (Black and Loeb, 1996; Black et al., 1996). Two of these HSV-1 TK mutants, termed TK30 and TK75, were highly sensitive to GCV when expressed in mammalian cells, resulting in more cell killing compared with cells expressing the wild-type HSV-1TK (Black et al., 1996; Kokoris et al., 1999). In this study, we hypothesized that the combination of the Asn mutations at Gln125 and the mutated regions in TK30 or TK75 would create a GCV-specific enzyme by eliminating the endogenous substrate competition of thymidine and improving the efficacy of GCV phosphorylation.

Based on this hypothesis, 16 mutant HSV-1 TK enzymes were generated using site-directed mutagenesis. These mutants and also wild-type HSV-1 TK, were expressed and partially purified from Escherichia coli, then screened for enzymatic activity with thymidine and GCV. Mutant HSV-1 TK enzymes deficient in thymidine phosphorylation activities, but active for GCV phosphorylation, were further characterized for their kinetic properties and expressed in human colon carcinoma cell lines for cell killing and drug metabolism studies. The cumulative substrate binding information generated from the enzymatic and cell culture studies were used in constructing an HSV-1 TK molecular model of active-site/substrate binding interactions with FlexiDock, a SYBYL 6.7-based program. Of the mutant enzymes evaluated, two were identified that possessed unique substrate properties consistent with the goal of generating HSV-1 TKs with minimal thymidine and maximal GCV phosphorylation activities. The correlation between the
functional enzymatic and cell culture data and the modeled ligand/protein structures for these HSV-1 TK mutants is discussed and has resulted in a new predictive structural modeling approach for generating substrate specific HSV-1 TKs.

Materials and methods

Materials

All reagents and nucleotides were purchased from Sigma Chemical unless specified otherwise. [Methyl-\(^3^H\)]Thymidine (69 Ci/mmol) and [8-\(^3^H\)]ganciclovir (17.4 Ci/mmol) were purchased from Moravek Biochemicals. DE81 filter-paper disks, isopropyl-\(\beta\)-d-thiogalactopyranoside (IPTG), dithiothreitol (DTT), heparin resin and DE-52 resin were purchased from Fisher Scientific. Automated DNA sequencing of plasmids was done using a Model 377 DNA sequencer from Applied Biosystems. Oligonucleotide primers were synthesized by Clontech. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs.

Site-directed mutagenesis

Site-specific mutations were introduced into the flanking regions of conserved sequences within the HSV-1 TK gene using the PCR-based, QuikChange Site-directed Mutagenesis Kit as per the manufacturer’s instructions (Stratagene). Four pairs of mutagenic primers were used to alter the amino acid sequence of three genes previously subcloned into a pET9a (Promega) expression construct: wild-type HSV-1 TK, Q125N TK and Q125E TK (Hinds et al., 2000). Two different sets of mutations, TK30 and TK75 (Black et al., 1996), were introduced at the flanking regions of conserved site 3 and site 4. Each of the site 3 (30-3 and 75-3) and site 4 (30-4 and 75-4) mutants were generated independently. For mutants incorporating both site 3 and site 4 mutations, the corresponding 3 mutant was used as the template. Specifically, in the TK30 set, three amino acid substitutions at site 3, L159I, I160L and F161A, and two mutations at site 4, A168Y and L169F, were introduced. These amino acid substitutions were incorporated into the vector templates using the following primers: CCGGCCCCCTACCC ATCCGCTGACCCAGGCATCCC and GGGATGCCGGTGACCCAGGATGAGGGGC for site 3 (30-3) and CATCCATCGCTATCCGTGCTAGCC and GTAGGGTGAGCCGATGAAGCAGCATGGG for site 4 (30-4). In the TK75 set, two amino acid substitutions at site 3, I160L and F161L, and two substitutions as site 4, A168V and A169M, were introduced. These substitutions were incorporated into the vector templates using the following sets of mutagenic primers: CCGGCCCCCTACCC ATCCGCTGACCCAGGCATCCC and GGGATGCCGGTGACCCAGGATGAGGGGC for site 3 (30-3) and CATCCATCGCTATCCGTGCTAGCC and GTAGGGTGAGCCGATGAAGCAGCATGGG for site 4 (30-4). The mutant plasmids generated by this procedure were transformed into JM109 competent E.coli cells. Positive transformants were identified by growth on LB medium containing kanamycin (50 \(\mu\)g/ml). Plasmid DNA was purified from positive transformants using the Qiagen Mini Prep kit as per the manufacturer’s instructions. To confirm the presence of the specified mutated residues and fidelity of the coding sequence of HSV-1 TK, automated sequencing of each construct was done using a primer directed at the T7 promoter located on the pET-9a expression vector.

Enzyme expression and purification

Following sequencing analysis, the mutant plasmids were transformed into competent E.coli SY211 TK cells and expression induced with IPTG as described previously (Rechtin et al., 1995; Hinds et al., 2000). Soluble protein lysate was loaded on to a DE 52 cellulose column coupled to a heparin column and purified as described previously (Hinds et al., 2000). This procedure results in 0.5–3 mg of total protein with a minimum purity of 70%. The mutant enzymes can be stored at –80°C for at least 6 months.

Photoaffinity labeling of latent HSV-1 TK enzymes

The TMP photoaffinity analog \([\(^{32}\)P]\)-5-azido-dUMP (5N3dUMP), was synthesized enzymatically using HSV-1 TK, 5-azidodeoxyuridine and [\(^{32}\)P]ATP as described previously (Rechtin et al., 1995). For photocrosslinking, 5 \(\mu\)g of partially purified enzymes were incubated with 10 \(\mu\)M \([\(^{32}\)P]-5N3dUMP, 10 mM MgCl\(\text{2}\), and 40 mM KCl for 10 s, followed by UV irradiation for 90 s with a hand-held UV lamp (254 nm UVP-11, Ultraviolet Products) at a distance of 3 cm. Samples were separated by SDS–PAGE and subsequent autoradiography as described previously (Rechtin et al., 1995).

Enzymatic assays

Partially purified HSV-1 TK mutants were screened in triplicate for thymidine and GCV phosphorylation using a standard reaction mix, 20 mM potassium phosphate, pH 7.6, 1 mM DTT, 5 mM ATP, 5 mM MgCl\(\text{2}\), 25 mM NaF, 40 mM KCl and 0.5 mg/ml bovine serum albumin (BSA) in a total volume of 25 \(\mu\)l and the following linear velocity conditions established for wild-type HSV-1 TK: for thymidine, 10 ng enzyme, 2 \(\mu\)M \([\(^3^H\)]\)thymidine, 10 min incubation at 37°C; for GCV, 250 ng, 40 \(\mu\)M \([\(^3^H\)]\)GCV, 20 min incubation at 37°C (Hinds et al., 2000). The entire reaction mixture (25 \(\mu\)l) was spotted on an ion-exchange filter, DE-81, washed and quantitated by scintillation counting as described previously (Hinds et al., 2000). Control reactions incubated without enzyme were spotted and processed to correct for backgrounds. These mutant enzymes were also screened for thymidylate kinase (TMPK) activities. For these experiments, 20 \(\mu\)M \([\(^3^H\)]\)TMP was substituted for deoxythymidine in the above assay mixture and \([\(^3^H\)]\)TDP product was separated from \([\(^3^H\)]\)TMP on thin-layer PEI-cellulose plates developed in 0.4 M LiCl (Hinds et al., 2000). The TDP product was quantified by scintillation counting of the excised TDP and TDP spots.

Those HSV-1 TK mutant enzymes that were active and demonstrated unique pharmacological properties were characterized further by determining the kinetic parameters (\(K_m\), \(k_{cat}\) and \(V_{max}\)) for each enzyme. The protein concentrations, substrate ranges and incubation times used in determining the \(K_m\) of thymidine and GCV for each mutant were done with linear velocity conditions established for each enzyme. Each \(K_m\) determination was assayed in triplicate using the following conditions: for \([\(^3^H\)]\)thymidine, wild-type HSV-1 TK, 10 ng, 0.25–5 \(\mu\)M, 15 min; Q30 TK, 500 ng, 2–60 \(\mu\)M, 10 min; Q75 TK, 50 ng, 0.5–40 \(\mu\)M, 10 min; Q75-3 TK, 100 ng, 0.5–40 \(\mu\)M, 5 min; N75-30 TK, 400 ng, 10–100 \(\mu\)M, 20 min; and Q75-30 TK, 100 ng, 10–100 \(\mu\)M, 5 min for \([\(^3^H\)]\)GCV, wild-type, 150 ng, 20–100 \(\mu\)M, 20 min; Q30 TK, 300 ng, 20–120 \(\mu\)M, 20 min; Q75 TK, 100 ng, 10–100 \(\mu\)M, 20 min; Q75-3 TK, 200 ng, 2–70 \(\mu\)M, 20 min; N75-30 TK, 400 ng, 20 min, 40–200 \(\mu\)M; and Q75-30 TK, 100 ng, 10–100 \(\mu\)M, 20 min. The kinetic values (\(K_m\), \(V_{max}\) and \(k_{cat}/K_m\)) for each enzyme and substrate were determined using Microsoft Kalidegraph software version 3.6.

Pseudo-first-order rate constants for wild-type HSV-1 TK and N30-3 TK were determined using thymidine and GCV as
substrates. Using the filter-binding assays described above, the experiments were carried out by preincubating a 50-fold excess of wild-type HSV-1 TK or N30-3 TK with 2 nM thymidine–5 μl [3H]thymidine or 80 nM GCV–5 μl [3H]GCV. Equal volumes of the enzyme–substrate mixture were added with assay buffer and incubated for a total of 10 min for thymidine or 20 min for GCV. Aliquots were taken at time zero as a baseline, then every 15–30 s and spotted on a DE81 filter. The filters were washed as above and radioactivity was quantitated by scintillation counting of the washed filters. The resulting data points were plotted and fitted to a best-fit single exponential with the Kaleidograph software, from which the pseudo-first-order rate constants were obtained. Wild-type HSV-1 TK had a pseudo rate of 0.5 nM/min for thymidine and 0.8 nM/min for GCV with product yields of 65 and 70%, respectively (data not shown). The rates for N30-3 TK were 1.2 nM/min for thymidine and 0.4 nM/min for GCV; however, the product yields were lower at 3% and 16%, respectively (data not shown).

**HSV-1 TK expressing cell lines**

The HCT116 and SW620 human colon carcinoma cell lines were maintained in RPMI 1640 supplemented with L-glutamine and 10% fetal bovine serum (Cellgro). To generate a Moloney murine leukemia virus-derived plasmid for each mutant, the unique BspEI–MluI restriction fragment that contains the mutated flanking regions of site 3 and site 4 were excised from the respective mutant pET-TK plasmids and ligated into the same site of pLENTK (McMasters et al., 1998) as described previously (Hinds et al., 2000). Each new pLEN-mutant TK construct was sequenced positive clones were screened, selected and expanded based on the results of [3H]GCV metabolic labeling, GCV sensitivity (10 μM) and western blot analysis (Hinds et al., 2000). Similar procedures were used to generate the wild-type HSV-1 TK and Q7530 TK expressing SW620 cell lines. The resulting clonal isolates from each mutant HSV-1 TK cell line were screened by western blot analysis to create a panel of representative cell lines that expressed their respective HSV-TK isoform.

**Molecular modeling of substrate interactions with mutant HSV-1 TKs**

All of the molecular modeling analyses were performed using SYBYL version 6.6/6.7 (Tripos, St. Louis, MO) on Silicon Graphics R4400 and R5000 computers in the UAMS Biomedical Visualization Laboratory. The coordinates of two X-ray crystal structures, HSV-1 TK bound with thymidine and HSV-1 TK bound with GCV (Champness et al., 1998), were downloaded from the Brookhaven protein database and loaded into the FlexiDock module of the molecular modeling program SYBYL 6.7/UNITY 4.2. The FlexiDock program consists of two main components, a genetic algorithm and an energy evaluation function for scoring the resulting solutions. The genetic algorithm continually evolves possible ligand–receptor interactions selecting for the structure with the lowest energy form. The energy evaluation scoring functions in the program are based on the Tripos force field, van der Waals interactions, electrostatic interactions and torsional and constraint energy terms. For simplicity, one monomer was used in these studies. Initially, all ligands and water molecules, except for four water molecules important in thymidine binding to the active site, were deleted from the wild-type structures before the amino acid substitutions for Q75-3 TK, Q30-4 TK and Q7530 TK were introduced separately. Next, the active site, designated by a 6 Å radius around Y101, site 3 (L159–H1164) and site 4 (Y168–P173), was fully minimized using an annealing function that relaxes the local strain introduced by the amino acid substitutions. Using the FlexiDock program, the ligand–receptor complex for wild-type thymidine–TK and GCV–TK were optimized according to the following parameters: initial population, generation number and flexible bonds. For thymidine, the Cα–Cβ bond for I160, F161, D162, A168, L169 and L170, the Cα–Cβ bond and the OH side chain for Y101, Y172 and Y132, the Cγ–Cβ bond for R163 and Q215 and the Cβ–Cγ bond for E83 and E225 were marked as flexible. For GCV, the Cα–Cβ bond for I160, F161, D162, A168, L169 and L170, the CαCβ bond and OH side chain for Y101, Y132 and Y172 and the Cγ–Cβ bond for R163 and Q125 were identified as flexible. The resulting ligand–receptor geometries obtained

**The metabolite numbers would reflect growth rates and therefore cellular kinase activities, not specific to HSV-1 TK activities. For both labeling experiments, an aliquot of each methanol-soluble supernatant was analyzed for radioactivity by scintillation counting.**

**Cell viability and clonal dilution assays**

For MTT cell viability assays, cells expressing wild-type, Q30 TK, Q75 TK, Q75-3 TK, N30-3 TK and N7530 TK were plated in 0.1 ml (5000 cells/well) in a 96-well microtiter plate. The following day, GCV was added to the cells within the dose range 0.01–10 μM. After 4 days, 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyltetrazolium bromide (MTT) was added to each well (50 μg/well) for 3 h followed by the addition of dimethyl sulfoxide to solubilize the precipitated crystals. Cell viability was determined by using a plate reader measuring optical density at 540 nm. For clonal dilution assays, cell lines were seeded (105 cells/well) in 24-well plates in 1 ml of medium. The following day, 0.0001, 0.001, 0.01, 0.1 or 1 μM GCV was added to the appropriate wells in triplicate. After 24 h, the cells in each well were sequentially diluted from 1:10 to 1:10 000 in 1 ml of fresh medium as described previously (Drake et al., 1999). The resulting cell colonies were counted after 7 days.
from a FlexiDock run using the above parameters were tested against the ligand–receptor geometry seen in the crystal structure. By using these parameters and the wild-type HSV-1 TK structure, the FlexiDock program was able to match the ligand–protein geometry and hydrogen-bonding pattern seen in the crystal for both substrates with root mean square values of 0.5048 and 0.5750 for thymidine and GCV respectively. These parameters for thymidine and GCV were then used to evaluate ligand binding in the active sites of mutant HSV-1 TK enzymes. An initial population of 2000 individuals, analyzed for 50 000 generations, was chosen as the optimal conditions for analyzing thymidine and GCV binding in the active site of HSV-1 TK. Because thymidine and GCV have different binding properties, the hydrogen donor–acceptor pairs and the flexible bonds varied. On average, these conditions required at least 15 h to complete one FlexiDock analysis per structure. Further details about this algorithm will be provided freely by the authors upon request.

### Results

**Enzymatic screening of the HSV-1 TK mutants**

To introduce amino acid substitutions in the flanking regions of site 3 and site 4 in HSV-1 TK corresponding to the TK30 and TK75 enzymes (Black et al., 1996), the coding sequences of three HSV-1 TK genes were used encoding wild-type HSV-1 TK, Q125N TK and Q125E TK that had been previously cloned into a bacterial expression vector, pET-9a (Hinds et al., 2000). Using the QuikChange site-directed mutagenesis protocol, 16 HSV-1 TK mutants were developed, expressed and purified from *E.coli* as described in Materials and methods (Hinds et al., 2000). Initially, the HSV-1 TK mutants containing the full set of mutations, Q30 TK, Q75 TK, N30 TK, N75 TK, E30 TK and E75 TK were assayed for phosphorylation of thymidine (2 µM) and GCV (40 µM) using enzymatic conditions optimized for wild-type HSV-1 TK (Table I). For comparative purposes, the results obtained for each mutant were normalized as a percentage relative to wild-type HSV-TK activities (set at 100%). As expected, wild-type HSV-1 TK and Q75 TK effectively phosphorylated both substrates and Q30 TK was more GCV-specific. However, substitution of Gln125 with Asn or Glu into Q30 TK or Q75 TK led to comparatively low phosphorylation activities for both substrates for the N30, E30, N75 and E75 HSV-1 TK mutants.

Next, the eight individual half-site HSV-1 TK mutants (Q30-3 TK, Q30-4 TK, Q75-3 TK, Q75-4 TK, N30-4 TK, N30-3 TK, N75-4 TK and N75-3 TK) were screened for activity. Because the determined activities of both E75 TK and E30 TK were so low for both substrates, the corresponding half-site enzymes were not evaluated further. Unlike the previous screen, these half-site HSV-1 TK mutants had a broad range of phosphorylation activities for thymidine and GCV, resulting in some enzymes with specific preferences for thymidine or GCV (Table I). One enzyme, Q30-4 TK, was inactive. Two additional hybrid mutants termed Q7530 TK and N7530 TK were generated containing mutations at site 3 (75-3) and site 4 (30-4). In the comparative activity screen, these HSV-1 TK mutants had a significant decrease in thymidine kinase activity while still maintaining GCV kinase activity (Table I).

Following the activity screens, $K_m$ (app) and $k_{cat}$ (app) for thymidine and GCV were determined for each of the following HSV-1 TK mutant enzymes: Q30 TK, Q75 TK, Q75-3 TK, Q75-4 TK, N30-3 TK, N7530 TK, N30-3 TK and N7530 TK. Linear velocity conditions for each individual enzyme and substrate were established prior to $K_m$ (app) determinations (see Materials and methods). The kinetic data obtained from each experiment were analyzed using a double-reciprocal plot and the $K_m$ (app), $k_{cat}$ and $k_{cat}/K_m$ (app) values were derived from these graphs and are compiled in Table II. For each mutant evaluated, the $K_m$ (app) values for thymidine were 7–100 times higher than those for wild-type HSV-1 TK. Of note for GCV, the Q75-3 TK and Q7530 TK enzymes had lower $K_m$ (app) values of 34 and 40 µM, respectively, combined with the higher $K_m$ (app) values of 15 and 53 µM, respectively, for thymidine. The Q7530 enzyme actually has a lower $K_m$ (app) for GCV than thymidine. The determined $k_{cat}$ values were also informative, in that for thymidine there were relatively minor changes compared with wild-type HSV-1 TK, suggesting alteration of thymidine binding as the effect of the mutations. In contrast, there was a large range (20–100-fold) of decreased $k_{cat}$ values for GCV as a substrate relative to wild-type HSV-1 TK. Although N30-3 TK had detectable activities, linear velocity conditions could not be established. Under the conditions described in Materials and methods, N30-3 TK was evaluated in comparison with wild-type HSV-1 TK to determine a pseudo-first-order rate for thymidine and GCV. Although their respective pseudo-first-order rates were not very different, the product yields were drastically changed, 65% for thymidine and 70% for GCV with wild-type HSV-1 TK compared with 3% and 16%, respectively, for N30-3 TK (data not shown). This apparent product inhibition for N30-3 TK is similar to that reported for the Glu125 TK mutant (Drake et al., 1999; Hinds et al., 2000).

A subset of the mutants (Q7530 TK, N7530 TK, N30-3 TK, Q75-3 TK Q30 TK and Q75 TK) were also assayed for thymidylate kinase (TMPK) activity using TMP as a substrate. The percentage conversion of TMP to TDP for each mutant was normalized to that determined for wild-type HSV-1 TK activity (Table II). As expected, those HSV-1 TK mutants that utilized thymidine poorly also had decreased TMPK activity.

### Table I. Amino acid substitutions of HSV-1 TK mutants and comparative activity screen

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<tr>
<th>Site 3</th>
<th>Site 4</th>
<th>% wild-typea</th>
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aPartially purified HSV-1 TK mutants were screened in triplicate for dT and GCV phosphorylation using a standard reaction mixture and the following linear velocity conditions established for wild-type HSV-1 TK: for dT, 10 ng, 2 µM [3H]dT, 10 min incubation at 37°C; for GCV, 250 ng, 40 µM [3H]GCV, 20 min incubation at 37°C (Hinds et al., 2000).
Further analysis of these mutants was done using the TMP photoaffinity analog \(^{32}\text{P}\)-5-azido-dUMP, which has proven useful in characterizing the thymidine/TMP binding site of HSV-1 TK (Rechtin et al., 1995, 1996; Hinds et al., 2000). As seen in the autoradiograph in Figure 1, \(^{32}\text{P}\)-5-azido-dUMP was photoincorporated efficiently into wild-type HSV-1 TK and Q75-3 TK, but there were only trace amounts of photoincorporation into the active sites of Q30 TK, Q7530 TK and N30-3 TK, indicative of a loss in the ability of these enzymes to bind pyrimidines (Rechtin et al., 1995; Hinds et al., 2000).

**Cellular expression of HSV-1 TK mutants and GCV sensitivity**

The cDNAs of a subset of HSV-1 TK mutants (Q30 TK, Q75 TK, Q7530 TK, N7530 TK and N30-3 TK) were subcloned into a retroviral plasmid, pLENTK, and used to transfect stably a human colon cancer cell line, HCT116. Additionally, wild-type HSV-1 TK and Q7530 TK retroviral plasmids were transfected into SW620, another human colon tumor line. From a panel of multiple HSV-1 TK expressing clones, a subset of HCT-116 clones expressing either wild-type HSV-1 TK, Q30 TK, Q75 TK, Q7530 TK N30-3 TK or N7530 TK were selected for equivalent levels of HSV-1 TK expression based on western blot determinations (data not shown). Despite numerous attempts, a clone expressing equivalent levels of N30-3 TK was not identified. Therefore, the N30-3 TK clone utilized in the following studies had 20% of the expression level of protein compared with the rest of the HSV-1 TK expressing cell panel.

The HCT116 cell lines expressing the different HSV-1 TK isoforms were evaluated for dose-dependent GCV sensitivity by using an MTT cell viability assay and a more sensitive clonal dilution assay. As shown in Figure 2, the Q30 TK, Q75 TK and Q7530 TK expressing cells were more sensitive to the lowest GCV concentration tested than wild-type HSV-1 TK expressing cells. At the 0.1–10 \(\mu\text{M}\) GCV doses, these same mutant TK expressing cell lines were similar in effectiveness to wild-type HSV-1 TK cells. The cell lines expressing N7530 TK and N30-3 TK, which were both poor GCV kinases in the activity screens, displayed only minor sensitivity to GCV at higher concentrations (1–10 \(\mu\text{M}\)). These cell lines were further evaluated using a more sensitive clonal dilution assay as described in Materials and methods. Each cell line was treated with low GCV doses (0.0001–1 \(\mu\text{M}\)) for 4 days, cell viabilities were determined with a plate reader measuring optical density at 540 nm following 2 h of MTT treatment. TK expressing cells. At the 0.1–10 \(\mu\text{M}\) GCV doses, these same mutant TK expressing cell lines were similar in effectiveness to wild-type HSV-1 TK cells. The cell lines expressing N7530 TK and N30-3 TK, which were both poor GCV kinases in the activity screens, displayed only minor sensitivity to GCV at higher concentrations (1–10 \(\mu\text{M}\)). These cell lines were further evaluated using a more sensitive clonal dilution assay as described in Materials and methods. Each cell line was treated with low GCV doses (0.0001–1 \(\mu\text{M}\)) for 4 days, followed by drug removal and sequential dilution. As shown in Figure 3, the number of surviving colonies plotted versus GCV doses indicated IC\(_{50}\) values of 0.01 and 0.008 \(\mu\text{M}\), respectively, for wild-type HSV-1 TK and Q7530 TK. Q30TK expressing cell lines had GCV sensitivities (0.009 \(\mu\text{M}\)) analogous to the Q7530TK and wild-type HSV-TK cell lines (data not shown). Even the poor GCV metabolizing N7530 TK and N30-3 TK HCT-116 cell lines had therapeutically low IC\(_{50}\) values of 0.08 and 0.5 \(\mu\text{M}\), respectively.

**Metabolic labeling**

These same HCT-116 cell lines were evaluated for intracellular metabolism of \(^{3}\text{H}\)thymidine and \(^{3}\text{H}\)GCV. Following an 18 h incubation with 4 \(\mu\text{M}\) \(^{3}\text{H}\)GCV, total methanol-soluble metabolites were extracted from each cell line. As shown in Table III, the Q7530 TK and Q75TK cell lines metabolized GCV at levels approaching those extracted from wild-type

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**Table II.** Kinetic constants of HSV-1 TK mutant enzymes for thymidine and ganciclovir

<table>
<thead>
<tr>
<th>HSV-1 TK</th>
<th>Thymidine</th>
<th>Ganciclovir</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_{\text{m app}} (\mu\text{M}))</td>
<td>(k_{\text{cat}} (\text{s}^{-1}))</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.9 ± 0.08</td>
<td>0.06</td>
</tr>
<tr>
<td>Q30 TK</td>
<td>28 ± 3</td>
<td>0.021</td>
</tr>
<tr>
<td>Q75 TK</td>
<td>7 ± 0.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Q75-3 TK</td>
<td>15 ± 0.9</td>
<td>0.023</td>
</tr>
<tr>
<td>Q7530 TK</td>
<td>53 ± 4</td>
<td>0.056</td>
</tr>
<tr>
<td>N7530 TK</td>
<td>90 ± 7</td>
<td>0.016</td>
</tr>
<tr>
<td>N30-3 TK</td>
<td>ND (^{b})</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^{a}\)TDP to TTP conversion was quantitated by separation of the product on PEI-cellulose TLC plates (Hinds et al., 2000). The amounts of product determined for wild-type HSV-TK reactions were normalized to 100% values. The estimated standard deviations (%) for each reaction are included in parentheses. \(^{b}\)ND, not determined.
Materials and methods.

Data presented in Figure 2. For [3H]thymidine, the same cell wild-type HSV-1 TK cells, consistent with the MTT cell killing GCV metabolite levels that were 15- and 200-fold lower than HSV-1 TK cells, whereas the Q30TK cells generated half of aHCT116 and SW620 cell lines expressing the indicated HSV-1 TK N30-3 TK 1.9 2.5 N7530 TK 1.7 56 Q75-3 TK 3.0 630 Q30 TK 1.4 260 Q7530 TK 1.9 520 3.7 190 Parent 1.7 0.8 3.1 2

Table III. Total methanol-soluble [3H]thymidine and [3H]GCV metabolites

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>HCT116</th>
<th>SW620</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>1.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Wild-type HSV-1 TK</td>
<td>3.0</td>
<td>594</td>
</tr>
<tr>
<td>Q7530 TK</td>
<td>1.9</td>
<td>520</td>
</tr>
<tr>
<td>Q30 TK</td>
<td>1.4</td>
<td>260</td>
</tr>
<tr>
<td>Q75 TK</td>
<td>3.2</td>
<td>501</td>
</tr>
<tr>
<td>Q75-3 TK</td>
<td>3.0</td>
<td>630</td>
</tr>
<tr>
<td>N7530 TK</td>
<td>1.7</td>
<td>56</td>
</tr>
<tr>
<td>N30-3 TK</td>
<td>1.9</td>
<td>2.5</td>
</tr>
</tbody>
</table>


Table III and SW620 cell lines expressing the indicated HSV-1 TK enzymes were labeled with [3H]thymidine and [3H]GCV as described in Materials and methods.

HSV-1 TK cells, whereas the Q30TK cells generated half of these levels. The N7530 TK and N30-3 TK cells generated GCV metabolite levels that were 15- and 200-fold lower than wild-type HSV-1 TK cells, consistent with the MTT cell killing data presented in Figure 2. For [3H]thymidine, the same cell lines were grown to confluence and labeled with 2 µM [3H]thymidine for only 1 h to minimize cellular thymidine metabolism associated with DNA replication. Under these conditions, wild-type HSV-1 TK and Q75 TK cells generated twice the levels of thymidine metabolites compared with parent HCT-116 cells. In contrast, the Q30 TK, Q7530 TK, N30-3 TK and N7530 TK cell lines generated thymidine metabolites at levels comparable to the parent HCT-116 cells. An additional metabolic labeling comparison was done in another colon tumor cell line, SW620, expressing wild-type HSV-1 TK and Q7530 TK. As presented in the second column of Table III, analogous results to the HCT-116 cell lines were obtained.

Molecular modeling

The FlexiDock component of the molecular modeling program SYBL 6.7 was used with the coordinates of two wild-type HSV-1 TK crystal structures (Champness et al., 1998) to dock thymidine or GCV in the active site of Q7530 TK and the corresponding half-site mutants, Q75-3 TK and Q30-4 TK. The FlexiDock program uses a genetic algorithm and energy scoring functions to evaluate thousands of potential ligand–receptor interactions, optimized for HSV-1 TK according to the following parameters: initial structure population, generation number, flexible bonds and hydrogen bonding interactions (see Materials and methods). When the native HSV-1 TK structures were modeled with thymidine (Figure 4A) or GCV (Figure 4B), there was a good correlation between the crystal and FlexiDock model. Root mean square (r.m.s.) values of 0.5594 for thymidine and 0.5750 for GCV were obtained, indicative of convergence in the thousands of structures evaluated by the genetic algorithm. As seen in Figure 5A, modeling of the full set of mutations in Q7530 TK with thymidine predicted that it would bind backwards in the active site relative to wild-type HSV-1 TK. Modeling of GCV with Q7530 TK generated conformations analogous to that of GCV bound to wild-type HSV-1 TK (Figure 5B). Modeling of the half-site Q75-3 TK bound with thymidine was similar to that of wild-type HSV-1 TK, which was expected since the Q75-3 TK possessed robust thymidine phosphorylation activities (Figure 6A). In contrast, for modeling of Q30-4 TK, an enzyme that lacked catalytic activity, the most stable predicted conformation positioned thymidine with its 5-methyl group rotated 180° with respect to its position in the wild-type enzyme (Figure 6B). Binding of GCV was not predicted to be significantly altered in the active site of Q75-3 TK or Q30-4 TK (data not shown). The cumulative modeling data indicate that the mutations near site 4 are most responsible for the observed loss of thymidine phosphorylation activities.

Discussion

Because the HSV-1 TK/GCV suicide gene therapy system could benefit from a GCV-specific enzyme, we proposed to combine previously described mutations of Gln125, which affected nucleoside base binding (Hinds et al., 2000), with mutations within conserved sites 3 and 4 that affected catalysis (Black et al., 1996). This could result in a novel GCV-kinase by eliminating endogenous pyrimidine utilization and improve catalytic efficiency so that GCV was the preferred substrate. Based on this hypothesis, 16 different HSV-1 TK mutant enzymes were developed and screened for improvements in GCV phosphorylation and the loss of thymidine and TMP metabolism. While results with Q30TK and Q75 TK were consistent with previous reports (Black et al., 1996; Kokoris et al., 1999), the mutant enzymes containing an Asn125 or Glu125 had low phosphorylation activities for both thymidine and GCV. Despite the flexibility found in the active site of HSV-1 TK, it cannot tolerate the combined mutations of TK30 or TK75 with the Asn or Glu mutations at Gln125 and still maintain wild-type level activities for GCV. The activity profiles of the half-site and combination half-site mutant enzymes demonstrated a wide range of phosphorylation rates for both thymidine and GCV, including two novel kinases, Q7530 TK and N7530 TK, which retained a greater proportion of GCV phosphorylation activity than for thymidine.

The Q7530 TK enzyme possessed the most interesting properties with regard to the development of a GCV-specific kinase. In comparison with wild-type TK, it had a decreased $K_m$ (app) for GCV to 40 µM and a greater than 50-fold increase in the $K_m$ (app) for thymidine to 53 µM. To our knowledge, the Q7530 TK is the first HSV-TK derived mutant to have a $K_m$ for GCV that is lower than that of thymidine. Q7530 TK...
Fig. 4. The predicted FlexiDock interactions of (A) thymidine and (B) GCV bound to wild-type HSV-1 TK.

Fig. 5. The predicted FlexiDock interactions of (A) thymidine and (B) GCV bound to Q7530 TK.

Fig. 6. The predicted FlexiDock interactions of thymidine bound to (A) Q75-3 TK or (B) Q30-4 TK.
also lacked TMPK activity and only trace amounts of [32P]-5-azido-dUMP were photoincorporated (Figure 1), results consistent with the inability of TMP to bind properly in the active site of this enzyme. In support of the kinetic data, Q7530 TK expressed in the HCT116 cell line generated levels of [3H]thymidine metabolites comparable to parental cells lacking HSV-1 TK, yet it was able to generate GCV metabolites comparable to those of expressed wild-type HSV-1 TK (Table III). In cell viability assays, Q7530 TK expressing cells were equally sensitive to low GCV concentrations as cells expressing wild-type HSV-1 TK or Q30 TK. A more rapid initial rate of GCV metabolism, incorporation into DNA and accelerated S-phase cell cycle arrest have been observed in the Q7530 TK cells relative to those expressing wild-type enzyme (data not shown). It remains to be determined with in vivo studies whether the properties of Q7530 TK confer any therapeutic advantage in ablating tumor growth, as has been reported previously for Q30 TK relative to wild-type HSV-1 TK (Kokoris et al., 1999). Another mutant HSV-1 TK possessing improved GCV metabolism, termed sr39 TK, has demonstrated improved efficacy of tumor regression in animal models and more sensitive killing of sr39TK transduced T-lymphocytes (Pantuck et al., 2002; Qasim et al., 2002). Hence it is feasible that Q7530 TK will function similarly in in vivo studies and experiments to evaluate these mutants in a murine prostate tumor model are in progress.

The other mutant enzyme with GCV kinase properties, N7530 TK, was not as efficient as Q7530 TK in utilizing GCV as a substrate. Despite a $K_m$ (app) of 71 µM for GCV similar to wild-type HSV-1 TK, N7530 TK possessed an 800-fold decrease in the catalytic efficiency for GCV phosphorylation. However, the change in the $K_m$ (app) of thymidine to 91 µM for N7530 TK was the most drastic change for any of the mutants analyzed. Like Q7530 TK, N7530 TK also lacked the ability to phosphorylate TMP (Table I, Figure 1). When expressed in the HCT116 cells, extracted GCV metabolites relative to wild-type HSV-1 TK for N7530 TK were 40-fold lower. In cell viability assays, the N7530 TK expressing cells were insensitive to lower GCV doses, but displayed limited cytotoxicity at higher doses (10 µM) (Figure 2). We hypothesize that these GCV cytotoxicities would not have been observed had the deoxypyrimidine activities of this enzyme not been decreased. This is because normal intracellular concentrations of thymidine exceed the $K_m$ for HSV-1 TK, while intracellular GCV concentrations are considerably lower than its $K_m$. Thus, thymidine would normally out-compete GCV for binding to the active site (Markham and Faulds, 1994; Kokoris et al., 1999).

In order to evaluate why the different structural mutations comprised in Q7530 TK resulted in a more GCV-specific kinase, a FlexiDock molecular modeling algorithm was generated and tested. This FlexiDock program allows both the ligand and binding pocket to flex during docking, so that an induced fit can be more thoroughly evaluated among the thousands of different possible structures analyzed. As shown in Figures 4–6, the structural differences caused by mutation at Ala168 were the primary contributors to the different thymidine activities observed between wild-type HSV-1 TK, Q7530 TK, Q75-3 TK and Q30-4 TK. For Q7530 TK, the loss in thymidine kinase activity was directly related to the Tyr168 mutation near site 4 (Figure 5A). In both Q7530 TK and Q30-4 TK, the bulky phenyl group of Tyr168 sterically blocks the binding pocket where the 5-methyl group of thymidine normally is bound. In a previous molecular modeling analysis of Q30 TK based on a temperature annealing algorithm (Kokoris et al., 1999), the same conclusion of a steric interference role for Tyr168 was drawn. Also, an early structural study examining HSV-1 TK mutants isolated from BVdU-resistant viruses revealed that a single amino acid substitution at A168T conferred BVdU resistance (Larder et al., 1983). The likelihood that thymidine actually is bound with its 5-methyl group flipped 180° relative to wild-type enzyme, seen in Q30-4 TK (Figure 6B), or rotated 180°, seen in Q7530 TK (Figure 5A), is unknown since the FlexiDock modeling program assumes that the substrate had entered the binding site. The most probable scenario is that thymidine has limited access to the active site of these particular enzymes, as supported by the activity, photoaffinity and cellular data. Although not shown or described, the molecular modeling algorithm could also accurately predict poor or altered thymidine binding in N7530 TK, N30-3 TK and Q30TK.

For modeling with GCV, more subtle variations in binding were detected. Although thymidine and GCV share the same binding site, GCV is positioned higher, closer to Arg176. As a result, the steric interactions between GCV and the phenyl group of Tyr168 were not significant enough to alter GCV binding in Q7530 TK (Figure 5B). For N7530 TK, the extra substitution of Asn125 may be a destabilizing factor to account for its decreased activity relative to wild-type or Q7530 TK. One inconsistency with the modeling and activity data is that of the Q30-4 TK enzyme. Even though the purified Q30-4 TK enzyme lacked apparent GCV phosphorylation activity, it was positioned in the modeled active site similar to that of the highly active Q7530 TK. One reason for this inconsistency may be that as determined in the kinetic evaluations for these mutants (Table II), the overall changes in the $K_m$ for GCV (less than 2-fold) were not as high as those for thymidine (15–90-fold). Hence there was a greater probability that the modeling program would more readily predict greater changes for thymidine binding. Also, the modeling program does not allow the visualization of how the substrate enters the active site, as the FlexiDock program makes an assumption that the substrate can gain access to the active site. It is possible that other structural perturbations within Q30-4 TK prevent both thymidine and GCV binding, and hence activity, in the purified enzyme. Lastly, the current HSV-1 TK model is weighted for the influence of hydrogen bonding on substrate binding, interactions which have consistently proven to be important for thymidine binding (Brown et al., 1995; Champness et al., 1998; Hinds et al., 2000) and is reflected in the models of the mutant enzymes. As additional HSV-1 TK mutants are generated, there are many parameters within the FlexiDock program that can be refined to improve analysis of GCV binding, especially an emphasis on hydrophobic and steric interactions.

In conclusion, from the 16 different HSV-1 TK mutants generated, purified and assayed, two mutant enzymes, Q7530 TK and N7530 TK, have been characterized as GCV-specific kinases. Originally we proposed to develop these novel GCV kinases by incorporating mutations known to minimize deoxypyrimidine substrate utilization (Hinds et al., 2000) with mutations known to improve the catalytic efficiency of GCV phosphorylation (Black et al., 1996). The Q7530 TK essentially achieved our initial goal by possessing a $K_m$ for GCV lower than that of thymidine. Introduction of an Asn or Gln for Gln125 in the Q75 TK or Q30 TK mutant backgrounds eliminated thymidine metabolism of HSV-1 TK and, with the
exception of N7530 TK, also proved to be equally detrimental for GCV phosphorylation. However, these studies do demonstrate that even though all nucleoside/nucleotide substrates bind in the same active site region, discrimination of phosphorylation of guanine analogs such as GCV can be achieved while eliminating activity for thymidine/TMP. Besides the well documented importance of Gln125 (Brown et al., 1994, Champness et al., 1998; Hinds et al., 2000), Ala168 was again identified (Kokoris et al., 1999) as being a critical participant in substrate binding in the active site. It is clear that the half-site mutations at site 3 in the 75–3 TK are responsible for the improved thymidine and GCV activities, while the site 4 half-site mutations (that included mutations to Ala168) were generally detrimental to substrate binding. By analyzing these new HSV-1 TK mutant enzymes with the FlexiDock program, we have refined a computer modeling algorithm that can be used to design and test substrate binding properties before generating the mutants. Our future plan is to use this modeling approach as a screen to identify new HSV-1 TK mutants, in an effort to develop enzymes that lack deoxypyrimidine binding and possess $K_m$s for GCV that are lower than 10 µM.

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References


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