FOR THE RECORD

Characterization of Herpes Simplex Virus type 1 thymidine kinase mutants engineered for improved ganciclovir or acyclovir activity

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Abstract

Herpes Simplex Virus type 1 (HSV-1) thymidine kinase (TK) is currently the most widely used suicide agent for gene therapy of cancer. Tumor cells that express HSV-1 thymidine kinase are rendered sensitive to prodrugs due to preferential phosphorylation by this enzyme. Although ganciclovir (GCV) is the prodrug of choice for use with TK, this approach is limited in part by the toxicity of this prodrug. From a random mutagenesis library, seven thymidine kinase variants containing multiple amino acid substitutions were identified on the basis of activity towards ganciclovir and acyclovir based on negative selection in *Escherichia coli*. Using a novel affinity chromatography column, three mutant enzymes and the wild-type TK were purified to homogeneity and their kinetic parameters for thymidine, ganciclovir, and acyclovir determined. With ganciclovir as the substrate, one mutant (mutant SR39) demonstrated a 14-fold decrease in K_m compared to the wild-type enzyme. The most dramatic change is displayed by mutant SR26, with a 124-fold decrease in K_m with acyclovir as the substrate. Such new "prodrug kinases" could provide benefit to ablative gene therapy by now making it feasible to use the relatively nontoxic acyclovir at nanomolar concentrations or ganciclovir at lower, less immunosuppressive doses.

Keywords: Herpes Simplex Virus type 1 thymidine kinase; ganciclovir; acyclovir; random sequence mutagenesis

Thymidine kinase (EC 2.7.1.21) is the key enzyme in the pyrimidine salvage pathway catalyzing the transfer of the γ -phosphate from ATP to thymidine to produce dTMP. Unlike the cellular thymidine kinase, Herpes Simplex Virus type 1 (HSV-1) thymidine kinase (TK) has a very broad substrate specificity including pyrimidines and pyrimidine analogs (thymidine, deoxycytidine, and AZT) as well as purine (guanosine) analogs (acyclovir, ganciclovir, buciclovir, and penciclovir) (Chen et al. 1979a, 1979b; Field et al. 1983; Furman et al. 1984; Gentry 1992; Balzarini et al. 1993). Once phosphorylated, these nucleoside analogs are

further phosphorylated by cellular kinases to the corresponding nucleoside triphosphates that inhibit DNA synthesis after incorporation into nascent DNA (Miller and Miller 1980; Boehme 1984; Reardon 1989). As such, HSV-1 TK can provide unique activities to cells that express it, and offers the prospect of rendering those cells susceptible to nucleoside prodrugs. HSV-1 TK has been used successfully in gene therapy in a wide variety of animal tumor models, and is currently in clinical trials for human cancers (Moolten and Wells 1990; Culver et al. 1992; Caruso et al. 1993). Some problems associated with high ganciclovir (GCV) doses used to achieve tumor cell killing include immunosuppression as well as other toxic side effects (Ram et al. 1993; Osaki et al. 1994; Scholz et al. 1994). It has been shown that not all cells need to express TK to be susceptible to prodrug-mediated killing. Known as the bystander effect,

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this phenomenon is thought to be due to several factors: transportation of activated prodrug through gap junctions formed by cell–cell contact, or via apoptotic vesicles and an immunologically mediated clearance mechanism (Bi et al. 1993; Freeman et al. 1993; Tapscott et al. 1994; Colombo et al. 1995). Because of the immunosuppressive action of GCV, this latter aspect of the bystander effect may not be fully realized. Another guanosine analog used as an anti-herpetic drug is acyclovir (ACV). Although acyclovir is relatively nontoxic even at high doses, HSV-1 TK displays a very high K_m towards ACV that precludes its application in gene therapy clinical settings. Novel HSV-1 thymidine kinases with increased specificity for phosphorylating GCV or ACV could benefit tumor ablation by enhancing cell killing and by reducing prodrug-mediated toxicity.

As a means to overcome the toxic side effects of GCV and the poor activity displayed towards ACV, we created a library of multiple amino acid containing-HSV-1 TK mutants that were screened for improved prodrug activities (Black et al. 2001). This is a second-generation library with restricted randomization (semirandom) at codons encoding substitutions that yielded desired traits in the first-generation library (Black et al. 1996a). From 512 possible substitution combinations, 71 clones were active. Sequence analysis revealed that all functional mutants contained three to five amino acid substitutions. Of these, seven were identified to have increased activity towards GCV and/or ACV in a negative selection system in *E. coli*. Based on direct enzyme assays, three mutants were identified for further analysis in vitro and for kinetic studies. Glioma cells expressing any of these three mutants, conferred substantial increased sensitivity to both GCV and ACV when compared to IC_{50} values of wild-type HSV-1 TK expressing transfectants (Black et al. 2001). In this report we describe the purification of wild-type and three semirandom mutant HSV-1 thymidine kinases using 3'-aminothymidine sepharose chromatography and kinetic evaluation with respect to thymidine, GCV, and ACV.

Results and Discussion

Through molecular evolution we sought to create new thymidine kinases with increased specificities towards ganciclovir and/or acyclovir (see Fig. 1 for substrate structures). This is particularly relevant when considering the limitations of pairing wild-type TK and GCV in current ablative gene therapy applications. The high K_m wild-type TK displays towards GCV (47 μM) in conjunction with the immunosuppressive side effects associated with high GCV administration doses is indicative of a less than optimal enzyme–prodrug combination. Furthermore, the reduced immune response caused by the immunosuppressive action of high GCV dosages may well impede the immune component of the bystander effect (Tapscott et al. 1994; Colombo et al. 1995; Dilber et al. 1997). One way to overcome these limitations is to alter the enzyme to increase its specificity towards GCV. This would allow administration of a lower and less toxic drug dose to provide similar levels of tumor ablation. Alternatively, a change in the substrate specificity to allow the use of a different, relatively nontoxic drug such as acyclovir would be advantageous. Acyclovir is presently not used in gene therapy as a prodrug due to the extremely high K_m value that the wild-type thymidine kinase displays towards it (319 μM). Mutant thymidine kinases with increased specificity and activity towards GCV or ACV could greatly improve the efficacy and safety of the enzyme prodrug approach for suicide gene therapy.

Previously, we described a library of HSV-1 TK mutants created by the insertion of semirandomized sequences (Black et al. 2001). In designing the oligonucleotides used to generate the semirandom library, substitutions identified in mutants from an earlier library that demonstrated enhanced sensitivity to the prodrugs were used as a template. The earlier library encompassed 64×10^6 possible amino acid substitution combinations (Black et al. 1996a). Only one million mutants from that library were screened. As a means to exploit the diversity of that library, we constructed

Fig. 1. Chemical structures of thymidine, ganciclovir (GCV) and acyclovir (ACV).

HSV-1 TK (wt) residues 159-174												
	A	L	I	F	<u>D</u>	<u>R</u>	<u>F</u>	P	I	A	A	L
												L
												<u>C</u>
												<u>Y</u>
												<u>P</u>
												A
Mutants												
SR11	-	F	L					F	N			
SR26	-	F	A					F	-			
SR39	I	F	L					F	M			

Fig. 2. Deduced amino acid sequence of three HSV-1 TK mutants derived from the semirandom library. The top line shows the amino acid sequence of the wild-type HSV-1 thymidine kinase (residues 158–174) with the deduced amino acid substitutions of mutants SR11, SR26, and SR39 shown underneath. The underlined residues represent the codons that were randomized in the creation of the library. All *Herpesviridae* thymidine kinases contain six highly conserved motifs; two such motifs are boxed (Balsubramaniam et al. 1990). All mutants were identified on the basis of increased sensitivity to GCV and/or ACV. Standard single letter amino acid nomenclature is used. The (-) denotes identity with the wild-type amino acid sequence.

the semirandom library based on the sequence of the best mutants identified from the large library. It was anticipated that this second-generation library would yield different combinations of substitutions with even greater abilities to ablate neoplastic cells than those identified in the first-generation library.

Mutants from the semirandom library were initially selected for enzyme function with thymidine and then subjected to a negative screen to identify mutants with enhanced sensitivity to GCV or ACV in *E. coli* (Black et al. 2001). Evaluation of three semirandom mutants for increased sensitivity to GCV or ACV in rat C6 glioma cells revealed that all three mutants conferred a substantially enhanced ability to kill cells compared to the wild-type TK (Black et al. 2001). IC₅₀ values of cells transfected with mutants were >3- to 294-fold lower than those of wild-type TK expressing cells.

To understand the biochemical nature of the changes in substrate specificities conferred by the multiple amino acid changes found in the three semirandom mutants (Fig. 2), SR11, SR26, SR39, and the wild-type TK were purified to near homogeneity using a novel single-step affinity chromatography procedure described in Materials and Methods, and their kinetic parameters determined (Table 1). This pu-

rification protocol differs from the thymidylyl-sepharose columns that have been used previously (Tung et al. 1996; Kokoris et al. 1999). The precursor reagents (CNBr sepharose and amidothymidine) are readily available and require minimal manipulation to create a high-affinity column suitable for purifying large amounts of thymidine kinase. All enzymes (wild-type, SR11, SR26, and SR39) displayed Michaelis-Menten kinetics throughout the concentration ranges used for each substrate, and no substrate inhibition or activation was observed. The K_m values were determined from Lineweaver Burk plots (data not shown), and are shown in Table 1. The Michaelis constants (K_m) of wild-type HSV-1 TK for the substrates thymidine, GCV, and ACV of 0.38, 47.6, and 417 μ M, respectively, are in close agreement with previously reported values (Munir et al. 1992; Balzarini et al. 1993; Black et al. 1996b; Kokoris et al. 1999).

Compared to the wild-type TK K_m values, all mutants display poorer K_m s for thymidine ranging from 2.5- to almost sevenfold higher values. When the K_m s for the prodrugs are considered, major differences are observed between the wild-type TK and all three semirandom mutants. The K_m values (GCV) for mutants SR11, SR26, and SR39 are 7.4-, 2.7-, and 14.3-fold lower, respectively, than that displayed by the wild-type TK. Most striking are the K_m values for ACV for all mutants with respect to wild-type TK values. Mutant K_m values for ACV (SR11, SR26, and SR39) are 43-, 75-, and 124-fold lower than that displayed by the wild-type enzyme (Table 1).

Although the change in K_m values of the mutants indicates a positive change in substrate affinity towards the prodrugs, the k_{cat} values suggest that catalysis is negatively impacted. The turnover numbers (k_{cat}) for SR11, SR26, and SR39 were substantially reduced from that of the wild-type on all substrates tested; thymidine (65-, 73-, and 27-fold less, respectively), GCV (18-, 11.5-, and 29-fold less, respectively), and ACV (32-, 54-, and 35-fold less, respectively).

The specificity constant, which reflects both k_{cat} and K_m (k_{cat}/K_m), for all mutants are orders of magnitude lower (164–190 fold) than the wild-type enzyme for thymidine. The specificity constants for the mutants are only two- to

Table 1. Kinetic parameters of mutant and wild-type thymidine kinases

	K_m (μ M)			k_{cat}^a (sec ⁻¹)			k_{cat}/K_m (sec ⁻¹ / μ M)		
	Thymidine	GCV	ACV	Thymidine	GCV	ACV	Thymidine	GCV	ACV
HSVTK	0.38	47.6	417	0.46	0.10	1.5×10^{-2}	1.2	2.1×10^{-3}	3.6×10^{-5}
SR11	0.95	6.41	5.60	7.0×10^{-3}	5.6×10^{-3}	4.7×10^{-4}	7.3×10^{-3}	8.7×10^{-4}	8.3×10^{-5}
SR26	1.3	17.55	3.37	6.2×10^{-3}	8.9×10^{-3}	2.8×10^{-4}	4.8×10^{-3}	5.1×10^{-5}	8.3×10^{-5}
SR39	2.64	3.33	9.79	1.7×10^{-2}	3.5×10^{-3}	4.3×10^{-4}	6.3×10^{-3}	1.1×10^{-3}	4.4×10^{-5}

^a The k_{cat} values were calculated using the equation $V_{max} = k_{cat} \cdot [E]$ where E = total enzyme concentration and is based on one active site/monomer. Assay conditions are described in Materials and Methods.

fourfold lower on GCV compared to the wild-type TK value. With respect to ACV, the k_{cat}/K_m for all three mutants was the same as wild-type TK or improved approximately twofold. From a comparison of the specificity constants between wild-type and mutant enzymes for GCV and ACV, one might not predict that the mutants would elicit hugely enhanced prodrug sensitivity to cells. However, because endogenous thymidine within the cell competes with the prodrug for the active site, it is important to consider the ratio of specificity constants for prodrug and thymidine. We used the equation $[k_{\text{cat}}/K_m(\text{prodrug})]/[k_{\text{cat}}/K_m(\text{thymidine}) + k_{\text{cat}}/K_m(\text{prodrug})]$ to take this into account. With GCV as the prodrug, mutants SR11, SR26, and SR39 display values 60-, 54-, and 80-fold higher, respectively, than the wild-type enzyme. With ACV as the prodrug, mutants SR11, SR26, and SR39 display 375-, 567-, and 239-fold higher values, respectively, compared to the wild-type enzyme. Clearly, when competition for the active site is considered, all three mutant enzymes reveal an enormous kinetic advantage for both prodrugs over the wild-type enzyme.

The goal for creating the semirandom library was to create better suicide genes than those previously identified. Two mutants, mutant 30 and 75, derived from the first-generation library, were shown to improve GCV- and ACV-mediated cell killing in mammalian cells (Kokoris et al. 1999; Black et al. 2001). To compare the kinetics of mutants from both the first- and second-generation libraries, we used the equation given above. With GCV or ACV as the prodrug, all mutants displayed values much higher than that of the wild-type HSV-1 TK. Compared to the wild-type enzyme on GCV, mutants 30, 75, SR11, SR26, and SR39 display values 66-, 11-, 60-, 54-, and 80-fold higher, respectively. When ACV values are compared to the wild-type TK, mutants 30, 75, SR11, SR26, and SR39 display 333-, 70-, 375-, 567-, and 239-fold higher values, respectively. Others have created mutants based on molecular modeling studies as a means to improve prodrug activity (Hinds et al. 2000). Kinetic comparison of their best site directed mutant, Q125N, with GCV revealed only a 2.3-fold higher value than the wild-type TK (Hinds et al. 2000). In light of these comparisons, mutants derived from the semirandom library demonstrate significant improvements over previous mutants. From a kinetic standpoint, SR39 is the best enzyme with GCV, and mutant SR26 displays the best kinetic advantage with ACV.

The semirandom mutants contain three to five amino acid substitutions located within or near the active site (Fig. 2). All the substitution combinations are unique and novel. We intentionally mutated codons that represent amino acids that are not conserved in alignments of *Herpesviridae* family thymidine kinases (Balasubramaniam et al. 1990) and were found in earlier random mutagenesis studies to accept a variety of different amino acid substitutions with maintenance of TK activity. Interestingly, all three semirandom

mutants contain a phenylalanine at position 160 (I160F) and a phenylalanine at position 168 (A168F). Molecular modeling of a six residue substituted mutant (mutant 30) derived from the first-generation library led us to suggest that the presence of tyrosine at alanine 168 (A168Y) caused neighboring side chains to be displaced and thereby enlarge the active site (Kokoris et al. 1999). Presumably, the more open active site can better accommodate access by the larger guanosine analogs such as GCV. The phenylalanine substitution at position 168 found in the semirandom mutants might achieve the same expansion of the active site as the A168Y-containing mutant.

Because isoleucine 160 lies outside the active site on a beta strand the influence of the I160F substitution is less clear. From sequence alignment of *Herpesviridae* family thymidine kinases there is a conservation of hydrophobic residues at that position with valine, leucine, or methionine at the corresponding position in other herpes thymidine kinases. However, in the first-generation library two of the 10 multiple amino acid substitution mutants with improved activities encode a phenylalanine at position 160 (Black et al. 1996a). Because I160 apparently displays no direct role in substrate binding by itself, the action of I160F is likely to be secondary in nature, possibly manifested by the presence of one or more additional substitutions. Evaluation of a single substituted I160F may facilitate elucidation of its role.

We have selected and identified new mutant HSV-1 thymidine kinase enzymes containing multiple amino acid changes at or near the active site. These mutants confer increased prodrug sensitivity to mammalian cells and display enzyme kinetics more related to "prodrug kinases" than to thymidine kinases. As such, these new enzymes could provide benefit to ablative gene therapy protocols in two important ways: by now making it feasible to use the relatively nontoxic ACV at clinically relevant doses, and by achieving tumor ablation using lower, less toxic doses of GCV.

Materials and methods

Materials

Radioisotopes for kinetic assays: [*methyl*-³H] thymidine (specific activity, 85 Ci/mmol) was purchased from Amersham and [8-³H]-acyclovir (specific activity, 16.9 Ci/mmol) and [8-³H]-ganciclovir (specific activity, 17.6 Ci/mmol) were purchased from Moravsek Biochemicals. All other chemicals used were purchased from Sigma.

Bacterial strain and expression vector

The *E. coli* strain BL21(DE3) *tdk*⁻ (F⁻ *ompT* *hsdSB*(rB-mB-) *gal dcm tdk* (DE3), a gift from Dr. William Summers (Yale University, New Haven, CT; F⁻ *ompT*[*lon*]*hsdS*_B(rB-mB-)), was used as a recipient for overexpression procedures. pET23d:HSVTK-

Dummy is a derivative of pET23d:HSVTK that contains a non-functional DNA fragment between the *SacI* and *KpnI* sites of the HSV-1 tk open reading frame (Black et al. 1996a). The semirandom library was constructed by replacement of the *SacI/KpnI* fragment of pET23d:HSVTK-Dummy with a pool of semi-randomized *SacI/KpnI* fragments as described in Black et al. (2001). Overexpression of wild-type HSV-1 TK and mutant thymidine kinases was done using pET23d:HSVTK, pET23d:SR11, pET23d:SR26, or pET23d:SR39 in BL21(DE3) *tdk⁻*.

AMT-sepharose affinity resin

A modified protocol for a thymidine kinase affinity matrix was made based on the description by Tung et al. (1996). The Tung et al. protocol (1996) was shortened and simplified by starting with 3'-aminothymidine rather than derivatizing it from azidothymidine and by altering the coupling reaction of the ligand to the resin as follows. Five grams of CH-Sepharose 4B were rehydrated in 30 mL of 1M HCl for 10 min and transferred to a sintered glass funnel. The gel was then washed with 2 L of 1 mM HCl. In 30 mL of Coupling Buffer (100mM NaHCO₃, pH8/500 mM NaCl) 50mg of 3'-aminothymidine (Sigma) was solubilized. The washed resin was added to the 3'-aminothymidine (AMT) and shaken gently at room temperature for 2 h. A 1:2 ratio of resin volume to Coupling Buffer was sufficient for binding. Excess ligand was removed by rinsing the resin with 5 gel volumes of Coupling Buffer. All remaining activated groups were blocked by rinsing the resin with 50 mL 100mM Tris, pH8. An alternating series of four 50-mL washes of high pH buffer (100 mM Tris, pH8/500 mM NaCl) and low pH buffer (100 mM acetate, pH4/500 mM NaCl) was performed. The resin is stored in 50 mM Tris, pH7.5, and 0.004% sodium azide at 4°C.

Protein overexpression and purification

Induction of TK expression from pET23d:HSVTK (BL21 (DE3) *tdk⁻*) using IPTG was as previously reported (Black et al. 1996b). Cells from a 1-L induced culture were pelleted by centrifugation and the supernatant removed. The pellets were lysed and a cleared lysate prepared as described in Black et al. (1996b).

Purification of wild-type and mutant TKs was performed by affinity chromatography on the AMT-Sepharose resin. All chromatography steps were done at 4°C. The 5-mL bed volume column was prepared by passing 10 mL of Absorption Buffer (50 mM Tris, pH7.5/10% sucrose/2 mM DTT/25 mM MgCl₂/10 mM ATP/1 mM PMSF) over the column. Prior to loading the lysate on the column, 1/1000 volume 1 M MgCl₂ and 1/200 volume 0.1 M ATP were added to the cleared lysate and the mixture filtered through a 0.45- μ m low-protein binding filter to remove any material that might clog the resin. The filtered lysate was passed over the column two to three times and the flow-through material collected. The column was washed with 2–15 mL aliquots of Buffer 1 (50 mM Tris, pH7.5/10% sucrose/2 mM DTT/1 mM PMSF) containing 1 M NaCl (wash fractions 1–2). Lower concentrations of NaCl were needed to purify mutant TK enzymes with poorer binding affinities. Thymidine kinase was eluted with 4–5 mL aliquots of Thymidine Buffer (300 mM Tris, pH7.5/10% sucrose/2 mM DTT/50 mM KCl/600 μ M thymidine) (elution fractions 1–4). The column was washed with 10 mL High Salt Buffer (50 mM Tris, pH7.5/10% sucrose/2 mM DTT/500 mM KCl) and stored in Buffer 1 containing 0.004% sodium azide. Elution fractions were extensively dialyzed against Buffer 1 without PMSF at 4°C to remove thymidine. Protein concentrations were determined using the Bio-

Rad Protein Assay Dye reagent according to the manufacturer's instructions. BSA supplied with the reagent was used as the protein standard.

Enzyme assays and kinetics

Thymidine kinase activity assays were carried out as previously described with varying substrate concentrations (Black et al. 1996b; Kokoris et al. 1999). Given the large variance in enzyme activities for the substrates assayed, a broad range of substrate concentrations (0.1 to 10 μ M) and incubation times (15 to 120 min) were necessary to achieve enough product formation for analysis. All assays were performed in covered 96-well microtiter plates at 37°C. Phosphorylation of the nucleosides, [*methyl*-³H]-thymidine, [8-³H]-ACV, and [8-³H]-GCV, was detected by a filter binding assay as described by Hruby and Ball (1981) with the exception that all washes were done at room temperature. All assays were performed in triplicate with water controls for each substrate concentration. The filter bound [³H]-nucleoside monophosphate was quantified using a Wallac 1409 liquid scintillation counter.

Data was plotted as the double reciprocal of velocity (min/ μ Mole $\times 10^4$) versus substrate concentration μ M⁻¹ (Lineweaver Burk) and the intercept values used to determine the *K_m* for each substrate. A conversion factor was established for each [³H]-nucleotide monophosphate by measuring the cpm for a known number of moles of each [³H]-nucleoside. The ratio of cpm to pmole nucleoside was used to determine the number of nucleotide monophosphate pmoles formed. These values were used to calculate the *k_{cat}* from the double reciprocal plot data as per active site (one active site per monomer).

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