

Creation of drug-specific herpes simplex virus type 1 thymidine kinase mutants for gene therapy

MARGARET E. BLACK*†‡, TERRY G. NEWCOMB*, HEATHER-MARIE P. WILSON*, AND LAWRENCE A. LOEB*

*Joseph Gottstein Memorial Cancer Research Laboratory, Department of Pathology, Box 357705, School of Medicine, University of Washington, Seattle, WA 98195-7705; and †Darwin Molecular Corporation, 1631 220th Street S.E., Bothell, WA 98021

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ABSTRACT Herpes simplex virus type 1 (HSV-1) thymidine kinase is currently used as a suicide agent in the gene therapy of cancer. This therapy is based on the preferential phosphorylation of nucleoside analogs by tumor cells expressing HSV-1 thymidine kinase. However, the use of HSV-1 thymidine kinase is limited in part by the toxicity of the nucleoside analogs. We have used random sequence mutagenesis to create new HSV-1 thymidine kinases that, compared with wild-type thymidine kinase, render cells much more sensitive to specific nucleoside analogs. A segment of the HSV-1 thymidine kinase gene at the putative nucleoside binding site was substituted with random nucleotide sequences. Mutant enzymes that demonstrate preferential phosphorylation of the nucleoside analogs, ganciclovir or acyclovir, were selected from more than one million *Escherichia coli* transformants. Among the 426 active mutants we have isolated, 26 demonstrated enhanced sensitivity to ganciclovir, and 54 were more sensitive to acyclovir. Only 6 mutant enzymes displayed sensitivity to both ganciclovir and acyclovir when expressed in *E. coli*. Analysis of 3 drug-sensitive enzymes demonstrated that 1 produced stable mammalian cell transfectants that are 43-fold more sensitive to ganciclovir and 20-fold more sensitive to acyclovir.

An underlying principle in cancer chemotherapy is the exploitation of minor biochemical and cellular differences that exist between normal and tumor cells by using drugs that selectively kill tumor cells. The major impediment is that cancer cells do not exhibit unique biochemical properties that distinguish them from normal cells. Even within the same tumor, malignant cells exhibit extensive genetic and biochemical heterogeneities. As a result, there are few chemicals affecting the majority of cancer cells that do not equally affect normal cells. Gene therapy offers the prospect of selectively introducing genes into cancer cells, rendering them susceptible to specific antimetabolites. Herpes simplex virus type 1 thymidine kinase (HSV-1 TK) is particularly attractive for gene therapy. In contrast to the endogenous human TK, HSV-1 TK phosphorylates a variety of nucleoside analogs (1). It is this relaxed substrate specificity that is the basis for the use of nucleoside analogs in the treatment of herpetic infections. Once phosphorylated, the analogs are analogized by cellular kinases to corresponding nucleoside triphosphates, which inhibit host cell DNA replication by chain termination (2–5). HSV-1 TK has been used successfully in gene therapy for a variety of animal tumor models. The use of HSV-1 TK is also being evaluated in the treatment of human brain tumors and is being considered for a variety of other human cancers (6–16). One introduces the HSV TK gene (*tk*) into a tumor and subsequently treats the individual systemically with a nucleoside analog that selectively inhibits DNA replication only in tumor cells. Novel HSV-1 TKs with increased specificity for phos-

phorylating ganciclovir (GCV) and/or acyclovir (ACV) could enhance tumor cell killing without increased toxicity.

HSV-1 TK has a high affinity for thymidine ($K_m = 0.5 \mu\text{M}$) (17), whereas the K_m values for GCV and ACV are much higher at $45 \mu\text{M}$ and $>400 \mu\text{M}$, respectively (18–19). We sought to remodel the active site of HSV-1 TK to increase the substrate specificity towards the guanosine nucleoside analogs GCV and ACV and concomitantly to decrease thymidine utilization. In the present study we have created over one million variants of the HSV-1 TK gene by random sequence mutagenesis and have used positive and negative genetic complementation in *Escherichia coli* to identify mutants that exhibit heightened GCV and/or ACV sensitivity when expressed in mammalian cells.

MATERIALS AND METHODS

Bacterial Strains. The *E. coli* strain BL21(DE3) *tdk*⁻ [*F*⁻ *ompT hsdSB*(rB⁻ mB⁻) *gal dcm tdk* (DE3)] used in the genetic complementation assays for TK activity was derived by repeated passages of SY211 [BL21(DE3) *tdk*⁻, pLysS] (20) on nonselective plates (no chloramphenicol) to cure the cells of pLysS. *E. coli* NM522 [*F*['] *lacIq* Δ (*lacZ*)M15*proAB*/*supE thi* Δ (*lac proAB*) Δ (*hdsMS*–*mcrB*)5(*rk*⁻ *mcrB*⁻)] (New England Biolabs) was used as a recipient in the cytomegalovirus pCMV clone constructions.

Vectors. pET23d (Novagen), the backbone for the construction of pET23d:HSVTK-Dummy vector, contains a nonfunctional DNA fragment between the sites in the HSV *tk* open reading frame used for insertion of random sequences. Briefly, a 1.7-kb *Nco* I/*Hin*DIII fragment was purified from a restriction digest of pT7:HSVTKII (21) and cloned into pET23d. The dummy vector was constructed by replacing the *tk* sequences between the *Kpn* I and *Sac* I sites with the *Kpn* I–*Sac* I fragment from pMDC (22–24).

The pCMV constructs were cloned as follows. DNA from select TK mutants and from the wild type were digested with *Nco* I and blunt-ended with DNA polymerase. The gel-purified fragments (*Nco* I-blunt) were ligated to pCMV (25) after restriction with *Not* I (blunt-ended). The wild-type *tk* gene in the wrong orientation relative to the CMV promoter served as an additional control. The sequence, orientation, and 5' junction of each inserted fragment were verified.

Library Construction and Selection. Six codons (encoding Leu-159, Ile-160, Phe-161, Ala-168, Leu-169, and Leu-170) were targeted for random sequence mutagenesis. For the construction of the random library, the *Sac* I/*Kpn* I fragment from pET23d:HSVTK-Dummy was replaced with a DNA fragment that had been prepared from the following oligonucleotides. Two oligonucleotides were synthesized by Operon: MB126 (a 58-mer), 5'-TGGGAGCTCATATGCCCCGCC-CCGGCCCTCACCNNNNNNNNNGACCGCCATCCC-

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Abbreviations: HSV-1, herpes simplex virus type 1; TK, thymidine kinase; GCV, ganciclovir; ACV, acyclovir; CMV, cytomegalovirus. ‡To whom reprint requests should be addressed.

ATC-3'; and MB127 (a 51-mer), 5'-ATAAGGTACCGCGC-GGCCGGGTAGCANNNNNNNNNGGCGATGGGATGGC-GG-3'; N designates an equimolar mix of all four nucleotides during synthesis (100% random). The purification of oligonucleotides, annealing, extension, and amplification by PCR were essentially as described (22).

Ligation and electroporation of pools of random-sequence-containing pET23d:HSVTKs is described elsewhere (22). Transformants able to confer TK activity to BL21(DE3) *tdk*⁻ were selected on TK selection plates as described (22) except that the 5'-fluorodeoxyuridine concentration was increased to 60 µg/ml. The total number of transformants was determined by counting the number of colonies on nonselective plates [2× YT (16 g of tryptone/10 g of yeast extract/5 g of NaCl/15 g of agar per liter) + carbenicillin at 50 µg/ml].

Selection of GCV- and ACV-Sensitive TK Mutants. Each of the 426 TK-positive mutants was picked and used to inoculate 200 µl of TK selection medium in a 96-well microtiter plate format. All 426 clones were then serially diluted 1:10⁴ in 0.9% NaCl (300 µl) with a 48-prong replicator (Sigma). Thirty microliters of the last dilution was spread onto TK selection plates containing 1 µg of thymidine per ml plus various concentrations of GCV or ACV. Initially 2 µg of GCV per ml was used, and the clones unable to grow were scored as positive results, since any mutant with increased conversion of the prodrug to its active compound results in lethality. The stringency for GCV or ACV over thymidine was increased by decreasing the concentration of the prodrug in the plates.

In Vitro TK Protein Synthesis and Enzyme Assays. Qiagen tip 100 or tip 500 columns were used to isolate mutant plasmid DNAs (Qiagen). *In vitro* transcription and translation of the 80 mutant DNAs were done as described by Black and Loeb (22) except that the isolated plasmids were not linearized prior to transcription. Cell-free translation products were assayed in duplicate for thymidine ([methyl-³H]thymidine at 87 Ci/mmol, Amersham; 1 Ci = 37 GBq), for ganciclovir ([⁸⁻³H]ganciclovir at 22 Ci/mmol, Moravex), and for acyclovir ([⁸⁻³H]acyclovir at 15 Ci/mmol, Moravex) phosphorylation at 1 µM, 7.5 µM, and 7.5 µM, respectively, and results were compared to those of pET23d:HSVTK mRNA translation product assays.

Mammalian Cell Transfections and Drug-Sensitivity Assays. Stable transformants expressing wild-type (pCMV:HSVTK) or mutant (pCMV:30, pCMV:75, pCMV:132) HSV-1 TKs were constructed in a *tk*⁻ background by using temperature-sensitive ts13 baby hamster kidney (BHK) cells. The ts13 BHK cells (American Type Culture Collection no. CRL-1632) were maintained in Dulbecco's modified Eagle's medium with 10% (vol/vol) calf serum in a humidified incubator at 37°C in an atmosphere containing 6% CO₂. They were transfected by the modified calcium phosphate precipitation method of Chen and Okayama (26). For each transfection, 5 × 10⁵ cells were cotransfected with 10 µg of either vector alone (pCMV), the pCMV vector with the HSV-1 TK gene in the wrong orientation relative to the promoter (pCMV:TK-wrong), the vector plus wild-type HSV-1 TK (pCMV:HSVTK), or one of three HSV-1 TK mutants (pCMV:30, pCMV:75, or pCMV:132) and 1 µg of pSV2neo (27) that encodes the aminoglycoside phosphotransferase gene as a selectable marker. Seventy-two hours after transfection, the cells were split 1:3 and then switched to selective medium containing 600 µg of G418 (Sigma) per ml. Cells were maintained in selective medium for 17 days. Individual G418-resistant colonies within each transfection group were harvested and pooled to yield populations of cells that contain the indicated construct randomly integrated. Approximately 30–40 clones were recovered and pooled for each construct containing a *tk* gene in the correct orientation and 130–140 clones for the cells transfected with the vector alone or with *tk* in the wrong orientation. Cells transfected with vector alone (pCMV) or with the wild-type HSV-1 TK gene in

the wrong orientation relative to the promoter (pCMV:TK-wrong) were also included as controls.

To determine the cytotoxic effect of GCV and ACV, cells were plated in 96-well microtiter plates at an initial density of 2000 cells per well in 100 µl of DMEM containing 10% calf serum. Sixteen hours later, either GCV (0.125, 0.5, 1.25, 5, 7.5, 10, and 20 µM) or ACV (0.5, 1, 2.5, 5, 10, 25, 50, 75, and 100 µM) was added to each plate. After 3 days in the presence of nucleoside analog, the fluorescent redox indicator alamar blue was added. Cell survival was determined 24 hr later according to the alamar blue manufacturer's protocol (Biosource International).

RESULTS AND DISCUSSION

Two highly conserved tripeptide motifs identified in alignments of *Herpesviridae* TKs have been postulated to participate in substrate binding (sites 3 and 4) (28). While the crystal structure of TK is not yet available to model potential amino acid interactions with the substrates, sequence analyses of functional clones isolated from two libraries containing random sequences in place of these nucleotide segments support the participation of these regions in thymidine binding (22–23). Further analysis of mutant TKs suggests that residues just N-terminal to these motifs participate in nucleoside binding, perhaps to mold the overall binding pocket structure and/or to maintain the hydrophobicity of the active-site cavity (22). Single amino acid substitutions within the active site of an enzyme often result in loss of protein stability, improper folding, reduced enzymatic function, or a combination of all three (29). Because these deleterious effects are magnified by the introduction of multiple amino acid changes, large populations of mutants need to be created and screened to identify a very small number of candidate clones with the desired properties. We constructed a DNA library of more than a million mutants in which random nucleotides were substituted for those that encode the three amino acid residues that are N-terminal to each of the two putative nucleoside binding sites.

The internal *Sac* I/*Kpn* I fragment of the HSV-1 *tk* open reading frame was substituted by oligonucleotides containing 100% random nucleotide sequences in place of six codons (encoding Leu-159, Ile-160, Phe-161, Ala-168, Leu-169, and Leu-170) (Fig. 1A). A small fraction of the transformation was plated onto antibiotic-containing plates (nonselective) and incubated at 37°C to determine the total number of transformants, and the rest was plated directly onto TK-selection plates to identify transformants that expressed active *tk* genes. From a library of ≈1.1 × 10⁶ transformants, 426 positive clones were identified. Thus, 0.039% of all transformants conferred TK activity to *E. coli* BL21(DE3) *tdk*⁻ cells.

To survey the spectrum of mutants produced, we sequenced the inserted segment on plasmids recovered from 17 clones that grew on nonselective plates and from 17 clones that grew on TK selection plates. The nonselected clone sequences encoded numerous charged and bulky residue replacements as well as proline residues and contained stop codons indicating that the substitutions at each of the designated positions were random (data not shown). A more limited repertoire of substitutions was obtained in clones that grew on the TK selection plates. The deduced amino acid sequences revealed that all clones from the selected set maintained the overall hydrophobic nature of this region, and each clone contained at least two amino acid changes. In both selected and nonselected *tk* clones, the introduction of mutations at sites distal to the randomized region was observed. Mutations were primarily confined to two codons, 155 and 156, and all changes at codon 155 were silent. Changes at codon 156 resulted in alanine replacement by valine, serine, or proline. Alignment studies indicate that position 156 is not conserved either for alanine or for the type of amino acid at that position. Therefore, it is

methyl group on the acyclic ribose of GCV. Those mutants with poor thymidine phosphorylation that are still able to phosphorylate GCV and/or ACV well are of particular interest because a loss in thymidine binding affinity could result in a reduction in competition between thymidine and nucleoside analog. We anticipate that these enzymes will have shifted their substrate specificities from thymidine towards GCV and ACV and will demonstrate an increased therapeutic value for mammalian cell killing. It is important to note that the mutants identified have between three and six amino acid alterations within the putative nucleoside binding site and that these combinations of substitutions would likely never have been chosen for insertion by site-directed mutagenesis. It is remarkable that such radical changes at the active site result in functionally active enzymes with dramatic alterations in substrate phosphorylation.

Three of the mutant TKs were selected for analysis in mammalian cells based on the rates of GCV or ACV phosphorylation compared to thymidine phosphorylation by using mutant enzymes synthesized in the rabbit reticulocyte lysate system. These ratios reflect the ability of the mutant enzymes to preferentially phosphorylate nucleoside analogs over thymidine relative to the wild-type enzyme. Wild-type HSV-1 TK preferentially phosphorylates thymidine that acts as a strong competitive inhibitor of GCV and ACV binding (M. Kokoris and M.E.B., unpublished results). Compared with HSV-1 TK, the ratios of GCV/thymidine and ACV/thymidine phosphorylation by mutants 30 and 132 are 31- and 32-fold higher and 13- and 20-fold higher, respectively. Mutant 75 has a similar GCV/thymidine ratio to that of HSV-1 TK and has a 7-fold increased ACV/thymidine phosphorylation ratio. DNA from mutant TKs (30, 132, and 75) and from the wild type was cloned into pCMV. The wild-type *tk* gene in the wrong orientation relative to the CMV promoter served as an additional control. The pCMV constructs were cotransfected with a neomycin-resistance marker plasmid (pSV2neo) (27) into ts13 BHK *tk*⁻ cells by modified calcium phosphate precipitation (26).

Cell extracts from stable BHK *tk*⁻ transfectants were assayed for their ability to phosphorylate thymidine, GCV, and ACV. The levels of nucleoside phosphorylation activity determined was proportional to the amount of protein expression as determined by Western blot analyses using polyclonal anti-TK serum (a gift from William Summers, Yale University) followed by densitometry of the scanned blot (data not shown). No immunoreactive band was seen in the lanes corresponding to pCMV or pCMV:TK-wrong (*tk* gene in the wrong orientation). Both the wild-type TK (pCMV:HSVTK) and pCMV:132 transfected cell lysates exhibited roughly equivalent band intensities. The immunoreactive band for pCMV:30 cell lysates was substantially more intense (10-fold) and that of pCMV:75 was approximately half that of the wild type.

Sensitivity to the guanosine analogs was determined after growth for 4 days in the presence of increasing concentrations of either GCV (Fig. 3A) or ACV (Fig. 3B). Cells transfected with pCMV:HSVTK displayed sensitivity to GCV and ACV with ED₅₀ values of 20 μM and 25 μM, respectively (Table 1). A repeat of this experiment gave similar ED₅₀ values of 13 μM and 20 μM for GCV and ACV, respectively. Cells transfected with pCMV:132 exhibited similar sensitivity to GCV and ACV as did wild-type thymidine kinase-transfected cells. Even though mutant 132 was not as active as the wild-type TK in phosphorylating GCV and ACV (Fig. 2), it exhibited a strong preference for phosphorylating GCV or ACV in BHK *tk*⁻ cells, which is in accord with the enhanced cell killing. This preferential killing is likely due to the increased GCV/thymidine and ACV/thymidine ratios, 13 and 20, relative to wild-type ratios of 1.

Cells stably transfected with pCMV:30 conferred a modest increase in GCV sensitivity (4.5-fold) and displayed a slight increase in sensitivity to ACV (1.4-fold) over pCMV:HSVTK-

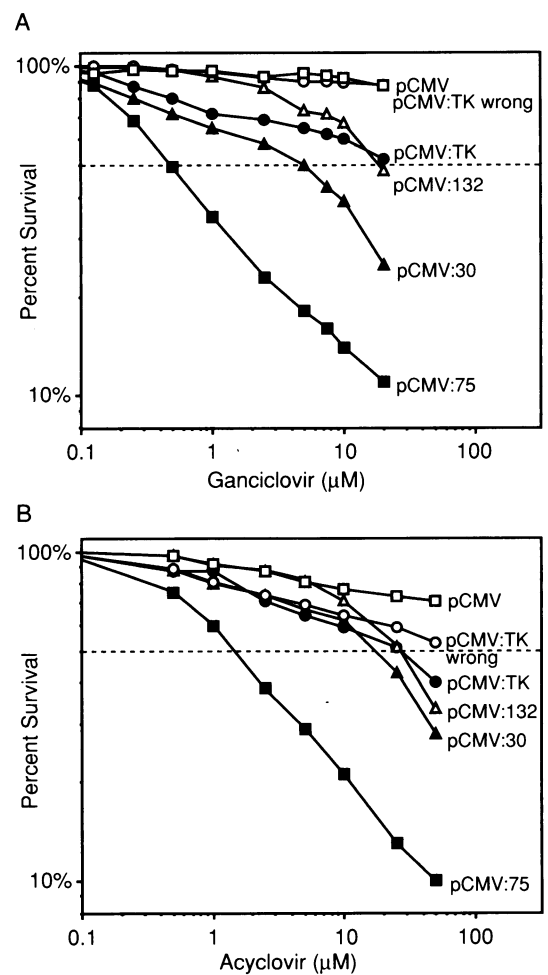


FIG. 3. Expression of mutant *tk* genes in mammalian cells. (A) GCV cytotoxicity. (B) ACV cytotoxicity. Stable transfectants expressing wild-type (pCMV:HSVTK) or mutant (pCMV:30, pCMV:75, and pCMV:132) HSV-1 TKs were constructed in ts13 BHK *tk*⁻ cells as described in the text. Cells transfected with vector alone (pCMV) or with wild-type HSV-1 TK in the wrong orientation relative to the promoter (pCMV:TK-wrong) were included as controls. The cytotoxic effect of GCV and ACV was determined as described in the text. After the addition of alamar blue, the plates were scanned in a fluorometer (Labsystems Fluoroskan II) with an excitation wavelength of 544 nm. The fluorescence intensity at 590 nm for each well containing nucleoside analog was recorded and expressed as a percentage of the value for control wells with no analog added ($n = 8$ for each prodrug concentration and $n = 16$ for controls). The dotted line represents the lethality of 50% of the cells.

transfected cells. This enhancement could be due to the increased phosphorylation of prodrugs or the 10-fold increased protein expression.

Clearly pCMV:75 renders the cells most sensitive to either GCV or ACV with a 43- and 20-fold difference in ED₅₀ (effective dose to kill 50% of the cells), respectively, compared with the wild-type TK-transfected cells. The ED₅₀ for pCMV:75-transfected cells compared with cells transfected with the wild-type TK was 0.47 μM and 20 μM for GCV and 1.25 μM and 25 μM for ACV. In a separate assay using pooled transfectants, the ED₅₀ for pCMV:75 was 0.7 μM with GCV and 0.8 μM with ACV. This enhanced killing is not due to overexpression. Kinetic evaluation of purified wild-type and mutant 75 TK enzymes were determined (M. Kokoris and M.E.B., unpublished results). The K_m of mutant 75 for GCV is 10 μM and is one-fifth of the wild-type K_m . Furthermore, the K_m ratio of GCV to thymidine for mutant 75 is 11, while that for HSV-1 TK is 10-fold higher at 120 (M. Kokoris and M.E.B.,

Table 1. Inhibition of mammalian cells transfected with wild-type or mutant TKs by ACV or GCV

TK	GCV		ACV	
	ED ₅₀ , μM	Factor decrease	ED ₅₀ , μM	Factor decrease
WT	20	1	25	1
Mutant				
30	4.4	4.5	18	1.4
75	0.47	43	1.25	20
132	18	1.1	25	1

The level of sensitivity to GCV or ACV displayed by BHK *tk*⁻ cells (pools) transfected with pCMV encoding either the wild-type (WT) or mutant TK (mutants 30, 75, or 132) is presented as the effective dose of drug required to inhibit 50% of the cell population (ED₅₀). The ED₅₀ values were taken from the graphs shown in Fig. 3. The factor decrease reflects the difference in ED₅₀ values of mutant TK relative to the wild-type TK transfectants.

unpublished results). The turnover rate or k_{cat} is the same between wild-type TK and mutant 75 for thymidine (0.23 and 0.21 sec⁻¹), GCV (0.05 and 0.05 sec⁻¹), and ACV (0.008 and 0.01 sec⁻¹) (M. Kokoris and M.E.B., unpublished results). Thus, it appears that the enhanced killing correlates with altered substrate specificity and indicates that the use of mutant 75 or similar mutant genes in gene therapy could facilitate a more effective killing of tumor cells at less toxic concentrations of GCV.

From more than one million mutant TK enzymes, we have isolated 10 mutants that demonstrate unique nucleoside phosphorylation characteristics. Without detailed knowledge of the three-dimensional structure, we have used random sequence mutagenesis to obtain a mutant with four amino acid substitutions that confers increased GCV and ACV sensitivity to mammalian cells. Two recent reports of the HSV-1 TK crystal structure place each of the amino acid substitution positions within the active site pocket (30, 31). A 43- and 20-fold increase in transfected mammalian cell killing by GCV and ACV, respectively, suggests that random sequence mutagenesis can be used to tailor HSV-1 TKs for gene therapy of cancers and for a wide variety of other applications, including cell lineage ablation and as negative selectable markers for homologous recombination events.

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