Herpes Simplex Virus-1 Thymidine Kinase Mutants Created by Semi-Random Sequence Mutagenesis Improve Prodrug-mediated Tumor Cell Killing

Margaret E. Black, Mark S. Kokoris, and Peter Sabo

ABSTRACT

Cancer suicide gene therapy affords the prospect of using the most optimal genes available because the source of the therapeutic gene is often irrelevant. Currently, there are numerous preclinical and clinical trials to develop tumor ablative therapies that use viral, yeast, or bacterial genes. One such gene, the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) is widely used as a suicide gene in combination with ganciclovir. In the study reported here, a restricted set of random sequences (semi-random) was introduced into the active site of HSV-1 TK, and the resulting variants were selected on the basis of their ability to confer increased ganciclovir or acyclovir sensitivity to Escherichia coli. Sequence analysis demonstrated that functional mutants contained three to five amino acid substitutions that are unique and novel combinations. On the basis of enzyme assay results, three mutants were identified for further analysis in vitro. These three mutants conferred substantial increased sensitivity to both ganciclovir and acyclovir when compared with 1C3,6 of wild-type TK expressing rat C6 glioma cells. One mutant, SR39, was further evaluated in a xenograft tumor model in nude mice. Expression of SR39 in tumors was shown to prevent tumor growth at produg dosages that did not affect wild-type HSV-1 TK-expressing tumors. The use of any of these mutants as a suicide gene should provide a more effective and safer alternative to wild-type TK, because lower, less immunosuppressive doses of ganciclovir will be necessary for tumor ablation, and the use of acyclovir may now be possible.

INTRODUCTION

Originally designed as antiviral drugs, ACV and GCV have found a new application as prodrugs in suicide gene therapy of cancer. The target for these antiviral drugs, the HSV-1 TK, is currently being used in numerous clinical trials in combination with GCV to ablate tumors in which the gene has been introduced (1–3). This approach functions on the basis of enzyme assay results, three mutants were identified for further analysis in vitro. These three mutants conferred substantial increased sensitivity to both ganciclovir and acyclovir when compared with 1C3,6 of wild-type TK expressing rat C6 glioma cells. One mutant, SR39, was further evaluated in a xenograft tumor model in nude mice. Expression of SR39 in tumors was shown to prevent tumor growth at produg dosages that did not affect wild-type HSV-1 TK-expressing tumors. The use of any of these mutants as a suicide gene should provide a more effective and safer alternative to wild-type TK, because lower, less immunosuppressive doses of ganciclovir will be necessary for tumor ablation, and the use of acyclovir may now be possible.

RESULTS

Mutagenesis. A semi-random mutagenesis approach was used to introduce combinations of amino acid substitutions within the HSV-1 TK active site. The possible codons for each semi-randomized codon are: L159, I or L; I160, L or F; F161, A, V, P, or L; A168, D, Y, V or F; and L169, F, Y, L, I, M, N, K, or stop. Because of codon usage, a number of undesired amino acids are also represented in the library, such as a stop codon at position 169. The total possible number of amino acid combinations is 512. To ensure that all mutants were present multiple times, 12,376 clones were plated onto TK selection plates. A total of 1717 colonies were TK positive, indicating that
TK variants were constructed and used to select mutants for prodrug sensitivity assays in mammalian cells. To evaluate the mutants, 3023 members of this library were screened. With the wild-type TK protein level set at 1, all of the mutants protein levels were present at higher concentrations of prodrug as described elsewhere.

Identification of Mutants with Increased Prodrug Sensitivity.

To identify the diversity of substitutions that provide functional activity, 64 mutants were sequenced. All mutants were novel combinations and contained three to five amino acid substitutions. Of the 64 sequences, 12 were duplicates and 3 were represented times. This level of multiple representatives indicates that most, if not all, of the 512 members of this library were screened.

In Vitro Prodrug Sensitivity Assays.

On the basis of the amount of product generated in GCV and ACV phosphorylation assays in bacterial lysates (data not shown), three mutants (SR11, SR26, and SR39) were selected for prodrug sensitivity assays in mammalian cells. To evaluate the mutants in vitro, mammalian vectors expressing the TK variants (pREP8Δ7:TK-GFP) were constructed and used to transfect rat C6 glioma cells as described previously. Pools of bulk-sorted transfectants were then assayed for the level of sensitivity to GCV or ACV (drug range, 0.001–10 μM for GCV and 0.01–20 μM for ACV). The results of a representative sensitivity assay of mutants SR11, SR26, and SR39 are shown in Fig. 2, along with the wild-type TK and a vector control.

In the presence of GCV, the wild-type IC₅₀ was 5 μM, whereas all three mutants displayed increased sensitivity to the prodrug. Mutant SR39 demonstrated an IC₅₀ of 17 nM, an almost 300-fold reduction in IC₅₀ compared with the wild-type TK-expressing transfectants. likewise, SR39-expressing transfectants were the most sensitive to ACV with an IC₅₀ of 0.11 μM. Because the wild-type TK-expressing transfectants were not very sensitive to ACV, even at 20 μM, no comparative IC₅₀ can be given. SR11 and SR26 were less sensitive to both GCV and ACV than SR39 but more sensitive than the wild-type TK.

TK protein concentration in the C6 cell lysates was determined using immunoblots and densitometry. With the wild-type TK protein level set at 1, all of the mutants protein levels were present at higher concentrations.
mice were s.c. injected with pools of transfectant cells (5 x 10^5 cells/mouse; n = 5). The cells used for tumor seeding had been transfected with the expression vector (pREPΔ7:dual-GFP), wild-type TK (pREPΔ7:TK-GFP), or mutant SR39 (pREPΔ7:SR39-GFP). After 5 days to allow for tumor formation, the mice were injected i.p. twice a day for 5 days (days 5–9). During this period (days 5–9) and for an additional 6 days (days 10–15), tumor diameter was measured every other day (n = 5 for each group).

In the xenograft tumor studies, nude mice were s.c. injected with pools of transfectant cells (5 x 10^5 cells/mouse; n = 5). The cells used for tumor seeding had been transduced with the expression vector (pREP8Δ7:dual-GFP), wild-type TK (pREP8Δ7:TK-GFP), or mutant SR39 (pREP8Δ7:SR39-GFP). After 5 days to allow for tumor formation, the mice were injected i.p. twice a day for 5 days with saline (control), GCV at 0.5 or 5 mg/kg, or ACV at 5 or 25 mg/kg. During the prodrug treatment period and for an additional 10 days, tumor size was monitored by caliper measurements on alternate days. The time course results are shown in Fig. 3.

At the start of the prodrug treatment, all tumors were between 1.75 and 3 mm in diameter. In both prodrug-treated and saline-treated non-TK-expressing groups (pREP8Δ7:dual-GFP), the tumors grew unchecked (Fig. 3 and Table 2). The mean tumor size for the vector-transfected tumor cells was 10.2 mm at the end of the time course. Wild-type TK and mutant SR39 transfectant tumor mean sizes were 7.9 and 6.15 mm, respectively. From analyses using the Fisher Protected LSD test, there was only a marginally significant difference (P = 0.031) in size of the saline-treated, wild-type TK-expressing and mutant SR39-expressing tumors at the end of the experiment.

Tumor cells transfected with vector only showed no statistically significant difference in tumor size between mice treated with saline or prodrug (Fig. 3). GCV-treated mice had a mean tumor size of 10.1 mm (GCV 5 mg/kg) and 9.2 mm (GCV 0.5 mg/kg) with P = 0.2915 (ANOVA). Mice treated with ACV also displayed only a marginal and not statistically significant difference in tumor sizes (P = 0.0891) with mean tumor sizes of 8.6 mm (ACV at 25 mg/kg), 10.25 mm (ACV at 5 mg/kg), and 10.2 mm for saline-treated mice. The reduced vector-transfected tumor size with 25 mg/kg ACV was unexpected and may reflect the general inhibition of proliferation that was observed in vitro with the C6 cell lines at very high ACV concentrations.

A comparison of GCV-treated versus saline-treated wild-type TK-expressing tumors indicates no difference between the low GCV dose (GCV 0.5 mg/kg) and saline treatment (P = 0.21; Table 2). However, the high dose of GCV (5 mg/kg) was able to partially limit tumor growth in the wild-type TK-expressing tumors (P = 0.0002). Mean tumor sizes were 7.9, 7.15, and 4.85 mm for mice treated with saline or GCV at 0.5 or 5 mg/kg, respectively. Mice bearing wild-type TK-expressing tumors showed no reduction in tumor size after 5 mg/kg ACV treatment compared with the saline-treated mice (7.8 versus 7.9 mm; P = 0.874). Mice treated with ACV at 25 mg/kg displayed only a slight, albeit significant, difference in mean tumor size of 6.19 mm (P = 0.024), similar to what was seen with vector-transfected tumors at the same ACV dose. There was no difference in mean tumor size in the 25 mg/kg ACV group when wild-type TK-expressing and vector-transfected tumors were compared (P = 0.351).

In contrast to tumors expressing wild-type TK where only the highest GCV dose substantially limited tumor growth, all prodrug treatments of mice bearing mutant SR39-expressing tumors elicited a strong response (Fig. 3 and Table 2). The mice bearing mutant SR39-expressing tumors that had been treated with GCV at 5 mg/kg displayed the greatest restriction in tumor growth (mean tumor size of 1.9 mm versus saline tumor mean of 6.15 mm; P = 0.0001). Even at 0.5 mg/kg GCV, mutant SR39 tumors were also dramatically smaller compared with the saline control group (2.2 versus 6.15 mm; P = 0.0001). Treatment of mice bearing mutant SR39-expressing tumors was also effective at both ACV doses compared with the respective saline control. The mean tumor size was 6.15 mm for the saline group, 3.65 mm for the ACV at 5 mg/kg group (P = 0.0005), and 2.45 mm for the ACV at 25 mg/kg treatment group (P = 0.0001). There was a significant difference in mean tumor size when mutant SR39-expressing tumors were compared with vector-transfected tumors (P = 0.0001).

To compare the effect of the different produgs on tumors expressing either wild-type TK or mutant SR39, the mean tumor size of the prodrug-treated group was divided by the mean tumor size of the saline control group for each TK set (Table 3). A value of 1 indicates the same mean tumor size as the saline-treated group. At all prodrug concentrations, there were large differences in tumor size between wild-type TK-expressing tumors and mutant SR39-expressing tumors. At 0.5 mg/kg GCV, the calculated values were 0.905 for wild-type TK and 0.3598 for mutant SR39 (P = 0.0001). At 5 mg/kg GCV, the calculated values were 0.614 for wild-type TK and 0.309 for mutant

Table 2 In vivo response of rat C6 tumors expressing wild-type TK or mutant SR39 to saline or prodrug treatment

<table>
<thead>
<tr>
<th>Prodrug tumor size (mm)</th>
<th>Saline tumor size (mm)</th>
<th>TK vs. SR39 P value</th>
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<tr>
<td>Saline</td>
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<tr>
<td>7.15 (0.21)^b</td>
<td>7.9 (0.031)^b</td>
<td>0.358</td>
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<tr>
<td>2.2 (0.0001)</td>
<td>6.15</td>
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^b Numbers in parentheses are P values (Fisher Protected LSD test) from comparisons between saline and prodrug treatment groups.

This P reflects a comparison between the TK and mutant SR39 tumor sizes treated with saline.

Table 3 Ratio of prodrug treated to saline treated mean tumor sizes with P values

<table>
<thead>
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<th>Prodrug tumor size (mm)</th>
<th>Saline tumor size (mm)</th>
<th>TK vs. SR39 P value</th>
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<tbody>
<tr>
<td>GCV 0.5 mg/kg</td>
<td>0.905</td>
<td>0.0001</td>
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<tr>
<td>GCV 5 mg/kg</td>
<td>0.614</td>
<td>0.0001</td>
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<td>ACV 5 mg/kg</td>
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<tr>
<td>ACV 25 mg/kg</td>
<td>0.784</td>
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^a A value of 1 indicates the same mean tumor size in both prodrug and saline treatment groups. P values were determined using the Fisher Protected LSD test.
SR39 ($P = 0.0001$). Clearly, tumors expressing mutant SR39 were much more sensitive to GCV than those expressing wild-type TK. Tumors expressing mutant SR39 displayed a significant response to GCV at both 0.5 and 5 mg/kg, whereas tumors expressing wild-type TK respond to GCV only at 5 mg/kg. This demonstrates that mutant SR39 was significantly more effective at limiting tumor growth with GCV at a dose 10-fold lower than seen with wild-type TK.

Wild-type TK-expressing tumor size was not effected by ACV at 5 mg/kg, whereas mutant SR39-expressing tumors were significantly smaller ($P = 0.0001$; Table 3). There was also a difference between wild-type TK and mutant SR39-expressing tumor sizes after treatment with the higher ACV level (25 mg/kg; $P = 0.0001$). These results indicate that mutant SR39 causes a significant growth restriction with ACV whereas wild-type TK-expressing tumors were only marginally restricted at the higher ACV dose.

**DISCUSSION**

The use of HSV-1 thymidine kinase as a suicide gene in combination with ganciclovir has demonstrated promise for tumor ablation in gene therapeutic settings (1–3). To ablate tumors, immunosuppressive doses of GCV are generally required. As a means to not only improve the overall efficacy of the HSV-1 TK/GCV system but to also reduce the requirements of high-dose GCV treatment, we sought to construct novel HSV-1 TKs with abilities to ablate tumors at lower GCV doses and/or to use ACV at clinically relevant levels. Currently, ACV is not effective with HSV-1 TK in gene therapy primarily because of the very high $K_m$ the enzyme displays toward the prodrug ($K_m = 417 \mu M$; Refs. 7 and 9). In this paper, we report the creation of a second-generation, semi-randomized library from which mutants with increased GCV and ACV activities were identified in *E. coli*, evaluated for the ability to confer increased prodrug sensitivity to tumor cells (one of which was further evaluated in a mouse xenograft tumor model) and examined for kinetic changes incurred by the introduction of multiple amino acid substitutions.

We described previously the creation and selection of mutant HSV-1 TKs that conferred enhanced prodrug sensitivity to *E. coli* from a random sequence-derived library of over a million members (5). Because the library was constructed to be randomized 100% at six codons, one would expect the diversity of this library to be on the order of $64 \times 10^6$. Clearly, with a library size of only one million variants, only a small sampling of the molecular diversity of the first library was examined. As a way to tap into the rest of the diversity pool, a semi-random library was constructed based on the amino acid substitutions found in the best TK mutants from the first library. This second-generation library is a scrambling of the substitutions that yielded the desired activities with the hope that combinations with even greater activities would be generated.

Of the mutants that complemented the tk-deficient *E. coli*, seven were identified to have increased activity toward GCV and/or ACV when bacterial lysates were assayed. Sequence analysis of these seven clones is shown in Fig. 1. Previously, we had used molecular modeling to analyze a mutant (mutant 30) created in the first generation library (5) and on which the semi-random library is based (7). Interestingly, mutant 30 contains six amino acid changes and appears to display altered substrate specificity because of the mutation A168Y (alanine to tyrosine at position 168). We suggested that the larger size side chain of the tyrosine causes the neighboring side chains to move and thereby enlarge the active site, presumably leading to greater accessibility of the analogues. Two of the seven mutants identified from the semi-random library contain a tyrosine at position 168. Substitutions at residues 159–161 are less likely to directly impact substrate specificity because those positions lie outside the active site. It is plausible that the phenylalanine substitution found in four of the seven mutants at residue 168 has a similar affect as the tyrosine substitution in mutant 30.

To compare the mutants to the wild-type HSV-1 TK in a tumor cell line, three mutants with improved GCV and/or ACV activity in *E. coli* were subcloned into mammalian expression vectors that were used to transfect rat C6 glioma cells. The results of *in vitro* prodrug sensitivity assays indicate that all three HSV-1 TK mutants substantially increase the sensitivity to both GCV and ACV compared with cells transfected with the wild-type TK (Fig. 2). Because the wild-type TK-expressing cells lack sensitivity to ACV such that an IC$_{50}$ could not be determined, the fold differences in IC$_{50}$ shown in Table 1 are likely an underestimation for all three mutant TK-expressing cells.

From the results of prodrug sensitivity assays, mutant SR39 displayed superior sensitivity to both ACV and GCV and was selected for *in vivo* evaluation. A xenograft tumor model was developed in which pools of transfectant cells were s.c. injected into nude mice. After 5 days to allow for visible tumor formation, the mice were treated by twice-daily injections of saline, GCV, or ACV for 5 days. Growth of individual tumors was monitored every other day during the treatment period and for an additional 9 days (Fig. 3). Treatment with 0.5 mg/kg GCV resulted in no difference in tumor growth of mice bearing wild-type TK-expressing tumors (Table 2). This is in contrast to the group of mutant SR39-bearing tumors that showed a dramatic impediment of tumor growth after treatment with the same GCV dose. Although both wild-type TK- and mutant SR39-expressing tumors responded to GCV at 5 mg/kg, growth of the SR39-expressing tumors was completely prevented, whereas wild-type TK-expressing tumors still grew, albeit somewhat checked. It appears that SR39 prevents tumor growth when GCV is administered at one-tenth the dose needed to slow wild-type TK-expressing tumor growth.

At the low ACV dose (5 mg/kg), used, only mutant SR39-expressing tumor growth was restricted (Fig. 3 and Table 2). Wild-type TK-expressing tumors were marginally restricted by ACV at 25 mg/kg, whereas the growth of mutant SR39-expressing tumors was substantially impeded. Although earlier *in vivo* studies with another HSV-1 TK mutant (mutant 30) demonstrated similar results (5), mutant SR39-expressing tumors were much more responsive to ACV at both prodrug concentrations.

Despite our attempts to isolate pools of stable transfectants with similar HSV-1 TK expression levels by cell sorting, the amount of protein expression for all of the mutants was higher than that of wild-type HSV-1 TK-expressing transfectants. Relative to the wild-type TK-expressing transfectants, the mutant TK transfectant protein levels ranged from 5- to 19-fold higher with SR39 transfectants containing the most TK protein. Chen *et al.* (10) compared the relationship between HSV-1 TK expression level and GCV cytotoxicity in 9L glioma tumor cells and found that clones with different enzyme levels displayed similar sensitivity to GCV. Three different 9L stable transfectants were shown to have 1-, 4.7- and 16.9-fold differences in protein expression levels. They note that the IC$_{50}$ for GCV for these clones are similar, regardless of the level of HSV-1 TK expression. Several investigators (10–13) suggest that there is a threshold level of GCV beyond which the rate of cell killing does not change. Shewach *et al.* (14) have reported enzyme level GCV sensitivity differences but at the 5000-fold range of enzyme level differences (14). Because the differences in protein expression level described here are in the range reported by Chen *et al.* (10), the protein level differences we observed are likely not responsible for the improved prodrug sensitivity of the mutant TKs.

In cell culture experiments, mutants SR11 and SR39 appear to grow at a rate 20–40% less than the wild type, but this difference is minor in comparison to the 33- and 294-fold enhanced susceptibility to GCV at a dose of 5 mg/kg.
GCV, respectively (Table 1). In the in vivo tumor studies, only a marginal difference in tumor size between saline-treated SR39- or wild-type TK-transfected pools was observed (P = 0.031; Table 2). Again, the difference between SR39- and wild-type-expressing tumor growth is minor in comparison with the differences in tumor size after GCV or ACV treatments (Fig. 3 and Table 2). Therefore, it appears that the enhanced prodrug sensitivity conferred by the mutants is a result of the characteristics of the mutants themselves rather than protein level or cell growth rate differences.

Previously, we reported the creation and characterization of two HSV-1 TK mutants (mutants 30 and 75) with enhanced prodrug activity (5, 7). Kinetic evaluations have been done on these mutants as well as the three mutants described in this report (7). 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. As a means to compare the earlier mutants (mutants 30 and 75) with those of the semi-random mutants reported here, we used the equation [kcat/Km(prodrug)]/[kcat/Km(thymidine)] + kcat/Km(prodrug), which takes the thymidine parameter into account. With GCV as the prodrug, mutants 30, 75, SR11, SR26, and SR39 displayed values 66-, 11-, 59-, 53-, and 83-fold higher, respectively, than the wild-type enzyme. With ACV as the prodrug, mutants 30, 75, SR11, SR26, and SR39 displayed 333-, 70-, 373-, 567-, and 233-fold higher values, respectively, compared with the wild-type enzyme. Clearly, when competition for the active site is considered, all five mutant enzymes reveal an enormous kinetic advantage for both prodrugs over the wild-type enzyme. From this vantage, SR39 is the best enzyme with GCV. With ACV, SR26 is the best enzyme, followed by SR11. The kinetic parameters displayed by the mutants reveal a biochemical basis for the enhanced prodrug sensitivity described here and elsewhere (5, 7). From this comparison, mutant SR39 would appear to ablate tumors more effectively in the presence of GCV, whereas mutants SR11 and SR26 might result in greater tumor sensitivity to ACV. Indeed, significant improvements at the kinetic level with the three new mutants compared with mutants 30 and 75 have been achieved.

Further evidence that mutant SR39 has the ability to increase tumor sensitivity to prodrugs is reported in Gambhir et al. (15). Rat C6 glioma cells stably transfected with mutant SR39 demonstrated better accumulation of penciclovir, an analogue closely related to GCV, in cell culture and in tumors than do cells expressing wild-type TK. Furthermore, when the wild-type TK or mutant SR39 is delivered to tumors using an adeno virus delivery system, mutant SR39 more effectively traps the analogue than the wild-type enzyme does. Analysis of mutant SR39 as an alternate to HSV-1 TK in positron emission tomography, a noninvasive imaging technique, demonstrated that mutant SR39 in the presence of 18F-labeled penciclovir significantly enhanced sensitivity for imaging reporter gene expression in vivo. Taken together, the multiple amino acid substitutions of the semi-random mutants described here result in enormous alterations in substrate specificities that, when expressed in vitro, display major differences in prodrug-mediated cell killing in transfected cell lines. In vivo, mutant SR39 demonstrates superior tumor ablation in the presence of GCV and ACV. The mutants described here (SR11, SR26, and SR39) should provide a significant advantage over the wild-type TK for suicide gene therapy applications in at least three important ways: (a) by enhancing prodrug-mediated cell killing; (b) by reducing the amount of immunosuppressive GCV required for effective cell killing; and (c) by bringing HSV-1 TK-mediated activation of ACV to a clinically relevant level. Furthermore, such mutants could replace the use of wild-type TK in gene therapy of other indications including restenosis, graft versus host disease, and HIV infections (16–18) as well as for noninvasive quantitative imaging of tumor cells with positron emission tomography (15).

REFERENCES


* Black and Kokoris, unpublished observation.
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