Enzymatic Therapeutic Index of Acyclovir

 VIRAL VERSUS HUMAN POLYMERASE $\gamma$ SPECIFICITY

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We have examined the kinetics of incorporation of acyclovir triphosphate by the herpes simplex virus-1 DNA polymerase holoenzyme (Pol-UL42) and the human mitochondrial DNA polymerase using transient kinetic methods. For each enzyme, we compared the kinetic parameters for acyclovir to those governing incorporation of dGTP. The favorable ground state dissociation constant ($6 \mu M$) and rate of polymerization ($10^{-4} \text{ s}^{-1}$) afford efficient incorporation of acyclovir triphosphate by the Pol-UL42 enzyme. A discrimination factor of ~50 favors dGTP over acyclovir triphosphate, mostly due to a faster maximum rate of dGTP incorporation. Once incorporated, acyclovir is removed with a half-life of ~1 h in the presence of a normal concentration of deoxynucleoside triphosphates, leading to a high toxicity index (16,000) toward viral replication. To assess the potential for toxicity toward the host we examined the incorporation and removal of acyclovir triphosphate by the human mitochondrial DNA polymerase. These results suggest moderate inhibition of mitochondrial DNA replication defining a toxicity index of 380. This value is much higher than the value of 1.5 determined for tenofovir, another acyclic nucleoside analog. The enzymatic therapeutic index is only 42 in favoring inhibition of the viral polymerase over polymerase $\gamma$, whereas that for tenofovir is greater than 1,200. Mitochondrial toxicity is relatively low because acyclovir is activated only in infected cells by the promiscuous viral thymidine kinase and otherwise, mitochondrial toxicity would accumulate during long term therapy.

Neonatal herpes simplex virus (HSV) infection is generally fatal if untreated and occurs at a rate of about 20–50 per 100,000 live births in the United States. Infection may occur in a variety of different locations including the skin, eye, mouth, central nervous system, and even simultaneously in multiple organs (1). A major breakthrough in management of HSV infections occurred more than two decades ago with the discovery of 9-(2-hydroxyethoxymethyl)guanine (acyclovir), which continues to be the leading treatment. Acyclovir triphosphate (TP) has been shown to compete with dGTP binding to the HSV-1 DNA polymerase and serves as an alternate substrate, resulting in the termination of DNA synthesis (2, 3). Previous studies using steady-state methods suggested that once incorporated onto the 3’ terminus of the primer strand, acyclovir monophosphate (MP) could not be removed by the 3’-5’ proofreading exonuclease function of HSV-1 DNA polymerase (4). It has been suggested that acyclovir-TP can be classified as a suicide inhibitor (5) although it is more accurately described today as a chain terminator for viral DNA replication.

The HSV-1 DNA polymerase holoenzyme (Pol-UL42) is a heterodimer composed of a 134-kDa catalytic subunit and a 51-kDa processivity factor (UL42) (6–10). Studies using a pre-steady-state single nucleotide incorporation assay suggested that Pol-UL42 has an extremely high misincorporation frequency of about 1 in 300 when comparing correct dTTP incorporation to incorrect dTPP incorporation (6). However, further studies revealed that the exonuclease proofreading function contributes greatly to the overall fidelity because Pol-UL42 is unable to extend beyond a mismatch efficiently, thereby altering the kinetic partitioning to favor excision rather than extension (11). In this study we aimed to more accurately define the kinetic parameters governing the incorporation and the exonuclease removal of acyclovir by Pol-UL42 using transient state kinetic methods (12) and to compare these kinetic parameters to those for the mitochondrial DNA polymerase (Pol $\gamma$). In these studies we provide a more accurate assessment of the toxicity of acyclovir triphosphate toward the viral enzyme and evaluate the potential for mitochondrial toxicity.

Most of the toxic side effects caused by nucleoside reverse transcriptase inhibitors (NRTIs) used for the treatment of human immunodeficiency virus (HIV) infection are a result of mitochondrial dysfunction due to the direct inhibition of Pol $\gamma$ (reviewed in Refs. 13–17). The kinetic parameters governing incorporation and excision correlate well with the severity of the clinically observed side effects for all FDA-approved nucleoside and nucleotide analogs (14) with the possible exception of 3’-azido-2’,3’-dideoxythymidine, which appears to act as a competitive inhibitor of thymidine kinase (18). A detailed evaluation of the ability of Pol $\gamma$ to discriminate against incorpora-
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Production of acyclovir-TP during polymerization plus a quantitative analysis of the excision of acyclovir-MP is necessary to fully understand its effectiveness and toxicity.

Activation of acyclovir to form the nucleoside triphosphate is dependent upon a promiscuous viral thymidine kinase; the low toxicity of acyclovir is due to the activation of acyclovir only in infected cells and the low inhibition of cellular replication (19). However, the mitochondrial toxicity of acyclovir triphosphate has not been evaluated. Similar acyclic guanosine analogs, penciclovir, used in the treatment of oral herpes simplex infections, and ganciclovir, used in treating cytomegalovirus infections, both rely upon viral encoded kinases for specificity (reviewed in Ref. 20). In cells expressing the viral thymidine kinase, ganciclovir induces significant mitochondrial damage (21, 22). In contrast, three acyclic nucleoside phosphonates are employed that bypass the first phosphorylation step and become activated in all cells: cidofovir, a cytidine analog used against cytomegalovirus, adefovir, an adenine analog used against hepatitis B, and tenofovir, a similar adenine analog used against HIV (23). Although tenofovir can be activated to the triphosphate form in any cell, its clinical toxicity is relatively low. Quantitative measurements of incorporation by HIV reverse transcriptase (RT) and the human mitochondrial DNA polymerase have revealed a human toxicity index of only 1.5 and a rather high therapeutic index toward HIV RT of 1200 (14).

It is generally assumed that the low toxicity of acyclovir is largely dependent upon the high specificity of the host thymidine kinase that precludes activation in uninfected cells, whereas its effectiveness relies upon the promiscuity of the viral thymidine kinase. However, there have been no quantitative measurements to evaluate the potential mitochondrial toxicity of acyclovir triphosphate or to test whether a phosphonate analog of acyclovir might be equally effective. Therefore, we have examined the relative effectiveness and potential for mitochondrial toxicity of acyclovir by comparing the kinetics of incorporation of acyclovir-MP versus excision of acyclovir triphosphate by the HSV and human mitochondrial DNA polymerases.

MATERIALS AND METHODS

Reagents—Buffers, salts, and dNTPs were obtained from Sigma. Acyclovir-TP was purchased from Moravek Biochemicals (Brea, CA). Radiolabeled [γ-32P]ATP was purchased from PerkinElmer Life Sciences.

Enzymes—HSV-1 Pol-UL42 holoenzyme was purified from S9 insect cells co-infected with recombinant baculovirus that express the genes encoding these proteins as detailed previously (24). HSV-1 Pol-UL42 used for the studies described herein was determined to be ≈95% pure, and all preparations contained 50–75% active enzyme, based on active site titrations. Exonuclease-deficient holoenzyme (D368A) was used to measure nucleotide incorporation reactions. Mutant D368A has been previously characterized and has been shown to lack any detectable exonuclease activity (11, 25, 26). The overexpression and purification of recombinant human Pol γ were carried out as previously described (27, 28). Holoenzyme was reconstituted at a 1:5 molar ratio of catalytic subunit to accessory subunit. For single nucleotide incorporation experiments, exonuclease-deficient holoenzyme (E200A) was used.

Preparation of DNA—All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The primer (25-mer) sequence for correct incorporation of both dGTP and acyclovir-TP was 5'-GGCTCGAGCGTCGACACACTGAGTTG-GTTGACGCTGCAGCC-3'. The 45-mer template sequence for incorporation reactions was 5'-GGACCGCATTTGAGCATCGACTAGTTG-GTTGAGCGCTGCAGCC-3'. The template sequence for the DNA containing eight different bases to create a frayed primer was 5'-GGACGCGATTGAGCATCGACTAGTTG-GTTGAGCGCTGCAGCC-3'. Primers containing 3'-terminal acyclovir-MP were created enzymatically using HIV-1 RT. The reaction was performed using 1 μM RT, 3 μM duplex DNA (coding for G incorporation), and 50 μM acyclovir-TP in the following reaction buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 2.5 mM MgCl2). The reaction mixture was incubated at 37 °C for 30 min, and then the product was purified using 15% denaturing PAGE to obtain the acyclovir-TP-terminated primer. All primers used were 5'-32P-labeled using T4 polynucleotide kinase, according to the manufacturer's instructions (Invitrogen). The reaction was terminated by incubation at 95 °C for 5 min, and excess nucleotide was removed using a Bio-Spin 6 gel filtration column (Bio-Rad). Primer was annealed to template by combining at an equimolar ratio, heating to 95 °C, and then slowly cooling to room temperature.

Reaction Conditions—A quench-flow apparatus (RQF-3) from KinTek Corporation (Austin, TX) was used for reactions too fast to mix and quench manually. All concentrations stated refer to the final concentrations after mixing. The buffer conditions for kinetic assays using HSV-1 Pol-UL42 were as follows: 50 mM Tris-Cl, pH 7.5, at 37 °C, 1 mM dithiothreitol, 1 mM EDTA, 125 mM KCl, and 400 μg/ml bovine serum albumin. The radiolabeled DNA substrate was then added to the holoenzyme and the mixture was incubated for at least 10 min on ice. Reactions for incorporation experiments were initiated by mixing holoenzyme-DNA complex with an equal volume of solution composed of the same buffer plus dGTP or acyclovir-TP and MgCl2 (6 mM Mg2+ after mixing). To start the exonuclease reactions Pol-UL42 holoenzyme-DNA complex was mixed with an equal volume of buffer plus MgCl2, and in two experiments, 100 μM dNTPs. The buffer conditions for kinetic assays using Pol γ were as follows: 50 mM Tris-Cl, pH 7.5, at 37 °C, 100 mM NaCl, and 100 μg/ml bovine serum albumin. The holoenzyme was reconstituted by incubating the two protein subunits for 5 min on ice. The radiolabeled duplex DNA was then added to the holoenzyme and the mixture was incubated for an additional 5 min on ice. Experiments using Pol γ were performed in the same fashion as with Pol-UL42 except that a final concentration of 2.5 mM Mg2+ was used. All reactions were terminated by the addition of 0.5 mM EDTA, pH 8.0. Products were separated from reactants using 15% denaturing PAGE, imaged on a Molecular Dynamics Storm 860, and quantified using ImageQuaNT software (Amersham Biosciences).
The slightly different buffer conditions for HSV-1 polymerase versus Pol γ, 125 mM KCl versus 100 mM NaCl, respectively, were chosen so that the results on each enzyme would be directly comparable with previous studies. However, these conditions are deemed to be sufficiently similar to afford a comparison between the two enzymes because the salt concentration optimum does not have a steep concentration dependence, and we have not seen any difference between potassium and sodium in previous measurements on Pol γ.

Pre-steady-state Single Turnover Reactions—Single nucleotide incorporation assays were performed with various concentrations of dGTP or acyclovir-TP to examine the nucleotide incorporation dependence of incorporation rate and amplitude using a KinTek RQF-3 rapid quench-flow instrument. Single-turnover conditions were employed, where the concentration of enzyme was greater than the concentration of DNA. All experiments were performed at least twice but, in all cases, error estimates are based upon nonlinear regression to the fitted curves as described below. These error estimates are comparable with the errors observed in repeating the experiment because other experimental errors (e.g. pipetting) are generally smaller in magnitude.

Specific reaction conditions are listed in each figure legend and under “Results.” A time course was performed for each concentration of dGTP or acyclovir-TP to examine the nucleotide concentration dependence of incorporation rate and amplitude using a KinTek RQF-3 rapid quench-flow instrument. Single-turnover conditions were employed, where the concentration of enzyme was greater than the concentration of DNA. All experiments were performed at least twice but, in all cases, error estimates are based upon nonlinear regression to the fitted curves as described below. These error estimates are comparable with the errors observed in repeating the experiment because other experimental errors (e.g. pipetting) are generally smaller in magnitude.

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To quantify the effectiveness of acyclovir-TP as an inhibitor of viral replication, the concentration dependence of its rate of incorporation was examined. A plot of the concentration of extended product (26-mer) as a function of time for various acyclovir-TP concentrations is shown in Fig. 2A. The concentration dependence of the rate (Fig. 2B) was fit to a hyperbola (Equation 2) to define a $k_{pol}$ of 10.1 ± 0.8 s$^{-1}$ and a $K_d$ of 6 ± 1 M. Each data set was fit by non-linear regression to a single exponential equation to obtain the observed rate of incorporation and standard error. The observed rates were plotted as a function of dGTP concentration. Specific values for $k_{pol}$ and $K_d$ for dGTP incorporation could not be obtained by fitting the data traditionally to a hyperbola because rates exceeding 600 s$^{-1}$ cannot be reliably measured using rapid quench methods. However, the data could be fit using Equation 3 to obtain a specificity constant ($k_{pol}/K_d$) of 8 ± 5 M$^{-1}$ s$^{-1}$.

To quantify the effectiveness of acyclovir-TP as an inhibitor of viral replication, the concentration dependence of its rate of incorporation was examined. A plot of the concentration of extended product (26-mer) as a function of time for various acyclovir-TP concentrations is shown in Fig. 2A. The concentration dependence of the rate (Fig. 2B) was fit to a hyperbola (Equation 2) to define a $k_{pol}$ of 10.1 ± 0.8 s$^{-1}$ and a $K_d$ of 6 ± 1 M. A comparison of the specificity constant for dGTP incorporation to that of acyclovir-TP incorporation (Table 1) demonstrates that the natural nucleotide is preferred by only 50-fold. With a specificity constant of 1.7 M$^{-1}$ s$^{-1}$, acyclovir-TP is a relatively efficient substrate for incorporation catalyzed by Pol-UL42. Interestingly, the apparent $K_d$ of dGTP and acyclovir-TP are not significantly different.

**Kinetics of Excision by Pol-UL4—**Exonuclease hydrolysis of both dGMP and acyclovir-MP from the 3’ end of the primer in duplex DNA were examined to determine the effectiveness of the exonuclease proofreading activity of Pol-UL42. The removal of dGMP was examined under single turnover conditions (100 nM wild type Pol-UL42 and 90 nM DNA). The reaction was initiated by rapidly mixing the preformed E-DNA complex with a solution containing Mg$^{2+}$ and various concentrations of acyclovir-TP (1 ( ), 6 ( ), 12 ( ), 20 ( ), and 35 ( )) μM). Each data set was fit by non-linear regression to a single exponential equation to obtain the rate and standard error. The observed rates were plotted as a function of acyclovir-TP concentration and fit using a hyperbola to obtain a $k_{pol}$ of 10.1 ± 0.8 s$^{-1}$ and a $K_d$ of 6 ± 1 μM.

**FIGURE 1.** Incorporation of dGTP by HSV-1 DNA polymerase. A, exonuclease-deficient HSV-1 holoenzyme (35 nM) was preincubated with 30 nM $^{32}$P-labeled 25/45-mer DNA duplex and then rapidly mixed with Mg$^{2+}$ and various concentrations of dGTP (0.75 ( ), 2 ( ), 6 ( ), and 18 ( )) μM). Each data set was fit by non-linear regression to a single exponential equation to obtain the observed rate of incorporation and standard error. B, the observed rates were plotted as a function of dGTP concentration. Specific values for $k_{pol}$ and $K_d$ for dGTP incorporation could not be obtained by fitting the data traditionally to a hyperbola because rates exceeding 600 s$^{-1}$ cannot be reliably measured using rapid quench methods. However, the data could be fit using Equation 3 to obtain a specificity constant ($k_{pol}/K_d$) of 80 ± 5 μM$^{-1}$ s$^{-1}$.

**FIGURE 2.** Acyclovir-TP incorporation by HSV-1 DNA polymerase. A, exonuclease-deficient HSV-1 holoenzyme (35 nM) was preincubated with 30 nM $^{32}$P-labeled 25/45-mer DNA duplex and then rapidly mixed with Mg$^{2+}$ and various concentrations of acyclovir-TP (1 ( ), 6 ( ), 12 ( ), 20 ( ), and 35 ( )) μM). Each data set was fit by non-linear regression to a single exponential equation to obtain the rate and standard error. B, the observed rates were plotted as a function of acyclovir-TP concentration and fit using a hyperbola to obtain a $k_{pol}$ of 10.1 ± 0.8 s$^{-1}$ and a $K_d$ of 6 ± 1 μM.
also biphasic, but in this case, the fast phase represented about 73% of the reaction amplitude and occurred at a rate of 125 ± 7 s⁻¹. The slow phase progressed 100-fold more slowly at only 1.3 ± 0.2 s⁻¹.

These results are comparable with those obtained previously with T7 DNA polymerase and Pol γ in that the rate and amplitude of the fast phase of excision from a frayed primer increases as a function of the number of 3’ mismatches (32, 33). The primer strand shuttles between the polymerase and exonuclease sites and greater fraying favors transfer to the exonuclease site. According to this interpretation, the rate of the fast phase approaches the rate of excision of DNA once it is in the exonuclease site, whereas the rate of the slow phase is a measure of the rate at which the primer migrates from the polymerase to the exonuclease site. Therefore, the amplitude of the fast phase corresponds to the fraction of primer strand present at the exonuclease active site at the start of the reaction. These data suggest that Pol-UL42 exhibits a relatively efficient exonuclease activity (k_{exo} ≥ 125 s⁻¹) that is limited by the slow rate of primer strand transfer (0.25 s⁻¹) with a properly paired terminus.

The kinetics of excision of acyclovir-MP from correctly paired and frayed DNA (Fig. 3B) were also examined. Given that extension cannot occur with a chain terminator, two additional experiments were performed in the presence of 100 μM of each of the four dNTPs to examine the effect of the binding of the next correct nucleotide, more accurately reflecting the physiological conditions. Unlike dGMP removal, all data for the removal of acyclovir-MP could be fit using a single exponential equation. The excision of acyclovir-MP from correctly paired DNA occurred at a rate of (5.1 ± 0.4) × 10⁻³ s⁻¹ and was slowed 25-fold to a rate of (2 ± 0.6) × 10⁻⁴ s⁻¹ in the presence of dNTPs.

The removal of acyclovir-MP from frayed DNA with and without the addition of dNTPs both proceeded at rates greater than 0.3 s⁻¹ (Fig. 3B). In both cases, the reaction was too fast to measure by manual mixing and was nearly complete by the first time point of 10 s. Although these data do not establish the rate of excision of acyclovir-MP once in the exonuclease site, they clearly demonstrate that primer strand transfer into the exonuclease site is responsible for the extraordinarily slow removal of acyclovir-MP. Strand transfer is further reduced 25-fold by the binding of the next correct nucleotide. The important conclusion is that the viral polymerase excises acyclovir at a very slow rate with a half-life of ~1 h.

**Kinetics of Incorporation by Pol γ**—Mitochondrial toxicity is the key limiting factor of highly active antiretroviral therapy used for the treatment of patients with HIV and each of the nucleoside analogs approved by the FDA have been scrutinized for this toxicity (14, 34). To evaluate the potential for mitochondrial toxicity, we inspected the kinetics of incorporation and excision of acyclovir-TMP by Pol γ. A concentration of 100 nM exonuclease-deficient (E200A) Pol γ holoenzyme was incubated with 90 nM 25/45-mer (as described in experiments with Pol-UL42) and then rapidly mixed with acyclovir-TMP/Mg²⁺ to start the reaction. The time dependence of product formation at various concentrations of acyclovir-TMP is shown in Fig. 4A. At all concentrations the reactions were monophasic and the rates depended on acyclovir-TMP concentration in a hyperbolic fashion (Fig. 4B), yielding a k_{pol} of 1.03 ± 0.07 s⁻¹ and a K_d of 6.0 ± 1.2 μM. The kinetics of incorporation of the natural nucleotide, dGMP, were previously examined (30) and are shown in Table 2 for direct comparison. Interestingly, dGTP is preferred by only 265-fold, relative to acyclovir-TMP.

**Kinetics of Excision by Pol γ**—The ability of Pol γ to excise acyclovir-MP was assessed in exactly the same manner as described for the above experiments with Pol-UL42. Initially, the removal of dGMP from both correctly paired and frayed DNA was examined. The time dependence of substrate depletion (26-mer primer) is plotted in Fig. 5A. Both reactions were biphasic and the amplitude and rate of the fast phase were greater in magnitude when dGMP was removed from frayed DNA (Table 2). In comparing frayed with non-frayed DNA, the rate of removal increased from 0.45 ± 0.13 to 2.6 ± 0.2 s⁻¹ and the amplitude increased from 16 ± 2 to 69 ± 4 nM, respectively. These data set a lower limit on the rate of hydrolysis of 2.6 s⁻¹ for DNA bound to the exonuclease site. Because the reaction was initiated by the addition of Mg²⁺ to a pre-formed enzyme-DNA complex, it is possible that other reactions limited the rate of excision (33). Pol γ does not rapidly hydrolyze single-stranded DNA, so we could not use the rate of hydrolysis of single-stranded DNA to provide an estimate for the rate of excision of DNA in the exonuclease site (32).
Excision of acyclovir-MP was examined with correctly paired and frayed DNA both in the presence and absence of 100 \(\mu M\) dNTPs (Fig. 5B). Similar to the observations with Pol-UL42 the reactions catalyzed by Pol \(\gamma\) were monophasic. The removal of correctly paired acyclovir-MP in the presence of dNTPs was extremely slow, with a half-life of over 2 h ((9.2 ± 1.0) \(\times 10^{-5}\) s\(^{-1}\)) and was slowed to a rate of (2 ± 0.6) \(\times 10^{-4}\) s\(^{-1}\) in the presence of dNTPs. The removal reactions of acyclovir-MP from frayed DNA with and without the addition of dNTPs both proceeded at rates of at least 0.3 s\(^{-1}\).

When nucleotides were excluded, the reaction proceeded about 20-fold faster ((2.1 ± 0.2) \(\times 10^{-3}\) s\(^{-1}\)), exhibiting a trend similar to that observed with Pol-UL42. When the reaction was performed using frayed DNA, there was a 4-fold reduction in excision rate in the presence of added dNTPs. This modest reduction in rate could be due in part to primer stabilization at the polymerase site.

**DISCUSSION**

In previous work, we demonstrated that the kinetics of incorporation of nucleoside analogs by HIV-1 RT versus Pol \(\gamma\) could be correlated with the clinically observed effectiveness versus mitochondrial toxicity of each of the FDA-approved nucleoside analogs (14, 36). Using a similar approach in this study, we measured the ability of HSV Pol-UL42 to discriminate against acyclovir-TP during polymerization and to excise acyclovir-MP after incorporation. These data can then be compared with the kinetics of incorporation and excision catalyzed by Pol \(\gamma\), the most likely site for toxic side effects.

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4. J. W. Hanes and K. A. Johnson, unpublished observations.
The observed 50-fold discrimination against acyclovir-TP by the HSV polymerase resulted from slower catalysis rather than weaker ground state binding, suggesting that the guanine moiety of acyclovir is able to hydrogen bond with the templating base and base stack with the adjacent residue in the primer strand affording approximately normal ground state binding. A detailed study by nuclear magnetic resonance (NMR) with a strand affording approximately normal ground state binding. A base and base stack with the adjacent residue in the primer ety of acyclovir is able to hydrogen bond with the templating weaker ground state binding, suggesting that the guanine moi- the HSV polymerase resulted from slower catalysis rather than alterations in structure could lead to an improved drug. Thus, discrimination against acyclovir-TP may reflect either a slower conformational change of the enzyme or suboptimal alignment of the reactive groups in the closed conformation.

HIV-1 RT discriminates against FDA-approved nucleoside analogs with values ranging from 0.6 to 60 in preferring the natural nucleotide over the analog while copying DNA (36). Thus, discrimination against acyclovir-TP by Pol-UL42 falls in the upper end of that range and it is reasonable to suppose that alterations in structure could lead to an improved drug. However, the selectivity imposed by the relative efficiencies of activation of acyclovir by viral versus host kinases limits the possible modification of acyclovir structure. Acyclovir is phosphorolyzed more efficiently by the promiscuous viral thymidine kinase than by the host kinases so it becomes activated primar- ily in infected cells (2, 38). Alterations in acyclovir structure to allow more efficient incorporation by the viral polymerase run the risk of leading to faster rates of phosphorylation by cellular kinases and increased toxic side effects. Alternatively, phosphonate analogs such as tenofovir, cidofovir, and adefovir could be developed to treat HSV infections without constraints imposed by the viral thymidine kinase.

The net effectiveness of acyclovir is a function of the kinetic parameters governing both its incorporation and its subsequent removal by the proofreading exonuclease of the HSV polymerase. We compute a toxicity index toward viral replication that represents the increase in the time it would take to replicate the viral genome due to incorporation and subsequent removal of a chain terminator (see Table 3). A toxicity index of 16,000 is calculated assuming equal concentrations of acyclovir-TP and dGTP, which is probably not the case. However, the toxicity index is a linear function of the actual concentration ratio, and our computed value of the toxicity index implies that even a low ratio of [acyclovir-TP]/[dGTP] would lead to significant poisoning of viral replication. Our measurements provide a quantitative basis for understanding the effectiveness of acyclovir in treating HSV-1 infections and provide a standard enzyme assay that can be used in the development of new drugs.

After the incorporation of a chain terminator, the next correct nucleotide is able to bind at the polymerase site and inhibit the transfer of the primer into the exonuclease site. The next correct base slows the rate of removal of the acyclovir by HSV Pol-UL42 ~20-fold, representing a significant contribution to further increase the toxicity of acyclovir toward viral replication. In contrast, the addition of a physiological concentration of the next correct base reduced the rate of excision of ddC by Pol γ only 4-fold. 4

Potential toxic side effects of acyclovir can be evaluated by measuring its kinetics of incorporation and excision catalyzed by the human mitochondrial DNA polymerase. Pol γ is susceptible to inhibition by other nucleoside analogs and so the potential for toxicity of acyclovir was assessed. In contrast to the serious and sometimes life threatening side effects caused by the more toxic nucleoside analogs used to treat HIV infection, acyclovir appears to be well tolerated clinically.

Pol γ exhibits a marginally higher degree of discrimination against acyclovir-TP than Pol-UL42 (~270 versus ~50). Moreover, by combining rates of incorporation and excision we compute a toxicity index of 380 defining the potential for acyclovir to slow mitochondrial DNA replication. Our results place acyclovir-TP below the levels seen for the most toxic NRTI with toxicity indices greater than 2000, illustrated by stavudine (2’,3’-didehydro-2’,3’-dideoxythymidine), which causes peripheral neuropathy with a high incidence and is no longer recom- mended for treatment of HIV infection (39). However, the toxicity index for acyclovir is considerably greater than that observed for the less toxic NRTI with toxicity indices less than 2 (14, 36).

The large 1000-fold gap in toxicity indices between those nucleoside analogs associated with clinical toxicity and those that are generally free of such side effects makes it difficult to define a threshold for toxicity. The toxicity index of 380 for acyclovir places it in the middle of this range, suggesting that it

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**TABLE 2**

Summary of kinetic parameters for Pol γ

| Incorporation  | $k_{pol}$ | $K_a$ | Specificity constant | Discrimination |
|---------------|----------|------|----------------------|---------------|
|               | $s^{-1}$ | μM   | $s^{-1}$ μM           |               |
| dGTP          | 37 ± 2   | 0.8  ± 0.13 | 45 ± 10               | 265 ± 86      |
| Acyclovir-TP  | 1.03 ± 0.07 | 6.0 ± 1.2 | 0.17 ± 0.04          |               |

| Excision b     | Fast rate | Fast amplitude | Slow rate | Slow amplitude |
|---------------|-----------|----------------|-----------|----------------|
|               | $s^{-1}$  | μM             | $s^{-1}$  | μM             |
| dG_correct    | 0.45 ± 0.13 | 16 ± 2        | 0.02 ± 0.001 | 74 ± 2         |
| dG_frayed     | 2.6 ± 0.2  | 69 ± 4        | 0.27 ± 0.07 | 27 ± 5         |
| Acyclovic_correct | (2.1 ± 0.2) | (9.1 ± 0.1) | (9.2 ± 0.01) | 45 ± 1         |
| Acyclovic_frayed | (1.3 ± 0.08) | (9.1 ± 0.1) | (9.2 ± 0.01) | 45 ± 1         |
| Acyclovic_correct + dNTPs | (2.1 ± 0.2) | (9.1 ± 0.1) | (9.2 ± 0.01) | 45 ± 1         |
| Acyclovic_frayed + dNTPs | (1.3 ± 0.08) | (9.1 ± 0.1) | (9.2 ± 0.01) | 45 ± 1         |

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a From Ref. 30.

b Shown are data obtained for rates of the removal of the natural nucleotide, dGMP, or the rates of removal of the nucleoside analog, acyclovir-TP, from either correctly paired or frayed DNA substrates (denoted in the left-hand column). In two experiments 100 μM dNTPs were added and are also denoted in the left-hand column. There are two rates given for the removal of dGMP because excision occurred in a biphasic manner while the removal of acyclovir-TP was monophasic under all conditions examined.

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TABLE 3

Calculation of the toxicity indices and the therapeutic index for acyclovir

|                 | $k_{pol, correct}$ | $k_{pol, correct}/k_{pol, incorrect}$ | Discrimination | Toxicity index$^a$ | Therapeutic index$^a$ |
|-----------------|---------------------|----------------------------------------|----------------|-------------------|----------------------|
| HSV-1 DNA Pol   | 6.40 x 10^{-4}      | 2.0 x 10^{-5}                          | 50             | 16,000            | 42                   |
| Pol γ           | 0.05 x 10^{-5}      | 9.2 x 10^{-5}                          |                |                   |                      |

$^a$ HSV-1 DNA Pol data are from data presented in Table 1. Pol γ data are from Table 2 and Ref. 30.

$^b$ Defined as $k_{pol, correct}/k_{pol, incorrect}$\times[acyclovir-TP]/dGTP]/4D, where it is assumed that the ratio of acyclovir-TP and dGTP concentration is 1.0.

$^c$ Defined as toxicity indexHSV-1 DNA Pol/toxicity indexPol γ.
associated with lower mitochondrial DNA levels and other mitochondrial abnormalities (21, 22).

The toxicity index computed here for acyclovir in killing HSV replication sets an easily quantifiable standard for evaluation of nucleoside analogs intended to prevent replication of a pathogen whose polymerase contains a proofreading exonuclease. By combining the contributions of polymerization selectivity and exonuclease efficiency, the toxicity index provides a quantifiable guideline for evaluating new drugs. The toxicity index of 16,000 computed here for acyclovir is consistent with the clinical effectiveness of the drug in treating HSV infections.

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