Insights into the Molecular Mechanism of Mitochondrial Toxicity by AIDS Drugs*

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Several of the nucleoside analogs used in the treatment of AIDS exhibit a delayed clinical toxicity limiting their usefulness. The toxicity of nucleoside analogs may be related to their effects on the human mitochondrial DNA polymerase (Pol γ), the polymerase responsible for mitochondrial DNA replication. Among the AIDS drugs approved by the FDA for clinical use, two are modified cytosine analogs, Zalcitabine (2',3'-dideoxyctydine (ddC)) and Lamivudine (β-D-(+)-2',3'-dideoxy-3'-thiacytidine ((−)3TC)). ((−)3TC) is the only analog containing an unnatural L(−) nucleoside configuration and is well tolerated by patients even after long term administration. In cell culture (−)3TC is less toxic than its D(+)-isomer, (+)3TC, containing the natural nucleoside configuration, and both are considerably less toxic than ddC. We have investigated the mechanistic basis for the differential toxicity of these three cytosine analogs by comparing the effects of dideoxy-CTP, (+)3TC-triphosphate (TP), and (−)3TC-TP on the polymerase and exonuclease activities of recombinant human Pol γ. This analysis reveals that Pol γ incorporates (−)3TC-triphosphate 16-fold less efficiently than the corresponding (+)isomer and 1140-fold less efficiently than dideoxy-CTP, showing a good correlation between incorporation rate and toxicity. The rates of excision of the incorporated analogs from the chain-terminated 3'-end of the DNA primer by the 3'-5'-exonuclease activity of Pol γ were similar (0.01 s⁻¹) for both 3TC analogs. In marked contrast, the rate of exonuclease removal of a ddC chain-terminated DNA occurs at least 2 orders of magnitude slower, suggesting that the failure of the exonuclease to remove ddC may play a major role in its greater toxicity. This study demonstrates that direct analysis of the mitochondrial DNA polymerase structure/function relationships may provide valuable insights leading to the design of less toxic inhibitors.

The in vitro and in vivo mechanisms of cytotoxicity of the nucleoside analogs used as anti-HIV1 drugs are not well understood. Various forms of toxicity, including peripheral neuropathy, myopathy, and pancreatitis, are observed after long term use of nucleoside analogs 3'-azido-3'-deoxythymidine (AZT) (1), 2',3'-dideoxynosine (ddI) (2), 2',3'-dideoxyctydine (ddC) (3, 4), and 2',3'-didehydro-3'-deoxythymidine (5). The profile for toxicity varies with each analog. For instance, whereas all of the three, AZT, ddI, and ddC, exert cytotoxic effects on human muscle cells and induce functional alternation of mitochondria, only AZT, but not ddI and ddC, can induce a myopathy in HIV-1-infected patients (6). The fact that toxicity of different drugs is cell-, tissue-, and organ-specific (6) indicates that the differences in subcellular bioavailability and pharmacokinetic profile involving the activation and deactivation of the drugs can all contribute to the clinical toxicity. Many mechanisms have been proposed for the origins of nucleoside analog toxicity, but the most obvious sites of action are the enzymes responsible for host cell DNA replication and repair: DNA polymerases α, δ, and ε, which are responsible for chromosomal DNA replication; DNA polymerase β, which is involved in DNA repair; and DNA polymerase γ (Pol γ), which carries out mitochondrial DNA synthesis (7–9). The toxicity toward proliferating tissues, such as the well documented bone marrow toxicity of AZT, is believed to be due to the inhibition of one or more of the chromosomal DNA polymerases (10). The toxicity manifested in many nonproliferating tissues is thought to result more specifically from the effects of inhibition of DNA Pol γ by nucleoside analogs (10–14). In support of this postulate, the recognizable clinical syndromes associated with nucleoside analog toxicity are reminiscent of mitochondrial dysfunctions associated with mitochondrial genetic disorders (12, 15–18); moreover, prolonged exposure of the cell to nucleoside analogs results in decreased mitochondrial DNA synthesis, decreased cell viability, and changes in the mitochondrial ultrastructure (19). Some in vitro studies have shown that for many of the nucleoside analogs tested, Pol γ is more sensitive than any of the other DNA polymerases (20), but there are conflicting reports (19, 21). Earlier studies to assess the interaction of nucleoside analogs have used partially purified preparations of native enzyme. However, recently, highly purified homogenous preparations of recombinant human Pol γ have become available through cloning and overexpression in a baculovirus expression system (22, 23–26). Human DNA Pol γ is composed of a catalytic subunit of 140 kDa and an accessory subunit of ⋍54 kDa (22). The large catalytic subunit provides both polymerase and 3'-5'-exonuclease activities (23, 24), whereas the smaller accessory subunit (25) facilitates substrate binding and dNTP incorporation, thereby improving the activity of the polymerase (23). The details of these interactions have been studied in cell-free extracts (23). The 3'-5'-exonuclease activity is important for the maintenance of balanced genome maintenance and repair (23).

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cessivity (26). The cloning, overexpression, purification, and reconstitution of the human Pol γ has now afforded a highly purified enzyme preparation, allowing a more rigorous analysis of the reactions governing nucleotide selectivity during incorporation and removal by the proofreading exonuclease (27).

Among the anti-HIV nucleoside analogs used clinically, the specific exonuclease activity, which is believed to perform a proofreading function (22, 37). It is possible that the failure of 3’-exonuclease activity, which is believed to perform a proofreading function (22, 37). Effective doses of (-)3TC are well tolerated during therapy (28–30), and there is little or no evidence of mitochondrial injury (13, 30). In contrast, ddC is one of the more toxic of the nucleoside analogs approved for AIDS therapy by the Federal Drug Administration. The mechanistic basis for observed stereocatalytic differential toxicity for the two 3TC isomers and enhanced toxicity of ddC are not understood, and a number of factors may come into play. The differences in toxicity may be a combination of factors including cellular uptake (31, 32), transport (10), metabolic activation (31–33), incorporation (31, 33, 34), and removal or degradation from the system (10, 27, 33, 35, 36). A quantitative analysis of each factor is clearly required.

The mechanism of inhibition of DNA Pol γ by the nucleoside analogs involves in vivo polymerase to form the triphosphate and subsequent incorporation into the DNA primer strand, resulting in chain termination. Thus in vitro studies have focused on evaluating the triphosphate form of analogues. Earlier steady-state kinetic studies have examined the effect of the (+) and (-)3TC-TP incorporation into DNA by Pol γ (21, 31) and suggest a 5-fold difference in $K_i$ values. However, these steady-state kinetic studies were conducted under conditions that employed a low concentration of Pol γ, and according to the $K_i$ value of 35 nM for formation of the heterodimer (26), the polymerase would not have existed as the holoenzyme complex. Indeed, the significance of earlier studies using native purified enzyme is unclear due to the variable activity resulting from the loss of the small subunit during purification.

Similar to many of the DNA polymerases, Pol γ has 3’-5’-exonuclease activity, which is believed to perform a proofreading function (22, 37). It is possible that the failure of 3’-5’-exonuclease of Pol γ to efficiently remove chain terminators may play a role in mitochondrial toxicity of nucleoside analogs. However, discrepancies exist in the literature concerning the efficiency of the exonuclease in removal of these analogs and correlation to drug toxicity. It has been proposed that efficient removal of 3’-terminal (-)3TC-monophosphates (MPs) by the 3’-5’-exonuclease activity of Pol γ could be responsible for the low toxicity of (-)3TC (33); however, similar rates of exonuclease removal were reported for the excision of dCMP, ddC-MP, and (-)3TC-MP from the 3’-terminus of a DNA primer, even though there are significant differences in mitochondrial toxicity (33). This assessment was made with a partially purified preparation of Pol γ that may not represent the holoenzyme complex due to the loss of the accessory subunit. Moreover, a novel cytosolic exonuclease was isolated from H9 cells that can remove chain-terminated dNMP and may be a contaminating activity in partially purified Pol γ (27). Finally, in cell culture studies, ddC showed higher inhibition of mitochondrial DNA synthesis than both the (+) and (-)3TC (31, 39). Thus, a detailed study using pure human Pol γ is needed.

Previously, we have studied the stereocatalytic selectivity between the (+) and (-)3TC triphosphate for inhibition of HIV-1 reverse transcriptase (RT) (40). We have also determined the detailed kinetic properties of human mitochondrial DNA polymerase for both the catalytic subunit and the holoenzyme (23, 26). We now describe our transient kinetic analysis of incorporation and excision of deoxycytidine triphosphate (ddCTP), (+)3TC, and (-)3TC, with the goal of understanding how these clinically important modified cytosine analogs interact with the Pol γ holoenzyme complex in comparison to ddTP.

An in-depth understanding of the mechanism of inhibition of the DNA γ polymerase is important in overcoming problems associated with cytotoxicity with nucleoside analogs. Knowledge of the mechanistic similarities and differences may identify key features important in selectivity for the interaction with the viral DNA polymerase (HIV-1 reverse transcriptase) over the DNA γ polymerase and thereby assist in the design of better AIDS drugs.

**Experimental Procedures**

The large and small subunits of Pol γ were purified as described (23, 26). The protein concentration was determined spectrophotometrically at 280 nm, with the extinction coefficients 234,420 and 71,894 M$^{-1}$ cm$^{-1}$ for the large and small subunits, respectively. A synthetic DNA duplex, 23-45 bp (40), was used in which the next correct base for incorporation was ddCTP. Concentrations of the oligonucleotides were estimated spectrophotometrically at 260 nm using the calculated extinction coefficients 226,750 M$^{-1}$ cm$^{-1}$ and 491,960 M$^{-1}$ cm$^{-1}$ for the 23- and 45-mer, respectively. Before annealing, the primer was 5’-labeled with T4 polynucleotide kinase and [$^{32}$P]ATP. The dCTP and ddCTP were purchased from Sigma, and the (+) and (-)3TC-TP isomers were the kind gift of Dr. Raymond Schinazi at Emory University.

Experiments to measure polymerization kinetics were performed as described (40). Rapid quench experiments were done using KinTek Instruments Model RQF-3 rapid quench-flow apparatus (41). Unless mentioned otherwise, all concentrations refer to concentrations during reactions after mixing on the quench apparatus. Typically, the experiments were done by first incubating the duplex DNA (250 nM) with pre-mixed (67 nM) and small subunits (440 nM) in reaction buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.8). A sample of this solution (15 μl) was rapidly mixed with an equal volume of a solution of dCTP analogs (at reaction concentrations) and MgCl$_2$ (2.5 mM) in reaction buffer. The reactions were quenched by --67 μl of 0.5 M EDTA at pH 8.0. All reactions were performed at 37°C. Single nucleotide incorporation was monitored by extension of 5’-32P-labeled 23-24 bp oligonucleotides. All reaction mixtures were analyzed on 20% denaturing polyacrylamide sequencing gels (8 M urea), imaged on a Bio-Rad GS-525 Molecular Imager System, and quantified using Molecular Analysis (Bio-Rad).

Data were fitted by nonlinear regression using the program KaleidaGraph (Synergy Software). The pre-steady-state burst experiments (Fig. 3) were fitted to a burst equation: [product] = $A_{\text{obsd}} - \exp(-k_{\text{burst}} t)$ + $k_f$[l], where $A_{\text{obsd}}$ represents the amplitude of the burst that correlates with the concentration of enzyme in active form, $k_{\text{burst}}$ is the observed first-order rate constant for dNTP incorporation, and $k_f$ is the observed steady-state rate constant.

The dissociation constant, $K_{\text{Diss}}$, for dNTP binding to the complex of Pol γ and 23-45 bp DNA duplex is calculated by fitting the data into the following hyperbolic equation: $k_{\text{off}} = (k_{\text{on}} \times [\text{dNTP}]/(K_{\text{Diss}} + [\text{dNTP}]))$, where $k_{\text{on}}$ is the maximum rate of dNTP incorporation, [dNTP] is the corresponding concentration of dNTP, and $K_{\text{Diss}}$ is the equilibrium dissociation constant for the interaction of dNTP with the enzyme-DNA complex.

The 24-mer primers were prepared by incorporating the corresponding dCTP analogs into the 23 bp DNA. A mixture of 23-45 DNA duplex (1000 pmol), HIV-1 RT (300 pmol), dCTP analogs (5–50 μM), and 10 μM MgCl$_2$ in reaction buffer (50 mM Tris-HCl, 50 mM NaCl, pH 7.8) was incubated at 37°C for 10–30 min. The 24-mer DNA products were purified by gel electrophoresis.

The exonuclease activity was studied by measuring the rate of formation of the cleavage products in the absence of dCTP or dCTP analogs. Products formed from the early time points were plotted as a function of time. The slope of the line was divided by the active enzyme concentration in the reaction, and a $k_{\text{exonuclease}}$ for exonuclease activity was obtained. The reaction was initiated by adding MgCl$_2$ (2.5 mM) to a solution of catalytic subunit (40 nM), accessory subunit (270 nM), and 1500 nM 24-45 bp DNA duplex in reaction buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.8) and quenched with 0.3 M EDTA. For exonuclease activity of the catalytic subunit, 16 nM catalytic subunit and 1000 nM 24-45 bp DNA duplex were used.

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RESULTS

The underlying mechanisms of toxicity were investigated by examining the kinetics of the two different reactions catalyzed by human mitochondrial DNA Pol γ: DNA-dependent DNA synthesis and 3′-5′-exonuclease cleavage. This analysis was carried out by examining the single nucleotide incorporation using synthetically prepared dCTP, ddCTP, and two modified deoxycytidine analogs, (+)3TC-TP and (−)3TC-TP, opposite a template deoxyguanosine for DNA-dependent DNA polymerization by the recombinant Pol γ holoenzyme complex. The exonuclease cleavage was investigated by evaluating the excision of dCMP, ddCMP, (+)3TC-MP, (−)3TC-MP, and dTMP from the 3′-terminus of the corresponding DNA 23- or 24-mer primer by the catalytic subunit alone and the holoenzyme complex of Pol γ. All of the incorporation studies were carried out at protein concentrations high enough to maintain the holoenzyme Pol γ complex and on a time scale short enough to follow a single turnover of enzyme.

Using a transient kinetic approach, we determined the rate of polymerization (kpol), the dissociation constant for the ground state nucleotide binding (Kd), and the incorporation efficiency or specificity constant (kcat/Km) for the natural substrate, dCTP, as well as for ddCTP, (+)3TC-TP, and (−)3TC-TP (shown in Fig. 1). Enzyme specificity is defined by the ratio kcat/Km, representing the apparent second-order rate constant for substrate binding. In the case of DNA polymerases, our previous work (42) has shown that the ground state binding of nucleoside triphosphates is in a rapid equilibrium, and accordingly, the Kd measured under single turnover conditions would equal the Km and the kpol would equal kcat. We employed a synthetic 23/45-mer DNA/DNA primer-template (Fig. 2) to perform a series of pre-steady-state burst and single turnover experiments.

In each case a pre-steady-state burst of product formation was observed, indicating that at saturating concentrations of dNTP, a step after the chemical step is rate-limiting (Fig. 3, A and B). The rate-limiting step after incorporation of a single nucleotide is the release of the DNA from the enzyme (26). In each panel, the solid line represents the best fit of the data to a burst equation, from which the burst amplitude, A, the observed rate of incorporation, kobsd, and the observed steady-state rate, kcat, were obtained.

A series of experiments were conducted using varying concent-

2 A. A. Johnson and K. A. Johnson, unpublished results.

DNA/DNA 23/45-mer:

\[
\begin{align*}
\text{GGGACCTGCAAGCTCGACCAACT} \\
\text{CAGGACGTCCGAGGTGGGACGTTAGGATTAGGCAG} \\
\text{CAGGACGTCCGAGGTGGGACGTTAGGATTAGGCAG} \\
\end{align*}
\]

DNA/RNA 23/45-mer:

\[
\begin{align*}
\text{GGGACCTGCAAGCTCGACCAACT} \\
\text{CAGGACGTCCGAGGTGGGACGTTAGGATTAGGCAG} \\
\text{CAGGACGTCCGAGGTGGGACGTTAGGATTAGGCAG} \\
\end{align*}
\]

Fig. 2. Sequence of the oligonucleotide substrates.

Fig. 3. Pre-steady-state burst experiments for incorporation of dCTP analogs into DNA/DNA 23/45 primer-template by human mitochondrial DNA polymerase. A, the pre-steady-state burst curve for incorporation of the natural substrate, dCTP (●). For this experiment, 67 nM catalytic subunit and 440 nM accessory subunit was preincubated with 200 nM 23/45-mer DNA duplex. The polymerization was initiated by adding an equal volume of solution containing dCTP (10 μM) and MgCl2 (2.5 mM). The reactions were quenched with 0.3 M EDTA at each indicated time. The formation of elongated product DNA was plotted as a function of time. The solid line represents the fit of data to the burst equation as described, and the curve shown represents fit with burst amplitude A = 34.5 ± 0.7 nM. The observed burst rate constant kobsd = 39 ± 3 s−1, and the observed steady-state rate constant kobs = 0.062 ± 0.007 s−1. B, the pre-steady-state burst curves for incorporation of (+)3TC-TP (15 μM) (○) and (−)3TC-TP (20 μM) (□) by human mitochondrial DNA polymerase. The reaction conditions are identical to those in A. For (+)3TC-CTP, the curve-fitting generated A = 34 ± 4 nM, kobsd = 0.31 ± 0.05 s−1, and kobs = 0.029 ± 0.01 s−1. For incorporation of (−)3TC-TP, the curve-fitting provided A = 30 ± 7 nM, kobsd = 0.12 ± 0.04 s−1, and kobs = 0.009 ± 0.008 s−1. Note the difference in time scale between panels A and B.

Fig. 1. Chemical structures of deoxycytidine analogs.
observed because the burst rate was comparable with the steady-state rate. Therefore, to measure the rate of incorporation, the reactions were studied under single turnover conditions with enzyme in excess of DNA as described in Johnson et al. (26) and Feng and Anderson (40).

The interaction of dCTP with the enzyme-DNA complex was assessed by fitting the concentration dependence of the burst rates to the hyperbolic equation \( k_{\text{obsd}} = \left( k_{\text{pol}} \times [\text{dNTP}] / [K_d + [\text{dNTP}]] \right) \), where \( k_{\text{pol}} \) is the maximum rate of incorporation, [dNTP] is the concentration of the nucleoside triphosphate, and \( K_d \) is the equilibrium dissociation constant for the ground state binding of dNTP with the enzyme-DNA complex. The concentration dependence of the observed polymerization rate for the incorporation of dCTP, ddCTP, (+)3TC-TP, and (-)3TC-TP into a DNA/DNA 23/45-mer primer-template by Pol \( \gamma \) holoenzyme is shown in Fig. 4, A–C.

A complete summary of \( k_{\text{pol}}, K_d \), and \( k_{\text{pol}}/K_d \) values for each dCTP analog is shown in Table I. The \( k_{\text{pol}} \) and the \( K_d \) value for dCTP are all in good agreement with previous studies (26).

A comparison of the analogs reveals that the natural substrate, dCTP, serves as the best substrate for human mitochondrial DNA polymerase, as illustrated by the observed tight binding and fast rate of polymerization. In contrast, ddCTP is incorporated 70-fold more slowly than dCTP, but binds 27-fold more tightly, so the overall incorporation efficiency is only 2.6-fold lower. The (-)3TC-TP and (+)3TC-TP analogs are incorporated 125- to 350-fold more slowly than dCTP, respectively. The dCTP and (+)3TC-TP share a similar high affinity for the enzyme-DNA complex, indicating chemistry or the conformational change has become rate-limiting for (+)3TC-TP incorporation. In contrast, the affinity of (-)3TC-TP for the enzyme-DNA complex is 6–8-fold weaker than that of the analog that of (+)3TC-TP, containing the natural nucleoside configuration. Accordingly, Pol \( \gamma \) incorporates the (-) isomer at least 16-fold less efficiently than the (+) isomer, as defined by the specificity constants summarized in Table I. This difference in incorporation efficiency is in good agreement with cell culture studies with the nucleoside form of the compounds where the (-)3TC isomer has been reported to be 25- and 12-fold more toxic than the (-)3TC in terms of inhibition of cell growth and mitochondrial DNA synthesis, respectively (36).

We conducted an in-depth analysis of Pol \( \gamma \) 3'-5'-exonuclease removal of ddC and (+) and (-)3TC MP's from duplex DNA with highly purified Pol \( \gamma \) catalytic subunit and reassociated holoenzyme complex. Our analysis was carried out using DNA substrates that were 3'-terminated with ddCMP (D24-ddC), (+)3TC-MP (D24-(+3TC)), or (-)3TC-MP (D24-(+3TC)) along with the corresponding unmodified DNA 23- and 24-mers containing 3'dTMP (D23-4T), and dCMP (D24-dC). We enzymatically synthesized the three DNA 24-mer primers that were 3'-terminated with dideoxy nucleoside analogs, ddCMP, (+)3TC-MP, and (-)3TC-MP, respectively. To eliminate the sample variability that may be introduced during preparation, the DNA 23- and 24-mers containing dTMP and dCMP, respectively, were prepared in parallel to those containing modified nucleosides.

A gel illustrating the time course for product formation during the exonuclease reaction is shown in Fig. 5. For clarity, only representative time points have been selected for dCMP (D24-ddC), ddCMP (D24-ddC), (+)3TC-MP (D24-(+3TC)), or (-)3TC-MP (D24-(+3TC)). A summary of the rates of exonuclease activity (\( k_{\text{end}} \)) for either the catalytic subunit or the holoenzyme complex of Pol \( \gamma \) using different D24-mer substrates is shown in Table II.

The unmodified correctly base-paired primers, D23-4T and D24-dC, exhibited the fastest excision rates; however, these rates are still small and consistent with the “cost of proofreading” due to the removal of ~1% of the correctly paired bases at the 3'-end of the primers (42). Excision of the (+) and (-) isomers of 3TC was 2-fold slower than removal of dCMP, and
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**Table I**

| dNTP | $k_{pol}$ | $K_d$ | $k_{exo}$ | $k_{pol} / K_d$ |
|------|-----------|-------|------------|----------------|
| dCTP | 44 ± 2    | 1.1 ± 0.1 | 0.06 ± 0.01 | 40 ± 5 |
| ddCTP | 0.66 ± 0.02 | 0.041 ± 0.007 | 0.03 ± 0.01 | 16 ± 3 |
| (+)3TC-TP | 0.35 ± 0.01 | 1.5 ± 0.1 | 0.04 ± 0.01 | 0.23 ± 0.02 |
| (-)3TC-TP | 0.125 ± 0.005 | 9.2 ± 0.9 | 0.02 ± 0.01 | 0.014 ± 0.002 |

**Figure 5.** Gel analysis comparing the nature and kinetics of the exonuclease activity of human mitochondrial DNA Pol γ using different DNA substrates. Four different kinds of 24/45-mer DNA duplex were tested. At the 3′-end of each of the DNA 24-mer, the dNMP was either an dCMP, a ddCMP, a (+)3TC-MP, or a (-)3TC-MP. The representative time courses are 0, 0.05, 0.5, 3, and 12 h. The reaction was initiated by adding MgCl₂ (2.5 mM)-containing buffer into a solution of catalytic subunit (40 nM), accessory subunit (270 nM), and 1500 nM D24/D45. The reaction was quenched at various times with 0.3 M EDTA, and products were quantified as described in Feng and Anderson (40).

**Table II**

| DNA substrates | Catalytic subunit $k_{pol}$ | Holoenzyme $k_{exo}$ |
|----------------|-----------------------------|---------------------|
| Primer | $s^{-1}$ | $s^{-1}$ |
| D23-dT | 0.24 ± 0.01 | 0.042 ± 0.001 |
| D24-dC | 0.098 ± 0.001 | 0.22 ± 0.001 |
| D24-ddC | <0.00002 | <0.00001 |
| D24-(+)+3TC | 0.020 ± 0.001 | 0.012 ± 0.001 |
| D24-(-)3TC | 0.015 ± 0.002 | 0.010 ± 0.001 |

* All of the templates used in this study are DNA 45-mers.

**Table III**

| dNTP | Relative incorporation | Relative exonuclease | Relative toxicity |
|------|------------------------|----------------------|------------------|
| ddCTP | 1.140 | <0.001 | 2300 |
| (+)3TC-TP | 16 | 1.2 | 12 |
| (-)3TC-TP | 1 | 1 | 1 |

* Calculated from $k_{pol} / K_d$ for each nucleotide from Table I.
* Calculated from the observed exonuclease rate from Table II.
* From Ref. 36, in which the nucleoside form of the analogs is assessed.

**Discussion**

Our studies have shown that (−)3TC-TP is less efficiently incorporated than (+)3TC-TP into DNA by Pol γ, and both are incorporated less efficiently than ddCTP. Moreover, the efficiency of incorporation by Pol γ is directly correlated with toxicity of each nucleoside analog. These observations point to incorporation by Pol γ into mitochondrial DNA as a major site of toxicity of nucleoside analogs. The availability of reliable preparations of Pol γ now affords a rapid method to screen drugs for toxicity and opens the path toward further structure/function studies to aid in the design of less toxic analogs.

3TC is well tolerated in the clinic (28, 29), and no subclinical signs of mitochondrial toxicity resulting from (−)3TC therapy for 6 months have been observed (30). Apparently to date (−)3TC has shown little evidence of mitochondrial injury (13). The unnatural l-(−) configuration of (−)3TC seems to play an important role in preserving the anti-viral activity while decreasing the inhibitory effect on the host Pol γ. This example suggests other unnatural nucleoside analogs may show similar effectiveness combined with low toxicity.

In contrast, the effects of inhibition on mitochondrial DNA synthesis in vivo and in vitro by ddC are profound (38, 45). The IC₅₀ value for ddC relative to the (+) and (−) isomers of 3TC indicated that ddC is 180- and 2300-fold more toxic than the (+) and (−) isomer, respectively (36). A number of factors may clearly come into play including uptake, transport, metabolic
activation, degradation, and removal from the system, but our studies have shown a lack of exonucleolytic removal of a 3'-ddC that may significantly contribute to drug toxicity. Conversely, analogs that activate the exonuclease removal by Pol γ may exhibit even lower toxicity, and this may provide a new avenue for exploration of less toxic drugs.

The accessory subunit appears to enhance discrimination by the proofreading exonuclease since slower rates of excision by the holoenzyme were observed for correctly base-paired DNA 23-dT- and 24dC-mers. When the 3'-terminus is occupied by a correctly base-paired natural dNMP with a 3'-OH, the accessory subunit may slow down the excision of this dNMP by facilitating the retention of the 3'-end at the polymerase site. Clearly, additional studies are required to identify the structural features of nucleoside analogs that may be utilized to obtain selectivity for HIV-1 RT over DNA Pol γ.

In summary, our studies have demonstrated that the (+)3TC-TP is a more potent inhibitor for human mitochondrial DNA Pol γ than the (−) isomer through tighter binding and faster incorporation rate into a DNA duplex, leading to greater toxicity. Using highly purified Pol γ, there is no significant difference in the excision of the incorporated (+)- and (−)3TCMP by the 3'-5'-exonuclease activity of Pol γ from the 3'-terminus of a DNA primer, indicating this step likely does not contribute to the low toxicity of (−)3TC. Additionally, our studies have shown that a terminally incorporated ddCMP is not efficiently removed by the 3'-5'-exonuclease activity of Pol γ, suggesting this may play a role in the high level of inhibition of ddC on mitochondrial DNA synthesis observed in vitro and in vivo. The overall toxicity of a nucleoside analog toward replication will be a function of the frequency of incorporation of the chain terminator and the kinetics of excision by the proofreading exonuclease relative to the rate of DNA replication required to sustain growth (Table III). We can now quantify these parameters and begin to assess the structural/functional determinants that govern nucleotide selectivity for Pol γ in each step.

An in-depth understanding of the mechanism of inhibition of DNA polymerase γ is important in defining the cytotoxicity of anti-HIV drugs toward mitochondrial replication. A knowledge of the mechanistic and structural similarities and differences between Pol γ and HIV-1 RT may identify key features important for selective drug incorporation by the viral HIV-1 RT but not by Pol γ and, therefore, facilitate the design of less toxic, viral-specific AIDS drugs.

REFERENCES

1. Wilde, M. I., and Langtry, H. D. (1993) Drugs 46, 515–578
2. Faulds, D., and Brogden, R. N. (1992) Drugs 44, 94–116
3. Whittington, R., and Brogden, R. N. (1992) Drugs 44, 656–683
4. Adkins, J. C., Peters, D. H., and Faulds, D. (1997) Drugs 53, 1054–1080
5. Hitchcock, M. J. M. (1991) Antiviral Chem. Chemother. 2, 125–132
6. Benbrik, E., Chariot, P., Bonavaud, S., Ammi-Said, M., Frisadal, E., Rey, C., Gherardi, R., and Barlovaz-Reinheit, M. (1997) J. Neurovirol. Sci. 149, 19–25
7. Frick, L. W., Nelson, D. J., St. Clair, M. H., Furman, P. A., and Krenitsky, T. A. (1988) Biochemistry 27, 5989–5995
8. Hall, E. T., Yan, J. P., Melancon, P., and Kuchta, R. D. (1994) J. Biol. Chem. 269, 14355–14358
9. Kew, S., Veal, G. J., Hoggard, P. G., Barry, M. G., and Back, D. J. (1997) Biochemistry 36, 588–593
10. Parker, W. B., and Cheng, Y.-C. (1994) J. Natl. Inst. Health Res. 6, 57–61
11. Horn, D. M., Nee, L. A., Colacino, J. M., and Richardson, F. C. (1997) Antiviral Res. 34, 71–74
12. Colacino, J. M. (1996) Antiviral Res. 29, 125–139
13. Honkoop, P., Scholte, H., de Man, R. A., and Schalm, S. W. (1997) Drug Safety 17, 1–7
14. Barile, M., Valenti, D., Quagliariello, E., and Passarella, S. (1998) Gen. Pharmacol. 31, 531–538
15. Lewis, W., and Dalakas, M. C. (1995) Nat. Med. 1, 417–422
16. Mroz, J., and Fried, M. W. (1997) Ber Bisseig, A. M., Park, Y., Savarese, B., Keiner, D., Toskos, P., Luciano, C. et al. (1995) N. Engl. J. Med. 333, 1099–1105
17. Simon, D. R., and Johns, D. R. (1999) Annu. Rev. Med. 51, 111–127
18. Pezeshkpour, G., Krajcer, C., Buchthal, F., DiMauro, S., Bresolin, N., and McBurney, J. (1987) J. Neurovirol. Sci. 77, 285–304
19. Medina, D. J., Tsai, C. H., Huang, G. D., and Cheng, Y. C. (1994) Antiinfect. Agents Chemother. 38, 154–158
20. Kukhanova, M., Liu, S.-H., Mozzerin, D., Lin, T.-S., Chu, C. K., and Cheng, Y.-C. (1995) J. Biol. Chem. 270, 23035–23039
21. Martin, J. L., Brown, C. E., Matthews-Davis, N., and Reardon, J. E. (1994) Antiinfect. Agents Chemother. 38, 2743–2749
22. Gray, H., and Wong, T. W. (1992) J. Biol. Chem. 267, 5835–5841
23. Graves, S. W., Johnson, A. A., and Johnson, K. A. (1998) Biochemistry 37, 6050–6058
24. Longley, M. J., Ropp, P. A., Lim, S. E., and Copeland, W. C. (1998) Biochemistry 37, 10529–10539
25. Lim, S. E., Longley, M. J., and Copeland, W. C. (1999) J. Biol. Chem. 274, 38197–38203
26. Johnson, A. A., Tsai, Y., Graves, S. W., and Johnson, K. A. (2000) Biochemistry 39, 1702–1708
27. Skalski, V., Liu, S. H., and Cheng, Y. C. (1995) Biochem. Pharmacol. 50, 815–821
28. Beach, J. W. (1998) Clin. Ther. 20, 2–25
29. Notermans, D. W., van Leeuwen, R., and Lange, J. M. (1996) Drug Safety 15, 176–187
30. Honkoop, P., de Man, R. A., Scholte, H. R., Zondervan, P. E., Van Den Berg, J. W., van Rijswijk, M., and van der Velden, W. (1999) Hepatology 26, 211–215
31. Chang, C. N., Skalski, V., Zhou, J. H., and Cheng, Y. C. (1992) J. Biol. Chem. 267, 22414–22420
32. Skalski, V., Chang, C. N., Dutschman, G. E., and Cheng, Y. C. (1993) J. Biol. Chem. 268, 23234–23238
33. Gray, N. M., Marr, C. L. P., Penn, C. R., Cameron, J. M., and Bethell, R. C. (1995) Biochemistry 34, 1043–1051
34. Hsu, G. J., Orr, D. C., Penn, C. R., Figuereido, H. T., Gray, N. M., Boehme, R. E., and Cameron, J. M. (1992) Antimicrob. Agents Chemother. 36, 1688–1694
35. Ciernik, R. A., Beaudoin, P., Marr, C. L., Reid, P. J., Boehme, R. E., Coates, J. A., Penn, C. R., and Cameron, J. M. (1992) Biochemistry 31, 2059–2064
36. Chang, C. N., Doong, S. L., Zhou, J. H., Beach, J. W., Jeong, L. S., Chu, C. K., Tsai, C. H., Cheng, Y. C., Lotta, D., and Schinazi, R. (1992) J. Biol. Chem. 267, 13938–13942
37. Olsen, M. W., and Kugler, D. P. (1992) J. Biol. Chem. 267, 23136–23142
38. Chen, C. H., and Vazquez-Padua, M., and Cheng, Y. C. (1991) Mol. Pharmacol. 39, 625–628
39. Chen, C. H., and Vazquez-Padua, M. (1991) J. Biol. Chem. 266, 11934–11937
40. Feng, J. Y., and Anderson, K. S. (1999) Biochemistry 38, 55–63
41. Johnson, K. A. (1990) Methods Enzymol. 154, 677–705
42. Johnson, K. A. (1993) Annu. Rev. Biochem. 62, 685–713
43. Longley, M. J., and Mosbaugh, D. W. (1991) J. Biol. Chem. 266, 24702–24711
44. Eriksson, S., Xu, B., and Clayton, D. A. (1995) J. Biol. Chem. 270, 18929–18934
45. Dubinsky, R. M., Yarcheo, R., Dalakas, M. C., and Broder, S. (1989) Muscle Nerve 12, 856–860