Differential Incorporation and Removal of Antiviral Deoxynucleotides by Human DNA Polymerase γ*

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Mitochondrial toxicity can result from antiviral nucleotide analog therapy used to control human immunodeficiency virus type 1 infection. We evaluated the ability of such analogs to inhibit DNA synthesis by the human mitochondrial DNA polymerase (pol γ) by comparing the insertion and exonucleolytic removal of six antiviral nucleotide analogs. Apparent steady-state $K_m$ and $k_{cat}$ values for insertion of $2',3'$-dideoxynucleotides (ddNTPs) were $2',3'$-dideoxy-CTP (ddCTP), $2',3'$-dideoxy-TTP (ddTTP), $2',3'$-dideoxy-AMP (ddATP), $2',3'$-dideoxy-UTP (ddUTP), $2',3'$-dideoxy-GTP (ddGTP), and carbocyclic $2',3'$-dideoxy-2',3'-dideoxy-CTP (CBV-TP). Apparent steady-state $K_m$ values for insertion of $3'$-dideoxynucleotides were $3'$-dideoxy-CTP, $3'$-dideoxy-TTP, $3'$-dideoxy-ATP, $3'$-dideoxy-UTP, and $3'$-dideoxy-GTP. Apparent steady-state $k_{cat}$ values for incorporation were $2',3'$-dideoxy-CTP $>$ $3'$-dideoxy-CTP $>$ $2',3'$-dideoxy-GTP $>$ $2',3'$-dideoxy-TTP $>$ $2',3'$-dideoxy-UTP $>$ $2',3'$-dideoxy-ATP. Apparent steady-state $k_{cat}$ values for removal of $2',3'$-dideoxynucleotides were $2',3'$-dideoxy-CTP $>$ $2',3'$-dideoxy-TTP $>$ $2',3'$-dideoxy-ATP $>$ $2',3'$-dideoxy-UTP $>$ $2',3'$-dideoxy-GTP. Apparent steady-state $k_{cat}$ values for removal of $3'$-dideoxynucleotides were $3'$-dideoxy-CTP $>$ $3'$-dideoxy-TTP $>$ $3'$-dideoxy-GTP $>$ $3'$-dideoxy-UTP $>$ $3'$-dideoxy-ATP. Apparent steady-state $K_m$ and $k_{cat}$ values for incorporation of $2',3'$-dideoxynucleotides and $3'$-dideoxynucleotides indicated incorporation of all six analogs, albeit with varying efficiencies. Dideoxynucleotides and d4t-Tp were utilized by pol γ in vitro as efficiently as natural deoxynucleotides, whereas AZT-TP, 3TC-TP, and CBV-TP were only moderate inhibitors of DNA chain elongation. Inefficient excision of dideoxynucleotides, D4T, AZT, and CBV from DNA predicts persistence in vivo following successful incorporation. In contrast, removal of 3'-terminal 3TC residues was 50% as efficient as natural 3'- termini. Finally, we observed inhibition of exonucleolytic activity by concentrations of AZT-monophosphate known to occur in cells. Thus, although their greatest inhibitory effects are through incorporation and chain termination, persistence of these analogs in DNA and inhibition of exonucleolytic proofreading may also contribute to mitochondrial toxicity.

More than 36 million people are infected by the human immunodeficiency virus worldwide, where 5.3 million new infections occurred during 2000 (1). Although antiviral therapy effectively extends the life of individuals, the death toll continues to rise; 3 million people, the highest number since the epidemic began, died from AIDS in 2000 (1). Nucleoside analogs utilized in antiviral therapy are readily incorporated into DNA by the HIV-1 reverse transcriptase. Although viral replication is effectively delayed cytotoxicity with a concomitant loss of mitochondrial DNA (8), indicating the cellular target is likely mitochondrial DNA replication. Treatment of human CEM cells with ddC results in delayed cytotoxicity with a concomitant loss of mitochondrial DNA (8), indicating the cellular target is likely mitochondrial DNA replication. Treatment of human CEM cells with ddC, D4T, and ddI results in a significant decrease of mtDNA and ultrastructural changes of the mitochondria (9). Both AZT and ddC treatment result in depletion of mitochondrial DNA, and both drugs have been shown to cause an increase in mtDNA deletions (10).

Mitochondrial DNA is replicated by an assembly of proteins and enzymes including DNA polymerase γ, single-stranded DNA-binding protein, DNA helicase, multiple transcription factors, and a number of accessory proteins (11). In vitro analysis from several laboratories has demonstrated that among the cellular replicative DNA polymerases, the mitochondrial DNA polymerase γ is the enzyme most sensitive to the antiviral nucleotide analogs currently approved to control HIV-1 infection (12–21).

As a thymidylate analog, AZT-TP is a competitive inhibitor for dTTP with pol γ (16). Partially purified human DNA pol γ is strongly inhibited by dideoxynucleotide triphosphates and D4T-TP, whereas AZT-TP, 3TC-TP, and CBV-TP inhibit pol γ to a lesser but significant degree (13). Purified recombinant yeast pol γ can readily incorporate dideoxynucleotides and dideoxyCTP, but this enzyme is less efficient in the incorporation of AZT (18). 3TC-TP has also been shown to be a substrate for human DNA pol γ as well as for HIV-RT (22). These results clearly show that pol γ is a primary cellular target for analog-induced mitochondrial toxicity. This acquired mitochondrial toxicity may be caused by 1) direct inhibition of DNA pol γ without incorporation, 2) chain termination by incorpor-
ration of these analogs into mitochondrial DNA by DNA pol γ, 3) alteration of the fidelity of DNA synthesis by pol γ, 4) the persistence of these analogs in mtDNA due to inefficient excision, or 5) any combination thereof. To understand the mechanism of this acquired mitochondrial toxicity, a thorough understanding of the interaction of nucleotides and analogs with pol γ is needed. Such information may help in the design of nucleotide analogs that selectively inhibit the HIV reverse transcriptase without inducing mitochondrial dysfunction.

We and others have cloned and overproduced the catalytic subunit of human DNA polymerase γ in insect cells via a recombinant baculovirus (23–25). In this report, we have determined the insertion efficiency of the currently approved anti-HIV analogs into DNA by purified recombinant human DNA polymerase γ and we have investigated the efficiency of removing these analogs from DNA by the intrinsic 3′-5′ exonuclease activity of pol γ.

**EXPERIMENTAL PROCEDURES**

**Materials—**Poly(rA)oligo(dT)$_{12-18}$, dideoxynucleoside triphosphates, dNTPs, and radioisotopes ([$\gamma$-$^{32}$P]dTTP, [α-$^{32}$P]dGTP, [α-$^{32}$P]dCTP, and [γ-$^{32}$P]ATP) were from Amersham Pharmacia Biotech. Oligonucleotides were purchased from Oligos Etc. or Life Technologies, Inc. DTMP and dTDP were purchased from United States Biochemical Corp. AZT-MP, AZT-DP, and AZT-TP were purchased from Moravek. CBV-TP and the minus endonuclease of 3TC-TP were generous gifts from GlaxoWellcome.

**D4T-TP was a gift from Triangle Pharmaceuticals, Inc.**

**Purification of Recombinant pol γ Enzymes—**The recombinant wild type histidine-tagged human DNA polymerase γ (wild type pol γ) was purified from baculoviral-infected insect cells as described (24). To make the histidine-tagged exonuclease-deficient DNA pol γ (Exo'-pol γ), the wild type BamHI-NotI fragment of pHuRTQ22 (24) was replaced with BamHI-NotI fragment of Exo'100/103huPV. The EcoRI-NotI fragment of the resulting plasmid was then inserted into the baculovirus transfer vector pVL1393 and resulting recombinant baculovirus, Exo'pQSVL11.4, selected. The Exo pol γ was purified from Exo'pQSVL11.4 baculoviral infected cells like the wild type pol γ.

**Polymerase Assays—**Reverse transcriptase activity of pol γ was determined using pol(rA)oligo(dT)$_{12-18}$ in reactions (50 μl) containing 25 mM Hepes-OH, pH 8.0, 1 mM 2-mercaptoethanol, 50 μg/ml acetylated bovine serum albumin, 5 μM MnCl$_2$, 25 μM [α-$^{32}$P]dTTP (2000 cpm/ pmol), 75 μM NaCl, 50 μg/ml poly(rA)oligo(dT)$_{12-18}$, and 2.5 ng of pol γ as described (24). One unit is the amount of enzyme required to catalyze the incorporation of 1 pmol of DTMP into trichloroacetic acid-pelletable DNA in 1 h at 37 °C using poly(rA)oligo(dT). Inhibition of reverse transcriptase activity of pol γ by antiviral nucleotides was determined in this standard assay in the presence of 0.2–1000 nM ddTTP or d4T-TP, or 0.2–438 μM AZT-TP.

**Exonuclease activity was determined by incubating 0.5 pmol of 5′-$^{32}$P-end-labeled 18-mer with 10, 40, or 120 ng of wild type pol γ or Exo'pol γ in 25 mM Hepes-OH, pH 7.5, 5 mM 2-mercaptoethanol, 1 μg of acetylated bovine serum albumin, and 5 mM MgCl$_2$ at 37 °C for 30 min.** Reaction was terminated at 90 °C for 3 min in 10 μl of formamide loading dye (95% deionized formamide, 0.1% w EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol). The reaction products were separated on a 20% polyacrylamide-urea gel, and products were visualized and quantified with a Molecular Dynamics PhosphorImager Storm860 and NIH Image 1.61 software.

**Incorporation of Antiviral Nucleotide Analogs into DNA by pol γ—**The kinetics of antiviral nucleotide analog insertion into DNA by Exo'pol γ was measured using the gel-based oligonucleotide extension assay (28, 27) as modified for incorporation of antiviral nucleoside analogs (17). The primer-template sets used for each type of analog follow.

**TTCP analog primer-template set:***

18-mer: 5′-TGG CAT TGG TCC TCT CTT ATT GCT TCT TTC TCT ACT-3′
36-mer: 5′-ACT GGT AGA TTT TCT CTT TCT TTC TCT TCT TCT-5′

**ddCTP analog primer-template set:***

18-mer: 5′-TGG CAT TGG TCC TCT CTT ATT GCT TCT TTC TCT ACT-3′
36-mer: 5′-ACT GGT AGA TTT TCT CTT TCT TTC TCT TCT TCT-5′

**dGTP analog primer-template set:***

18-mer: 5′-TGG CAT TGG TCC TCT CTT ATT GCT TCT TTC TCT ACT-3′
36-mer: 5′-ACT GGT AGA TTT TCT CTT TCT TTC TCT TCT TCT-5′

SEQUENCES 1–3

The positions for analog insertion are in bold type. The gel-purified 18-mer was labeled at the 5′ end with [γ-$^{32}$P]ATP and annealed in a 1:1.4 ratio to the respective templates in 10 mM Tris-HCl, pH 7.5, by heating to 90 °C for 5 min followed by slow cooling to room temperature. One pmol of primer-template ([γ-$^{32}$P]-end-labeled primer) was incubated with 1.4 ng of Exo'pol γ (10 fmol of enzyme) in a 10-μl reaction containing 25 mM Hepes-OH, pH 7.5, 2 mM 2-mercaptoethanol, 0.1 mM EDTA, 5 mM MgCl$_2$, 50 μg/ml acetylated bovine serum albumin, and variable dNTP concentrations (3 nM to 1.0 mM). Reactions were incubated at 37 °C for 10 min and stopped on ice by the addition of 10 μl of formamide loading dye. The products were separated on a 15% polyacrylamide-urea gel and visualized as described above. Ten fmol of 3′-$^{32}$P-end-labeled 38-mer was added to all reactions and used to normalize the products from gel loading error.

**Exonucleolytic Removal of Antiviral Analogs from the 3′-Terminus of DNA—**To produce single-stranded strands for exonuclease assay with various antiviral analogs at the 3′ terminus, the 18-mer primer was annealed on the designated template promoter with T4 polynucleotide kinase and then extended at the 3′ terminus with the different analogs using terminal deoxynucleotidyltransferase (TdT) in one-Phor-all buffer (Amersham Pharmacia Biotech). The products of the reaction were desalted and purified on a Sephadex G-25 column followed by centrifugation in a Microcon-3 microconcentrator. Purified HIV-RT was used to label the 3′ termini of the 18-mer in the 18/36-mer duplex with analog by incubating with 1 μM of the indicated analog triphosphate for 1 h at 37 °C. The reactions were heat-inactivated and duplex DNA purified on a Sephadex G-25 spin column and washed in a Microcon-3 microconcentrator with six volumes of distilled H$_2$O. To examine pol γ exonuclease activity with these analog-containing primer-templates, 0.2 pmol of 3′-$^{32}$P-end-labeled oligonucleotide containing the designated analog at the 3′ end was incubated with 70–840 fmol of wild type pol γ, as indicated. The exonuclease activity was carried out in 35 mM Hepes-OH, pH 7.5, 13 mM KCl, 1.3 mM DTT, and 3.3 mM MgCl$_2$ at 37 °C for 30 min. The products were separated by denaturing PAGE and visualized as described above.

**Inhibition of Exonuclease Activity of pol γ by AZT Mono- and Diphosphate—**To determine the inhibition of pol γ exonuclease activity by AZT mono- and di-phosphate, 21 fmol of wild type pol γ was incubated with 0.5 pmol of 3′-$^{32}$P-end-labeled 18-mer primer in a reaction containing 25 mM Hepes-OH, pH 7.5, 50 μg/ml acetylated bovine serum albumin, 2 mM 2-mercaptoethanol, 0.01 mM EDTA, and 10 mM MgCl$_2$ at 37 °C for 15 min in the presence of nucleotide mono- or diphosphate, as indicated. The reaction was stopped by heat denaturation and separated on a gel as described above.

**RESULTS**

We sought to identify the mechanisms by which AZT-TP, ddCTP, 3TC-TP, D4T-TP, and carbovir-TP inhibit the human DNA polymerase γ. All of these analogs lack the 3′ hydroxyl group and consequently act as chain terminators once incorporated into DNA. For comparison and reference the structures of these analogs are shown in Fig. 1. We used the purified recombinant human DNA polymerase γ overproduced in baculovirus-infected insect cells. This recombinant DNA polymerase γ and an exonucleolytic-deficient catalytic subunit has been characterized previously in our laboratory and shown to possess polymerase properties identical to the native catalytic subunit of DNA polymerase γ (24). To simplify the analysis of analog incorporation into DNA, without the complication of proofreading, we generated histidine-tagged exonuclease-deficient DNA polymerase. The specific polymerase activity of both the wild type and exonuclease-deficient histidine-tagged pol γ was 32 units/ng in the pol(γ)oligo(dT) assay (data not shown). We first addressed the inhibition and incorporation of these antiviral nucleotide analogs into DNA, and then we tested the efficiency of excising analogs from DNA by the 3′-5′ exonuclease activity.
Inhibition of Human DNA pol γ by Antiviral Nucleotide Analogos—As a first approximation, the IC₅₀ values for inhibiting DNA synthesis were determined with two different assays. We designed these assays to specifically measure the ability of analogs to inhibit the incorporation of the cognitive nucleotide. First, the incorporation of a single normal α-³²P-labeled dNMP into an 18/36-mer primer-template was assayed in the presence of increasing concentrations of competing antiviral nucleotide analog. Inhibition was monitored by gel electrophoresis and quantified to determine the IC₅₀ concentrations. Graphical results for all five analogs are shown in Fig. 2A. These results demonstrated that both dideoxycytidine and D4T-TP had strong inhibition profiles, whereas AZT-TP, 3TC-TP, and CBV-TP showed modest inhibition. The IC₅₀ for ddNTP and D4T-TP was 8 and 20 μM, respectively, while 3TC-TP and CBV-TP had an IC₅₀ of 80 μM. The analog AZT-TP had an IC₅₀ of 130 μM.

The severity of inhibition is better demonstrated in the second assay, which measures multiple incorporation events. Inhibition by the thymidine analogs was determined here in our standard poly(rA)·oligo(dT) assay using 25 μM dTTP. On this substrate pol γ has a Kₘ for ddTTP of 4.5 μM (28). Results were shown in Fig. 2B and demonstrate that ddTTP and D4T-TP are potent inhibitors in vitro while AZT-TP required higher levels to inhibit pol γ. Addition of AZT-TP resulted in an IC₅₀ of ~25 μM, which was also the concentration of normal dTTP in this assay. Dideoxythymidine triphosphate and D4T-TP showed IC₅₀ at ~15 and 150 nm, respectively, more than 2 orders of magnitude lower than AZT-TP. Given this relative ranking as inhibitors, we wanted to determine the mode of inhibition for each analog. We specifically wanted to determine whether chain termination was the primary mechanism of inhibition or whether inhibition of polymerase activity could occur prior to incorporation of the analog into DNA. Additionally, once incorporated into DNA how efficiently could the analogs be removed by the 3’-5’ exonuclease activity of pol γ?

Incorporation of Antiviral Nucleotide Analogos into DNA by DNA Polymerase γ—To determine relative efficiencies with which these analogs could be incorporated into DNA, we performed primer extension reactions and analyzed the products by gel electrophoresis. We used the exonuclease-deficient DNA polymerase γ in this assay to avoid degradation of the primer by the proofreading function and to simplify interpretation of results. This strategy became imperative due to the relatively high amount of enzyme and the longer incubation times required to detect incorporation with some of these analogs. Fig. 3 depicts the incorporation of dTMP, ddTMP, AZT-MP, and D4T-MP into DNA. Rate was determined as the fraction of primer extended by one nucleotide per unit time, and Michaelis-Menten kinetic constants were determined by plotting the rate as a function of nucleotide analog concentration (26). Human pol γ displayed high affinity (low apparent Kₘ) for normal nucleotides (Tables I–III), which is in agreement with other kinetic studies of pol γ (13–15, 18). All the analogs could be incorporated into our DNA substrate, but different concentrations of the analog were required. The dideoxynucleotide analogs ddCTP and ddTTP were the easiest to incorporate and had
$k_{cat}$ values similar to their normal nucleotide counterpart (Tables I–II). The apparent $K_m$ was 2–5-fold higher than the normal nucleotide. The effect of these analogs on competitive incorporation can be assessed by taking the ratio of the kinetic constants, ($f_{in}$). This value is equivalent theoretically to measurements made using competing substrates. Thus, ddTTP would get incorporated one in four incorporation events if the concentration of TTP and ddTTP were equal (Table I). The apparent $K_m$ for D4T-TP incorporation was similar to dideoxynucleotides but had a slightly decreased $k_{cat}$ (Table I).

These results predict D4T to be as inhibitory as the dideoxynucleotides. The $f_{in}$ values for these analogs determined with pol $\gamma$ followed the general trend of inhibition observed in Fig. 2. Pol $\gamma$ exerted most of its discrimination through $K_m$ effects, whereas the $k_{cat}$ was only modestly reduced for most of these analogs. Incorporation of AZT-TP, 3TC-TP, and CBV-TP required much higher concentrations than ddNTP or D4T-TP. However, apparent $K_m$ values were still in the micromolar range (Tables I–III), indicating AZT, 3TC, and CBV are only moderately incorporated as compared with

**Fig. 2. Inhibition of nucleotide insertion by antiviral nucleotide analogs.** A, graph of the inhibition of single nucleotide insertion for each of the analogs. One pmol of primer-template was incubated with 27 fmol of Exo $\gamma$ pol in the presence of 100 nM dCTP and in the presence of increasing concentration of analog as described under “Experimental Procedures.” Activity is presented as fraction of remaining activity relative to no added analog. *Filled squares*, inhibition from AZT-TP; *open circles*, inhibition from 3TC-TP; *filled circles*, inhibition from D4T-TP; *open squares*, inhibition from ddCTP; *filled triangles*, inhibition from CBV-TP. B, inhibition profile of pol $\gamma$ using the standard poly(rA)-oligo(dT) assay with 25 $\mu$M dTTP in the presence of the dTTP analogs. *Open squares* show the inhibition by ddTTP, *filled squares* depict the AZT-TP inhibition, and *filled circles* depict the D4T-TP inhibition.
Mechanism of Nucleotide Selectivity by DNA Polymerase γ

Table I

<table>
<thead>
<tr>
<th>Nucleoside triphosphate</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ (min$^{-1}$ μM$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>TTP</td>
<td>0.038</td>
<td>2.9</td>
<td>76</td>
</tr>
<tr>
<td>ddTTP</td>
<td>0.060</td>
<td>1.1</td>
<td>18</td>
</tr>
<tr>
<td>AZT-TP</td>
<td>94</td>
<td>0.56</td>
<td>0.006</td>
</tr>
<tr>
<td>D4T-TP</td>
<td>0.05</td>
<td>0.65</td>
<td>13</td>
</tr>
</tbody>
</table>

* $f_{in} = (k_{cat}/K_m)(h_{cat}/K_m$(analog)).

Table II

<table>
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<tr>
<th>Nucleoside triphosphate</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ (min$^{-1}$ μM$^{-1}$)</th>
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<tr>
<td>dCTP</td>
<td>0.025</td>
<td>2.9</td>
<td>116</td>
</tr>
<tr>
<td>ddCTP</td>
<td>0.130</td>
<td>2.1</td>
<td>16</td>
</tr>
<tr>
<td>3TC-TP</td>
<td>10.8</td>
<td>0.11</td>
<td>0.011</td>
</tr>
</tbody>
</table>

* $f_{in} = (k_{cat}/K_m$(dCTP))(h_{cat}/K_m$(analog)).

Table III

<table>
<thead>
<tr>
<th>Nucleoside triphosphate</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ (min$^{-1}$ μM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dGTP</td>
<td>0.020</td>
<td>2.8</td>
<td>143</td>
</tr>
<tr>
<td>CBV-TP</td>
<td>4.3</td>
<td>0.24</td>
<td>0.056</td>
</tr>
</tbody>
</table>

* $f_{in} = (k_{cat}/K_m$(dGTP))(h_{cat}/K_m$(CBV)).

dideoxynucleotides and D4T. The dCTP analog, 3TC-T, had the lowest $k_{cat}$ as well as a high apparent $K_m$ (Table II).

Exonucleolytic Removal of Antiviral Analogs from DNA by DNA Polymerase γ—The inhibitory effect of a chain terminator is limited by its ability to persist in DNA once incorporated. The persistence of all of these analogs in DNA has largely been ignored in the literature. This concept is critical to the understanding the toxicity because, even though many of these analogs are only moderate inhibitors in vitro and are not readily incorporated, their resistance to exonucleolytic removal increases their ability to thwart DNA replication and presumably increase cytotoxicity. Since DNA polymerase γ has an intrinsic 3'→5' exonuclease function, we investigated whether human DNA polymerase γ could remove these antiviral nucleotide analogs from DNA termini. Single-stranded DNA substrates bearing the analog at the 3' terminus were constructed with TdT. Since TdT did not effectively add AZT-MP to the ends of DNA we used HIV-1 reverse transcriptase to insert AZT-MP onto the 3' end of the 18/36-mer substrate. The exonuclease activity by pol γ on this dsDNA substrate was compared with the degradation of the normal 18/36-mer substrate, as well as a 19/36-mer dsDNA bearing either D4T-MP or ddTMP. At an equal molar ratio of wild type pol γ and either ssDNA or dsDNA, we observed efficient exonucleolytic removal of normal nucleotides, but very little detectable removal of the analogs with the exception of 3TC. At this stoichiometric level of polymerase and substrate, 10–20% of the 3TC was removed from the 3' terminus as compared with the control. Pol γ did not remove detectable amounts of the other analogs at these enzyme concentrations (data not shown). However, when enzyme concentrations exceeded substrate concentrations, we detected removal of the analogs from the 3' terminus (Fig. 4A). A 1.3-fold molar excess of enzyme was only able to remove 10% or the terminally incorporated analogs for single or double-stranded substrate. The exception was 3TC, where >50% of the analog was removed in 30 min (Fig. 4B). The remaining terminally incorporated analogs required a 3–4-fold molar excess of pol γ to remove >50% in 30 min.

AZT-monophosphate Inhibits pol γ Exonuclease Function—Through uptake and phosphorylation, AZT-MP is known to accumulate in millimolar concentrations in cells (29, 30). Since normal deoxynucleoside monophosphates inhibit the 3'→5' exonucleolytic activity by product inhibition, we tested the ability of the mono- and diphosphate forms of AZT to inhibit the exonuclease activity of pol γ. Inhibition of exonuclease digestion of the 18-mer by increasing concentrations of TMP, AZT-MP, TDP, or AZT-DP was monitored by gel electrophoresis. The fraction of 18-mer was plotted for the indicated concentration of each analog (Fig. 5). The TMP inhibited the human pol γ exonuclease activity at similar concentrations to what has been observed for the inhibition of Drosophila pol γ by AMP (31). AZT-MP and TMP inhibited pol γ exonuclease at similar concentrations, indicating an IC$_{50}$ of 2.5 μM. AZT-DP and TDP caused some inhibition of exonuclease but only at high concentrations. AZT-TP and TTP did not inhibit the exonuclease activity in this assay (data not shown). Thus, the inhibition of exonuclease activity was specific to either dTMP or AZT-monophosphate and occurred at concentrations similar to those observed in vivo (29, 30).

DISCUSSION

Antiviral nucleoside analogs have been implicated to cause mitochondrial toxicity in patients being treated for the HIV-1 viral infection. The cause of this toxicity is the inhibition or perturbation of mitochondrial DNA synthesis. We sought to determine the mode of inhibition of DNA polymerase γ, the only known polymerase in animal cell mitochondria. We found that all of the currently approved antiviral nucleoside analogs that we tested were incorporated into DNA by pol γ, and all inhibited DNA synthesis by pol γ in vitro to varying degrees. The dideoxynucleoside triphosphates and D4T-TP were incorporated most readily into DNA by pol γ and also exerted the strongest inhibition.

Pol γ was able to incorporate the analogs in the following order of efficiency: ddNTP > D4T-TP > CBV-TP > 3TC-TP > AZT-TP. Our data indicate that 3TC-TP was one of analogs least likely to be incorporated and yet was one of those most efficiently removed. Taken together, this may explain the low
with a glutamic acid side chain (Glu-200 in human pol)
the exonucleolytic mechanism by forming a hydrogen bond
the 3'-OH group of the terminal nucleotide plays a key role in
9
release of the terminal nucleoside monophosphate. We sought
ester bond, inverting the configuration of the phosphate to
5-cation analog substrate, respectively).
B
280 fmol of wild type enzyme in 30 min.
9
152 fmol of 3'
or single-stranded substrates represents
9
for wild type with either double-stranded
18/36-mer primer-template. 100% activity
enzyme.
Open bars
9
280 fmol of wild type pol γ for the 3'-terminal analog substrates (lanes 0–3 under the indicated analog substrate, respectively).
B, graph of the relative efficiency of exonucleolytic removal of antiviral analogs from the 3' terminus of DNA with 280 fmol of enzyme. Open bars represent the exonuclease activity on the single-stranded oligonucleotide substrates, and filled bars represent the exonuclease activity with the double-stranded primer-template substrates. Activities on the single-stranded and double-stranded primer-templates were normalized to wild type pol γ activity with the control 18-mer or 18/36-mer primer-template. 100% activity for wild type with either double-stranded or single-stranded substrates represents 152 fmol of 3' terminus degraded with 280 fmol of wild type enzyme in 30 min.

mitochondrial toxicity induced by 3TC in vivo. The low incorporation of 3TC may also be attributed to its existence as the
(−)-isomeric form. Our data also suggest that AZT-TP is the least likely to be incorporated into DNA by pol γ but once incorporated, it was not efficiently removed from DNA. The inefficiency of pol γ to remove AZT from DNA may help to explain the AZT-induced mtDNA depletion observed in vivo. The largest determinant in pol γ discrimination against these analogs, especially AZT, was through apparent Km effects with only modest changes in kcat. Although the exact level of AZT-TP in mitochondria is unclear, it appears that the concentration required for incorporation can be obtained in vivo (29, 30).

Exonucleolytic Removal of Analogs from DNA—Human DNA polymerase contains an intrinsic 3'-5' exonuclease active site in the 140-kDa polypeptide (23, 24). DNA polymerase γ is a family A polymerase, which is best typified by the Escherichia coli pol I (32). Mutagenesis studies and the three-dimensional structure of the E. coli pol I exonuclease active site show that the 3'-OH group of the terminal nucleotide plays a key role in the exonucleolytic mechanism by forming a hydrogen bond with a glutamic acid side chain (Glu-200 in human pol γ) facilitating this residue to make an ionic bond to the catalytic Mg2+ and a hydroxide anion (33–35). Stabilized by the metal cation, the activated hydroxide anion attacks the phosphodiester bond, inverting the configuration of the phosphate to release of the terminal nucleoside monophosphate. We sought to determine if the absence of the hydroxyl group in these chain terminators would affect their excision. We found that the pol γ exonuclease was inefficient at removing these chain terminators at molar equivalents and removal required a 3–4-fold molar excess of polymerase over 3' termini. Excision of ddNMP and other analogs from the 3' terminus by yeast and porcine DNA pol γ has also been shown to be inefficient when enzyme is the limiting component in the reaction (18, 36). The exception was the excision of 3TC, which was only 2-fold less efficient than a normal nucleotide with our highly active pol γ. Gray and colleagues (22) have also demonstrated that 3TC-MP can be removed from the 3' terminus of DNA, but only after 2 h of incubation. 3TC is in the (−)-enantiomer form; therefore, the possibility exists for the ribose oxygen to maintain hydrogen bonding with the active site glutamic side chain through a water molecule, accounting for its efficient excision. Due to the role of the 3' OH in the exonucleolytic catalysis, the inefficient excision of chain terminators by polymerases with intrinsic exonucleases may be a general phenomena. Indeed, the nuclear DNA polymerases δ and ε have also been shown to remove AZT-MP inefficiently from the 3' termini (19). Clearly, the 3'-azido group in AZT cannot substitute for the 3'-OH group in H-bonding Glu-200. The monophosphate inhibition experiment shown in Fig. 5 suggests, at least with AZT, that these analogs bind with similar affinity in the exonuclease active site. Thus, the 3'-azido group may interfere sterically with the ability of...
the exonuclease-deficient yeast and mouse DNA polymerase γ 20-fold decrease in replication fidelity inactivation of exonuclease activity results in as much as a 4-fold decrease in replication fidelity. The exonuclease activity of pol γ was determined by incubation of 0.5 pmol of 32P-end-labeled 18-mer with 21 fmol of pol γ and increasing amount of nucleoside mono- or diphosphate. 100% activity represented 0.4 pmol of 32P end removed in 15 min with no added mono- or diphosphate. Open and filled squares depict the inhibition with TMP and TDP, respectively; open and filled circles depict the inhibition with AZT-MP and AZT-DP, respectively. The concentrations of nucleoside mono- or diphosphate are indicated in the x axis.

Glu-200 to hydrogen bond with the attacking OH anion, and provide added resistance to exonucleolytic cleavage.

Besides the antiviral nucleoside triphosphates, the precursor forms of these analogs have also been implicated in altering cellular DNA replication (37, 38). Furman et al. measured the intracellular concentrations of AZT and the various phosphorylated forms. They found that AZT accumulates at high level in the monophosphate form (>1 mM), whereas the concentration of the triphosphate was found to be only 2 μM (29, 30). We found that AZT-MP inhibited the exonuclease function as efficiently as its normal counterpart, dTMP. This was surprising, since AZT was the least favored substrate to be removed from DNA, and suggests that free AZT-MP or terminally incorporated AZT binds readily in the exonuclease active site preventing efficient catalysis. Thus, intracellular levels of AZT-MP are expected to inhibit the exonuclease activity of mitochondrial DNA polymerase and possibly lower its fidelity. Although AZT-MP has been shown to inhibit the exonuclease function of DNA polymerase δ (38) and SV40 replication in vitro (37), it was not shown to increase the mutation frequency in the SV40 replication-fidelity assay (37). However, this assay was not specific for pol γ and did not score mutations in mitochondrial DNA. Inhibition of pol γ proofreading by monophosphates or inactivation of exonuclease activity results in as much as a 20-fold decrease in replication fidelity in vitro (39, 40). In vivo, the exonuclease-deficient yeast and mouse DNA polymerase γ transgenes have conferred a mutator phenotype in mitochondrial DNA (41, 42). Thus, inhibition of pol γ exonuclease function by AZT-MP is likely to result in mutations within the mitochondrial DNA. Mutations in mitochondrial DNA cause a wide range of mitochondrial diseases due to the resulting defects in oxidative phosphorylation (43, 44). When oxidative phosphorylation is disrupted electrons can leak into the mitochondrial matrix, react with oxygen, forming reactive oxygen species (45). We find it intriguing that both an increase in reactive oxygen species and oxidative DNA damage have been noted in patients treated with AZT (46, 47). AZT incorporation and chain termination may also produce reactive oxygen species as a result of mtDNA depletion and loss of oxidative-phosphorylation function. Interestingly, AZT has been shown to be mutagenic in animal and cellular models (48–54). Future studies of the fidelity of pol γ in the presence of nucleoside monophosphate analogs may offer insight into the mechanism of AZT induced mitochondrial toxicity seen in treated patients.

Relevance to Clinical Symptoms and Observed Mitochondrial Toxicity—Acquired mitochondrial toxicity in patients taking antiviral nucleoside analogs is generally accepted to occur as a consequence of incorporation into mitochondrial DNA and/or inhibition of mitochondrial DNA replication (55). This toxicity requires five steps to present a clinical phenotype: uptake of analog into the cell, conversion to the triphosphate form, transport into the mitochondria, incorporation into mitochondrial DNA, and persistence in mitochondrial DNA. The transport of these analogs into the mitochondria may occur before or after phosphorylation, but one study suggests that intramitochondrially phosphorylated analogs are preferentially incorporated into DNA (56). In contrast to this finding, another study suggests that dDC is phosphorylated in the cytoplasm and transported into mitochondria prior to exerting its inhibitory effect on mtDNA synthesis (57). Our present study focused only on the latter steps leading to toxicity: the incorporation, inhibition, and persistence in DNA. If all of the analogs we studied showed equivalent cellular adsorption, phosphorylation and transport, then we could draw a direct correlation with cellular toxicity. However, the intramitochondrial concentration and phosphorylation state of these analogs is unclear. One of these analogs, 3TC, may not be transported efficiently into the mitochondria and may even block transport of other dCTP analogs (58–61). For carbovir, no mitochondrial toxicity has been observed in tissue culture cells (62), and it remains to be seen whether it will cause any mitochondrial toxicity in patients. Our results suggest that the observed AZT toxicity in vivo may be the combined effect of moderately efficient incorporation and very inefficient removal, resulting in persistence in mtDNA. Additionally, a high in vivo concentration of AZT-MP may inhibit pol γ proofreading, thereby causing an increase in mtDNA mutations. Our data suggest that, unlike AZT, the cytotoxicity from dideoxynucleosides and d4T is primarily due to incorporation and persistence in mtDNA.

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