Interaction of 2′-deoxyguanosine triphosphate analogue inhibitors of HIV reverse transcriptase with human mitochondrial DNA polymerase γ

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Mitochondrial toxicity is a limiting factor in the use of some nucleoside reverse transcriptase inhibitors of HIV. To further understand the impact of structural features on the incorporation and exonuclease removal of nucleoside monophosphate (MP) analogues by human mitochondrial DNA polymerase (pol γ), transient kinetic studies were done with analogues of 2′-deoxyguanosine triphosphate. The kinetic parameters for the incorporation and removal of carbovir (CBV)-MP, dioxolane guanosine (DXG)-MP and 2′,3′-dideoxy-2′,3′-didehydroguanosine (d4G)-MP were studied with pol γ holoenzyme. The importance of the ribose oxygen in incorporation by pol γ was illustrated by an approximate 3,000-fold decrease in the incorporation efficiency of an analogue lacking the ribose oxygen (CBV-TP) relative to those containing a ribose oxygen (DXG-TP and d4G-TP). As a result, a comparison with previous data for the incorporation by HIV reverse transcriptase showed CBV-TP to be approximately 800–8,000-fold more selective for its antiviral target over pol γ relative to the other guanosine analogues. However, DXG-TP and d4G-TP were found to be much more selective than previously reported values for mitochondrial toxic nucleoside analogues. Structural modelling based on sequence homology with other polymerase A family members suggests that an interaction between the ribose oxygen and arginine 853 in pol γ may play a critical role in causing this differential incorporation. Exonuclease removal of a chain-terminating CBV-MP was also found to be more efficient by pol γ. These results help to further elucidate the structure activity relationships for pol γ and should aid in the design of more selective antiviral agents.

Keywords: abacavir, cyclo-d4G, DAPD, HIV, mitochondrial DNA polymerase γ

Introduction

Despite the success of antiviral therapies at decreasing the morbidity and mortality associated with HIV infection, antiviral treatment is still limited by drug interactions (Back et al., 2005; Ray, 2005), development of resistance (Larder, 1995; Naeger & Miller, 2001; Ray et al., 2002b) and toxic side effects (Nolan & Mallal, 2004). Mitochondrial toxicity has been identified as a key mediator of many unwanted side effects associated with nucleoside/tide reverse transcriptase inhibitors (NRTIs; Parker & Cheng, 1994; White, 2001).

The 2′-deoxyguanosine triphosphate (dGTP) nucleotide analogues carbovir-triphosphate (CBV-TP), dioxolane guanosine ([2R,4R]-4-(2-amino-6-oxo-9H-purin-9-yl)-1,3-dioxolan-2-yl)methanol-triphosphate; DXG-TP) and 2′,3′-dideoxy-2′,3′-didehydro guanosine-triphosphate (d4G-TP, structures shown in Figure 1) have been found to be potent inhibitors of HIV reverse transcriptase (RT; White et al., 1989; Ray et al., 2002b; Jeffrey et al., 2003). To improve pharmacokinetic parameters, six substituted prodrugs of the nucleoside forms of these analogues have been explored as drug candidates (Daluge et al., 1997; Furman et al., 2001; Ray et al., 2002a). Abacavir ((-)-(1S,4R)-4-[(2-amino-6-(cyclopropylamino)-9H-purin-9-yl)]-2-cyclopentene-1-methanol; Ziagen®), DAPD ((-)β-2,6-diaminopurine dioxolane or (2R,4R)-4-(2,6-diamino-9H-purin-9-yl)-1,3-dioxolan-2-yl)methanol; Amdoxovir®) and cyclo-d4G (9-amino-6-cyclopurapymolino-(9)-(2,3-dideoxy-β-D-glycero-pent-2-enofuranosyl) purine) are dependent on deamination as part of the meta-
bolic processes involved in the formation of their respective biologically active dGTP analogue species (Faletto et al., 1997; Furman et al., 2001; Ray et al., 2005). Abacavir has received approval by the U.S. Food and Drug Administration as part of current anti-HIV treatment regimens, and DAPD has been the subject of several clinical trials and is currently in Phase IIb.

A good correlation between incorporation by the human mitochondrial polymerase γ (pol γ) in enzymatic assays (Johnson et al., 2001; Lim & Copeland, 2001; Lee et al., 2003) and depletion of mitochondrial DNA in *in vitro* whole-cell systems (Martin et al., 1997; Birkus et al., 2002; Ray et al., 2002a; Ray et al., 2005) has been observed for most NRTIs. Due to the difficulty in purifying the large quantities of pol γ needed for structural elucidation, our understanding of its active site is limited to the following: (i) analysis of sequence homology with other polymerase A (pol A) family members, (ii) mutagenesis and (iii) the structure activity relationships for the incorporation of alternative substrates. A better understanding of pol γ interaction with alternate nucleotide substrates is needed to aid in the design of antiviral therapies with improved toxicity profiles.

An important part of predicting the toxicity profile of potential NRTI drug candidates is by assessing their interaction with pol γ. Furthermore, building structure activity relationships for modified nucleotide analogues with pol γ may help in the design of less toxic and more specific inhibitors of HIV RT and other viral polymerases. Here, we used in depth kinetic analyses with purified pol γ holoenzyme to study the interaction of three dGTP analogue inhibitors of HIV RT with the pol γ polymerase and exonuclease active sites. We also employed molecular modelling based upon sequence homology and the known crystal structures of other polymerases in the same family as pol γ to provide a structural insight into our biochemical data.

**Materials and methods**

**Reagents**

dGTP was purchased from Amersham Biosciences (Pittsburgh, PA, USA). Redivue [γ-32P]ATP was obtained from Amersham Biosciences. d4G-TP and DXG-TP were synthesized as previously described (Furman et al., 2001; Ray et al., 2002b). Oligonucleotides used as primers and templates were obtained from the Keck DNA synthesis facility at Yale University and purified using 20% polyacrylamide denaturing gel electrophoresis.

**Purification of pol γ and its accessory subunit.**

Wild-type and exonuclease-deficient pol γ catalytic subunits were expressed using a baculovirus expression system in insect Sf9 cells essentially as previously described (Graves et al., 1998). An accessory subunit to pol γ has been identified (Wang et al., 1997) and shown to markedly alter the kinetics of incorporation. Over-expression of the accessory subunit of pol γ was carried out as described (Carrodeguas et al., 1999; Johnson et al., 2000). The holoenzyme was reconstituted by incubating a 1:5 mixture of the catalytic and accessory subunits (between 50–100 nM and 250–500 nM, respectively) on ice for 10 min.

**DNA prime-templates**

Studies were done using the following 30-mer DNA primer and 45-mer DNA template: 5′-GGCTCGACGCGGC-GCGCAGCTC-CACAAAACTCAACCTC-3′ and 3′-CGGAGCGGTCGGCAGGTTGGTTGAGCATTAGTTACGGCAG-5′.

The dCMP templating dGMP incorporation is highlighted in bold italics. The 5′-end of the primer was radio-labelled using T4 polynucleotide kinase (obtained from New England Biolabs, Ipswich, MA, USA) and [γ-32P]ATP as previously described (Kati et al., 1992). The primer/template was annealed by adding a 1:1.4 molar ratio...
of radiolabelled D30-mer primer to D45-mer template at 90°C for 5 min, at 50°C for 10 min and in ice for 10 min. Complete annealing was verified by analysing an aliquot of the annealed material on a 15% non-denaturing polyacrylamide gel to insure complete annealing. For studying exonuclease removal, primers were generated containing a 3′-terminal DXG-monophosphate (MP) or d4G-MP as previously described (Johnson et al., 2001) using exo- polymerase γ holoenzyme and standard reaction conditions (described below). For comparison, a D31-mer primer was synthesized with a terminal dGMP to study the removal of a natural nucleotide.

**Pre-steady-state kinetic incorporation assays**

Incorporation studies were done with either the wild-type or exonuclease-deficient holoenzyme. The exonuclease-deficient enzyme has been shown to be kinetically similar during the polymerase reaction to wild type (Johnson et al., 2001) and can be used to simplify kinetic observations when slow incorporation rates are encountered. Pre-steady-state kinetics were performed using a KinTek Corporation (Austin, TX, USA) model RQF-3 rapid-quench-flow apparatus essentially as previously described (Johnson et al., 2001). Incorporation studies were done at 37°C in buffer containing 50 mM Tris-Cl (pH 7.5), 100 mM NaCl and 2.5 mM MgCl₂. Either pre-steady-state burst or single turnover conditions were used to determine the incorporation of DXG-MP or d4G-MP into the D30-mer/D45-mer primer/template. Pre-steady-state burst experiments were done with a fivefold excess of D30-mer/D45-mer (250 nM final) to pol γ holoenzyme (50 nM final). Single-turnover conditions were done using pol γ holoenzyme concentrations of 100 nM and 90 nM D30-mer/D45-mer. Reactions were initiated by rapidly mixing a solution of nucleotide and magnesium with pol γ and primer/template. Reactions were quenched at desired times by the addition of 0.3 mM ethylenediaminetetraacetic acid (EDTA). Elongated primer was separated from the substrate by gel electrophoresis and phosphorimaging as described for the polymerase reaction.

**Sequence alignment and structural analysis**

Sequences of members of the pol A family including pol γ from distant organisms were obtained from the ExPASy proteomic server (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_prosite.html). Dual and multiple protein sequence alignments were done using the SIM alignment tool (Swiss Institute of Bioinformatics; available from http://au.expasy.org/tools/sim-prot.html) and DIALIGN on the Bielefeld University Bioinformatics Server (available from http://bibiserv.techfak.uni-bielefeld.de/dialign/submission.html), respectively. Structural analysis of the pol A family active site was done for residues with observed sequence homology using the structure of the ternary complex of T7 DNA polymerase with primer-template and ddGTP (Double & Ellenberger, 1998). Structural features were viewed using Swiss PDB Viewer (GlascoSmithKline & Swiss Institute of Bioinformatics; available from http://www.expasy.org/spdbv/) and images generated using Mega-Pov (Persistence of Vision Raytracer Pty. Ltd; available from www.povray.org).

**Data analysis**

Data from incorporation experiments were fit by non-linear regression using the program KaleidaGraph (Synergy Software, Reading, PA, USA). The observed first-order rate constants for incorporation (k_{obs}) at different nucleotide concentrations were determined using the equation [D31-mer] = A[1-exp(-k_{obs}t)] for single-turnover experiments. The k_{obs} and steady-state rate (k_s) of nucleotide incorporation were determined from pre-steady-state burst experiments using the equations [D31-mer] = A[1-exp(-k_{obs}t) + k_s t]. In these equations, A represents the amplitude of product formation and t equals time. The observed first-order rate constants for each nucleotide at different concentrations were then used to determine the maximum rate of incorporation (k_{max}) and the dissociation constant for the nucleotide (K_d) by fitting the data to the hyperbolic equation k_{max} = (k_{pol}[dNTP])/(K_d + [dNTP]). The efficiency of incorporation was calculated by dividing k_{pol} by K_d. The selectivity of the enzyme for the natural dGTP substrate over the analogue was calculated by comparing the efficiency of incorporation of dGTP with that of the analogue (selectivity = efficiency_{dGTP}/efficiency_{analogue}). A comparison of the selectivity of pol γ to that of RT was then calculated (therapeutic index = selectivity_{pol}/selectivity_{HIV RT}) as previously described (Johnson et al., 2001).
Rate of exonuclease removal ($k_{\text{exo}}$) were determined by plotting the amount of full length substrate versus time and fitting the data to a single exponential decay ($[\text{D}31\text{-mer}] = A \cdot \exp(-k_{\text{exo}}t)$) for single turnover conditions or a line ($[\text{D}31\text{-mer}] = -(\text{enzyme}) [k_{\text{exo}}]t + 1,500 \text{ nM}$) for steady-state conditions. Reported error represents the deviation of points from the curve fit generated by KaleidaGraph.

**Results**

**Incorporation of the natural substrate, dGTP and its analogues by pol $\gamma$**

Pre-steady-state kinetics were used to gain an understanding of the incorporation of DXG-MP and d4G-MP (triphosphate structures shown in Figure 1) into a D30-mer/D45-mer primer/template by pol $\gamma$ holoenzyme. Data collected
incorporated approximately 3,000-fold more efficiently than the carbocyclic analogue, CBV-TP (Table 1). However, these guanosine nucleotide analogues were incorporated 278- and 282-fold less efficiently, respectively. Dideoxyguanosine triphosphate (dGTP), both DXG-TP and d4G-TP were found to be incorporated similarly with kpol, Kd and efficiencies of incorporation within twofold of one another. Relative to determination of kpol and Kd for both analogues (Figures 2 and 3). Results showed that DXG-TP and d4G-TP are incorporated similarly with kpol, Kd and efficiencies of incorporation within twofold of one another. Relative to DNA-directed RT incorporation of dGTP and its analogues, an analysis of the selectivity for their viral target relative to polγ was obtained (which was calculated as a therapeutic index). Despite similar incorporation by polγ, it was found that d4G-TP was 2- and 8.5-fold more selective for HIV RT than DXG-TP during DNA- and RNA-directed RT incorporation, respectively. The enhanced selectivity of d4G-TP was due to its improved incorporation efficiency by HIV RT. The selectivity of both DXG-TP and d4G-TP were much less than that observed for CBV-TP (Table 2).

Pre-steady-state burst and single turnover experiments allowed for measurement of incorporation rate at different concentrations of DXG-TP and d4G-TP. Plotting the observed rate (kobsd) versus DXG-TP or d4G-TP concentration, and fitting to a hyperbolic equation led to the determination of kpol and Kd for both analogues (Figures 2 and 3). Results showed that DXG-TP and d4G-TP are incorporated similarly with kpol, Kd and efficiencies of incorporation within twofold of one another. Relative to dGTP, both DXG-TP and d4G-TP were found to be incorporated 278- and 282-fold less efficiently, respectively. However, these guanosine nucleotide analogues were incorporated approximately 3,000-fold more efficiently than the carbocyclic analogue, CBV-TP (Table 1).

Using previously obtained kinetic values for DNA- and RNA-directed incorporation by HIV RT for DXG-MP (Jeffrey et al., 2003) and d4G-MP (Ray et al., 2002b), their selectivity for their viral target relative to polγ was obtained (which was calculated as a therapeutic index). Despite similar incorporation by polγ, it was found that d4G-TP was 2- and 8.5-fold more selective for HIV RT than DXG-TP during DNA- and RNA-directed RT incorporation, respectively. The enhanced selectivity of d4G-TP was due to its improved incorporation efficiency by HIV RT. The selectivity of both DXG-TP and d4G-TP were much less than that observed for CBV-TP (Table 2).

**Active site residues involved in differential incorporation of dGTP analogues by polγ**

To understand the structural basis for differences in incorporation of dGTP and its analogues, an analysis of the polγ active site based on homology modelling and protein sequence alignment with other pol A family members with available structural information was used. Previous sequence homology and mutagenesis studies have shown a similar role for residues in the O-helix of pol A family members including polγ (Ponamarev et al., 2002; Graziewicz et al., 2004). The similar incorporation of DXG-MP and d4G-MP, despite differential modification of the 2’ and 3’ positions of the ribose ring, and the markedly decreased incorporation of the carbocyclic analogue CBV-TP led us to look for differential contacts between active site residues and the ribose oxygen. Polymerase structures have shown a general topology

### Table 1. Kinetic parameters for the incorporation of dGTP analogues into DNA/DNA 30/45-mer primer/template by mitochondrial polγ

<table>
<thead>
<tr>
<th>dNTP</th>
<th>kpol, s⁻¹</th>
<th>Kd, μM</th>
<th>Efficiency, μM⁻¹s⁻¹</th>
<th>Selectivity¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>dGTP‡</td>
<td>0.44 ±0.10</td>
<td>0.07 ±0.09</td>
<td>0.45</td>
<td>278</td>
</tr>
<tr>
<td>DXG-TP</td>
<td>0.27 ±0.02</td>
<td>0.61 ±0.13</td>
<td>0.44</td>
<td>282</td>
</tr>
<tr>
<td>d4G-TP</td>
<td>0.0018 ±0.0001</td>
<td>13 ±1.5</td>
<td>0.00014</td>
<td>892,857</td>
</tr>
</tbody>
</table>

*Efficiency = maximum rate of incorporation (kpol) divided by dissociation constant for the nucleotide (Kd).* ¹Selectivity = efficiency of incorporation of dGTP/efficiency of incorporation of analogue. ‡Values reported previously (Johnson et al., 2001). CBV-TP, carbovir-triphosphate; dGTP, 2′- deoxyguanosine triphosphate; dNTP, deoxyribonucleotide triphosphate; DXG-TP, dioxolane guanosine-triphosphate; d4G-TP, 2′-dideoxy-2,3′-dideoxyguanosine-triphosphate. Polγ, polymerase γ

### Table 2. Comparison of the kinetic parameters for the incorporation of dGTP analogues by HIV-1 RT and polγ

<table>
<thead>
<tr>
<th>dNTP</th>
<th>Polγ</th>
<th>HIV RT DNA-dependent</th>
<th>HIV RT RNA-dependent</th>
<th>Therapeutic index †</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DNA-dependent</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RNA-dependent</td>
</tr>
<tr>
<td>DXG-TP</td>
<td>278</td>
<td>2.9</td>
<td>18</td>
<td>96</td>
</tr>
<tr>
<td>d4G-TP</td>
<td>282</td>
<td>1.5</td>
<td>2.2</td>
<td>188</td>
</tr>
<tr>
<td>CBV-TP</td>
<td>892,857</td>
<td>34</td>
<td>7.4</td>
<td>26,261</td>
</tr>
</tbody>
</table>

*Selectivity = efficiency of incorporation of dGTP (efficiencydGTP) divided by efficiency of incorporation of analogue (efficiencyanalogue). †Therapeutic index = selectivity of polγ-selectivity of HIV reverse transcriptase (RT). Values for HIV RT selectivity reported previously (Ray et al., 2002b; Jeffrey et al., 2003). CBV-TP, carbovir-triphosphate; dGTP, 2′- deoxyguanosine triphosphate; dNTP, deoxyribonucleotide triphosphate; DXG-TP, dioxolane guanosine-triphosphate; d4G-TP, 2′-dideoxy-2,3′-dideoxyguanosine-triphosphate; polγ, polymerase γ.
commonly described based on the analogy of a right hand with fingers, thumb and palm subdomains (Joyce & Steitz, 1994). Residues from the palm β-sheets 7 and 8, and residues from the fingers O-helix in pol A family members make contacts with the incoming nucleotide. In the T7 polymerase active site, two residues are found within 4 Å of the 4′ ribose oxygen including Glu 480, the steric gate residue known to limit rNTP incorporation in pol A family members (Astatke et al., 1998), and Arg 429 extending between β-sheets 7 and 8 (Figure 4A). Similarly, sequence alignments suggest that pol γ Glu 895 plays a homologous role as T7 Glu 480 (alignment not shown) and that pol γ residue Arg 853 plays a homologous role as T7 Arg 429 (Figure 4B).

Exonuclease excision of terminal dGMP, and its analogues by the pol γ exonuclease proofreading domain.
Pol γ has a 3′ to 5′ exonuclease domain and when considering the toxicity of chain-terminating nucleotide analogues it is important to assess the ability of pol γ’s exonuclease to remove a chain-terminator, thereby, potentially rescuing mtDNA synthesis. The removal of terminal DXG-MP and d4G-MP were studied using the D31-mer/D45-mer primer/template. As summarised in Table 3, removal rates of >1,400-, 52- and 14-fold slower than the natural analogue dGMP were observed for DXG-MP, d4G-MP and CBV-MP, respectively.
Table 3. Kinetic parameters for exonuclease removal of terminal dNMPs from DNA/DNA 30-dNMP/45-mer primer template by mitochondrial pol γ

<table>
<thead>
<tr>
<th>dGMP</th>
<th>DXG-MP*</th>
<th>d4G-MP</th>
<th>CBV-MP**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4 ±0.1</td>
<td>&lt;0.001</td>
<td>0.027 ±0.0002</td>
<td>0.098 ±0.001</td>
</tr>
</tbody>
</table>

*Rate based on the lack of cleavage product observed after a 12 h incubation. †Value reported previously (Johnson et al., 2001). CBV-MP, carbovir-monophosphate; dNMP, deoxyribonucleoside monophosphate; dGMP, 2′-deoxyguanosine monophosphate; DXG-MP, dioxolane guanosine-monophosphate; d4G-MP, 2′,3′-dideoxy-2′,3′-didehydroguanosine monophosphate; kexon rate of exonuclease removal; pol γ, polymerase γ.

Discussion

Structural studies of pol γ have been hampered by the difficulties encountered when trying to produce the large quantities of protein required for structural elucidation. A high degree of structural homology observed within the active sites of other pol A family members suggest that pol γ may have a similar active site topology (Delarue et al., 1990; Joyce & Steitz, 1994). A structural model, based upon sequence homology between pol γ and other pol A family members for which structural information is available, has allowed for many insights into residues critical in determining the substrate specificity of pol γ. For example, an understanding of the underlying molecular mechanism for a mutant form of pol γ containing a mutation of Tyr 955, which is associated with the heritable disorder progressive external ophthalmoplegia, has been established by a combination of sequence/structural homology analysis and mutagenesis. Sequence alignment showed that Tyr 955 is homologues to a residue in the O-helix that is highly conserved in pol A family members and contacts the base of the incoming nucleotide. Mutagenesis of this residue caused error prone incorporation by pol γ (Ponomarev et al., 2002; Graziewicz et al., 2004). Tyr 955 has also been implicated in the poor selectivity against incorporation of 2′,3′-dideoxynucleotides by pol γ by being able to partially compensate for the lack of a 3′-hydroxyl (Lim et al., 2003).

Here we show that pol γ incorporates a chain-terminating DXG-MP and d4G-MP more than 3,000-fold more efficiently than the carbocyclic analogue CBV-MP. The difference in the interaction of pol γ with d4G-TP and CBV-TP is especially surprising because the only difference between the two analogues is the isosteric replacement of the ribose oxygen with carbon. This result is in contrast to the smaller difference observed for HIV RT where d4G-MP is incorporated 5- to 10-fold more efficiently than CBV-MP (Ray et al., 2002b). Taken together, these results indicate that pol γ may have a specific mechanism for recognizing the ribose oxygen not present in HIV RT. Structural analysis of the region around the ribose oxygen in pol A family members with available crystallographic data showed the presence of a potential hydrogen bond with the Arg side chain arising from the linker between sheets 7 and 8 in the polymerase palm. While sequence homology is not as strong between pol γ and other pol A family members in this region as for the catalytic Asp residues and the O-helix, it is proposed that Arg 853 in pol γ may play a homologous role.

Mutation of this Arg to Ala in the Klenow fragment of DNA pol I (residue 668) causes a large decrease in the rate of deoxyribonucleoside monophosphate incorporation (Polesky et al., 1990; Polesky et al., 1992). Similarly, data generated for the incorporation of guanosine nucleotides shows a 150-fold decrease in the incorporation rate between CBV-MP and d4G-MP. Taken together these results suggest that loss of the interaction between this Arg and the ribose oxygen in Pol A family members either by mutagenesis or removal of the ribose oxygen causes a marked decrease in the rate of catalysis. Similar decreases in incorporation rates between D- and L-nucleotide analogues have been noted previously (Feng et al., 2001; Feng et al., 2004; Murakami et al., 2004) and may be related to the loss of the interaction of this Arg with the ribose ring or a steric clash. These data suggest that the poor incorporation observed for CBV-MP and L-nucleotide analogues may in part be mediated by Arg 853. Another factor that may play a role in the selectivity of pol γ for nucleotide analogues is the conformation of the ribose ring. The potential for ribose conformation to have an effect on incorporation of d4G-MP and CBV-MP by HIV RT has been discussed in detail elsewhere (Ray et al., 2002b).

An interesting parallel has been observed for some NRTIs between incorporation by pol γ and sensitivity to a specific resistance mutation within the HIV RT active site. Better substrates for pol γ including DXG-TP and d4G-TP have not been found to be affected by the Met to Val mutation at position 184 (M184V) in HIV RT, whereas exceedingly poor substrates for pol γ including CBV-TP and 3TC-TP are sensitive to the M184V mutation (Schinazi et al., 1993; Tisdale et al., 1993; Tisdale et al., 1997; Ray et al., 2002b; Jeffrey et al., 2003). The mechanism of resistance for the M184V mutation has been shown to be a steric hindrance for analogues with structural features extending beyond the normal position of the ribose oxygen in the HIV RT active site (Sarafianos et al., 1999). The similarities in HIV RT M184V resistance and pol γ incorporation further support the hypothesis that contacts around the ribose oxygen may be critical in dictating incorporation by pol γ.

Inhibition of mitochondrial DNA synthesis by chain-terminating nucleotide analogues is likely a balance...
between incorporation efficiency by the polymerase active site and removal by the exonuclease active site. As these two enzymatic activities are catalysed by distinct regions of the protein, differences in substrate specificity are likely. For example, the high mitochondrial toxicity of 2′,3′-dideoxycytidine is likely due to its efficient incorporation and poor excision, causing it to be a highly effective inhibitor of mitochondrial DNA synthesis (Feng et al., 2001; Johnson et al., 2001). Therefore, it is critical when assessing the potential for mitochondrial toxicity of a chain-terminating analogue to study both the processes of incorporation and excision. Here we demonstrated that in contrast to incorporation, CBV-MP is removed more efficiently than either d4G-MP or DXG-MP. The poor incorporation and efficient removal of CBV-MP is likely the reason for the lack of observed mitochondrial damage with abacavir.

It should be noted that while DXG-TP and d4G-TP were more efficiently incorporated and their respective monophosphates less efficiently removed by pol γ relative to CBV-TP, these dGTP analogues did not show nearly the potential for inhibition of pol γ as the triphosphate forms of NRTIs with clinically manifested mitochondrial toxicity: ddC-TP, ddA-TP or d4T-TP, shown in previous transient kinetic studies (Feng et al., 2001; Johnson et al., 2001; Lim & Copeland, 2001). Consistent with the findings from these enzyme assays, cyclo-d4G has shown reduced potential for mitochondrial DNA depletion relative to ddC and d4T (Ray et al., 2002a; Ray et al., 2005), and DAPD has shown no mitochondrial depletion in cellular assays (Furman et al., 2001). The low capacity for DAPD to cause mitochondrial damage has been further supported by findings in clinical studies (Thompson et al., 2005; Gripshover et al., 2006).

In conclusion, a study examining the incorporation of these dGTP analogues by pol γ, has implicated the importance of alterations around the ribose oxygen in dictating the efficiency of incorporation. The high sensitivity of pol γ to these structural changes is illustrated by the approximate 3,000-fold decrease in incorporation efficiency observed between DXG-TP/d4G-TP (analogues containing the ribose oxygen) and CBV-TP (an analogue lacking the ribose oxygen). It was also found that the 3′ to 5′ exonuclease possesses unique structure activity relationships and that its catalytic rate is highly sensitive to structural alterations present in these dGTP analogues. Furthermore, we suggest a key role of residue Arg 853 in substrate specificity for incorporation by the polymerase active site of pol γ. These results combined with previous observations help elucidate and extend the structure activity relationship for pol γ’s interaction with alternative substrates. These analyses serve to provide a better understanding of the mechanism of toxicity caused by some current nucleoside analogues and may potentially aid in the design of future more selective antiviral agents.

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