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Investigating the effects of stereochemistry on incorporation and removal of 5-fluorocytidine analogs by mitochondrial DNA polymerase gamma: comparison of D- and L-D4FC-TP

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Abstract

Enantiomers of β-2′,3′-didehydro-2′,3′-dideoxy-5-fluorocytidine (D/L-D4FC) are nucleoside analog reverse transcriptase inhibitors (NRTIs) currently under investigation as antiviral agents. One of the major problems of NRTIs is toxicity to mitochondria. It has been shown that mitochondrial toxicity of NRTIs can correlate with incorporation and removal of these compounds by mitochondrial DNA polymerase (Pol γ). Mechanistic studies have shown that, if activated, NRTIs are incorporated *more* efficiently by HIV-1 reverse transcriptase (RT) and *less* efficiently by Pol γ, the corresponding nucleosides are considered to be more selective. In the present study, in order to predict potential DNA Pol γ-related mitochondrial toxicity of D- and L-D4FC, the incorporation and removal of the monophosphate form of these compounds by Pol γ were studied using transient kinetic methods. Our cell-free results showed that Pol γ incorporated the natural D-isomer significantly more efficiently than the unnatural L-isomer. However, the removal rates of these enantiomers from the chain-terminated primers were almost identical. While these results suggest that D-D4FC may present more mitochondrial toxicity than L-D4FC in cell-free assays, we have previously shown that HIV-1 RT prefers D-D4FC-TP as a substrate over the L-isomer, particularly in the case of mutant forms of RT associated with nucleoside drug resistance such as M184V. Since the effectiveness of NRTIs is a balance between efficiency of incorporation by wild-type and drug-resistant forms of HIV-1 RT and mitochondrial toxicity, our kinetic results suggest that both enantiomers may show promise as potential therapeutics.

Keywords: Mitochondrial DNA polymerase; β-2′,3′-Didehydro-2′,3′-dideoxy-5-fluorocytidine; Nucleoside reverse transcriptase inhibitors; Mitochondrial toxicity; Human immunodeficiency virus (HIV)

1. Introduction

In current anti-human immunodeficiency virus (HIV) chemotherapies, several steps in HIV replication, including viral cell entry, reverse transcription, DNA integration, and proteolytic processing are targeted (De Clercq, 2002; Gulick, 2003). At the cornerstone of highly active antiretroviral therapy are the nucleoside analog reverse transcriptase inhibitors (NRTIs) which lacks a 3′-hydroxyl group on the ribose ring and, as a result, terminate elongation of DNA. However, these NRTIs often show mitochondrial toxicity which is associated with mitochondrial DNA polymerase (Pol γ) because phosphorylated NRTIs are incorporated into mitochondrial DNA (mtDNA) by Pol γ. This causes replication of mtDNA to be inhibited and can lead to mitochondrial dysfunction (White, 2001).

Both enantiomers of β-2′,3′-didehydro-2′,3′-dideoxy-5-fluorocytidine (D/L-D4FC) are currently in clinical trials targeting viral reverse transcription; D-D4FC (Reverset™, Pharmasset, Inc.) (Ma et al., 1999; Shi et al., 1999; Schinazi et al., 2002; Geleziunas et al., 2003) is in Phase II as an anti-HIV agent and L-D4FC (elvucitabine, Achillion Inc.) (Zhu et al., 1998; Le Guerhier et al., 2000, 2001; Krishnan et al., 2002) is in Phase I/II studies as an anti-HIV and -HBV agent. The structures of the triphosphate forms of the compounds are shown in Fig. 1. Recently, it has been shown at a molecular level that HIV-1 reverse transcriptase (RT) incorporates D- and L-D4FC-MP less efficiently than dCMP by 2- to 3-fold and 20- to 30-fold, respectively (Ray

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[1] Author receives royalties for the sale of 3TC and is entitled to royalties from sales and marketing of nucleosides discussed in this article (FTC, and D-D4FC) as recognition for his contribution to the discovery and development of these drugs.

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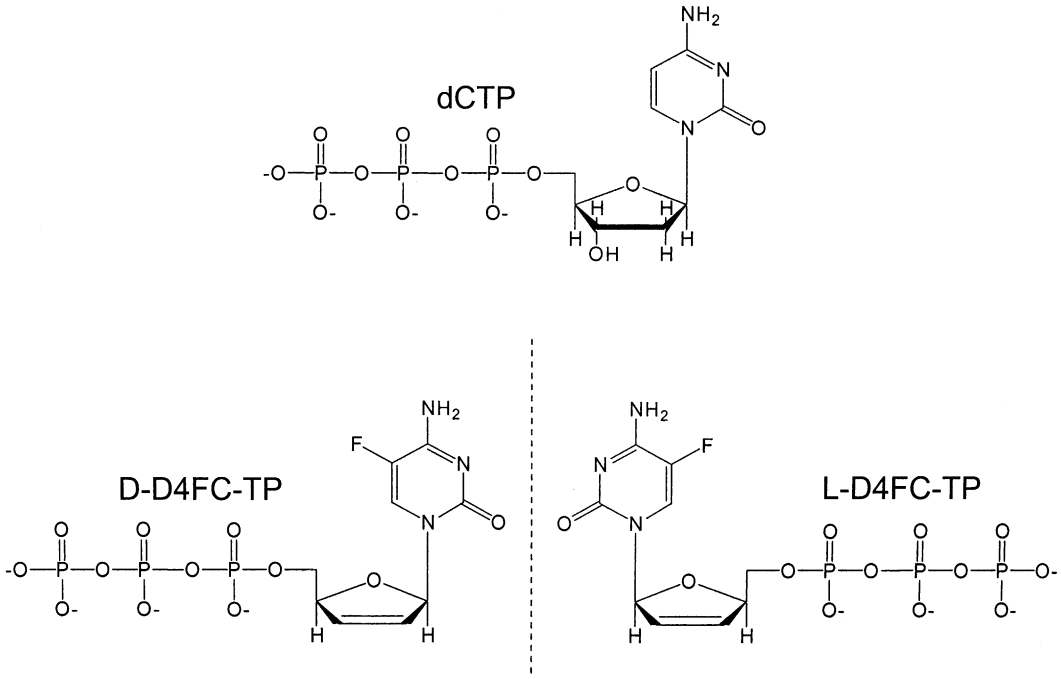

Fig. 1. Structure of dCTP, and D/L-D4FC-TP.

et al., 2002, 2003). Thus, HIV-1 RT incorporates D-D4FC-MP 10-fold more efficiently than L-D4FC-MP, suggesting that D-D4FC may exhibit higher potency against HIV than the corresponding L-isomer. However, evaluation in cell culture indicates that the potency of the two enantiomers against wild-type HIV is similar (Schinazi et al., 2002; Lin et al., 1996), suggesting that perhaps other cellular factors such as transport or metabolism of these analogs may also contribute to the antiviral efficacy (Ray et al., 2003).

Pol γ is a multifunctional enzyme which catalyzes DNA- and RNA-directed DNA synthesis, 3′ → 5′ DNA excision, apurinic-apyrimidinic lyase, and other RNA-associated reactions (Johnson and Johnson, 2001a,b; Pinz and Bogenhagen, 2000; Murakami et al., 2003). Since Pol γ is the only DNA polymerase found in mitochondria, inhibition of this enzyme could be a crucial determinant of unwanted side effects. Efficient incorporation by Pol γ leads to chain-termination, inhibition of mtDNA synthesis, and potential mitochondrial toxicity. Mechanistic studies have shown that mitochondrial toxicity from cell culture studies may be correlated with efficiency of incorporation of phosphorylated NRTIs by Pol γ (Johnson et al., 2001). Therefore, examining the effects of NRTIs on Pol γ at a molecular level offers an efficient and convenient method to understand and predict the propensity of a nucleotide to induce mitochondrial toxicity related to Pol γ.

HIV-1 RT incorporates nucleotide analogs containing an oxathiolane ring with unnatural L-configuration relatively efficiently (Feng and Anderson, 1999; Feng et al., 1999). On the other hand, Pol γ is more stereospecific and poorly incorporates unnatural L-isomers as compared with the corresponding natural D-isomers as previously shown for two sets of enantiomers of β-2′,3′-dideoxy-3′-thiacytidine monophosphate, (+)/(−)-3TC-MP,[2] and β-2′,3′-dideoxy-5-fluoro-3′-thiacytidine monophosphate, (+)/(−)-FTC-MP (Feng et al., 2001, 2004). Since additional analogs with the unnatural L-configuration have not been tested, it is not known whether or not the high stereospecificity is limited to analogs with the oxathiolane ring. Thus, in order to understand if the high stereospecificity of Pol γ is a general behavior, it would be of considerable interest to study the incorporation of a pair of enantiomers with other structural modifications of the ribose ring, such as the 2′,3′-unsaturation found in D- and L-D4FC-MP.

The 3′ → 5′ DNA excision reaction for proofreading by Pol γ is also an important factor in considering mitochondrial toxicity of NRTI's, because once a nucleotide analog is incorporated, the primer is chain-terminated, and if the analog is removed efficiently, the primer can be used for DNA replication again. Thus, it is important to study both incorporation and removal of the drugs. Therefore, in this study, both incorporation and excision reactions of two enantiomers of D- and L-D4FC-MP catalyzed by Pol γ were investigated using kinetic approaches. The results were compared with other nucleotide analogs and evaluated to predict the level of Pol γ-related mitochondrial toxicity of D- and L-D4FC relative to the other analogs.

[2] (+)3TC is also commonly referred to as BCH-189. 3TC generally refers to the unnatural (−)-b-isomer of 2′,3′-deoxy-3′-thiacytidine which is in current clinical use.

2. Materials and methods

2.1. Materials

D- and L-D4FC-TP were synthesized as described previously (Ray et al., 2002). The [γ-^{32}P]ATP was obtained from Amersham Biosciences. Deoxyoligonucleotides were synthesized by Keck Oligonucleotide Synthesis Facility at Yale University and purified by 20% polyacrylamide gel electrophoresis.

2.2. Overexpression and purification of enzymes

Two different subunits of Pol γ were overexpressed separately. Both wild-type (wt) and exonuclease deficient (exo$^-$) mutant catalytic subunit were expressed in Sf9 insect cells using the baculovirus expression system as described previously (Graves et al., 1998). The accessory subunit was overexpressed in *E. coli* as described previously (Johnson et al., 2000). The holoenzyme was reconstituted by mixing the catalytic and accessory subunits at 1:5 ratio and incubating for 10 min on ice prior to the experiments. HIV-1 RT was overexpressed and purified as described previously (Kati et al., 1992).

2.3. Preparation of DNA substrates

The 5′-end of the primer was radiolabeled with ^{32}P using T4 polynucleotide kinase and [γ-^{32}P]ATP as previously described (Kati et al., 1992). For annealing, the labeled primers and desired templates were mixed at 1:1.4 ratio and heat-treated at 90 °C for 5 min followed by slow cooling, typically 50 °C for 10 min and on ice for 10 min. The complete annealing was confirmed by a native gel electrophoresis containing 15% polyacrylamide. DNA primers chain-terminated with D- and L-D4FC-MP (D24-mer) were prepared by elongating D23 using HIV-1 reverse transcriptase (Johnson et al., 2001). All the primer-template sets used in this study are shown in Fig. 2.

2.4. Incorporation of D- and L-D4FC-MP

All concentrations of reactants are reported as the final concentrations after mixing in the rapid chemical quench apparatus (see below). For burst experiment analyses, time-dependent product formation was fitted to an equation; $[\text{product}] = A(1 - \exp(-k_{\text{obs}}t)) + Ak_{\text{ss}}t$, where A is amplitude, k_{obs} is observed burst rate, t is time, and k_{ss} is observed steady-state rate. The program, Kaleidagraph (Synergy Software, Reading, PA) was used for the data analyses.

A pre-steady-state kinetic analysis was used to examine incorporation of a single nucleotide into a DNA primer/template substrate using a KinTek Corporation (Austin, TX) Model RQF-3 rapid-quench flow apparatus as described previously (Kerr and Anderson, 1997; Kati et al., 1992).

Incorporation of D-D4FC-MP into D23/D45 by Pol γ was studied under burst conditions in which a slight excess primer/template substrate was mixed with the enzyme. Since a pre-steady-state burst of product formation was not observed during L-D4FC-MP incorporation, the experiments were performed under single-turnover conditions, where the enzyme concentration was slightly in excess over the DNA substrate. The reaction time course for D-D4FC-MP incorporation was initiated by mixing a reactant solution containing varying concentrations of D-D4FC-TP, 2.5 mM $MgCl_2$ in 50 mM Tris–HCl/100 mM NaCl buffer pH 7.8 and another reactant solution containing 300 nM D23/R45, 80 nM exo$^-$ Pol γ holoenzyme. For L-D4FC-MP incorporation, all the experimental conditions were identical except D23/D45 and exo$^-$ Pol γ holoenzyme concentrations were 50 and 200 nM, respectively. The reaction was rapidly quenched using 0.3 M EDTA at desired times. Products were separated on a 20% polyacrylamide gel containing 8 M urea and analyzed on a BioRad GS-525 Molecular Imager System.

2.5. Removal of D- and L-D4FC-MP

Removal of D- and L-D4FC-MP by Pol γ was studied as described previously (Johnson et al., 2001; Feng et al., 2001) except that the concentrations of the chain-terminated

DNA 23mer
5′-*GCCTCGCAGCCGTCCAACCAACT -3′
3′- CGGAGCGTCGGCAGGTTGGTTGAGTTGGAGCTAGGTTACGGCAGG -5′
DNA 45mer

DNA 24mer
5′-*GCCTCGCAGCCGTCCAACCAACTX -3′ (X:D-/L-d4FC)
3′- CGGAGCGTCGGCAGGTTGGTTGAGTTGGAGCTAGGTTACGGCAGG -5′
DNA 45mer

Fig. 2. Sequence of primer-template.

primer-template substrate and the holoenzyme concentrations were much higher to ensure that binding was not limiting. The reaction mixture contained 1 μM D24/D45, 2 μM Pol γ, and 2.5 mM $MgCl_2$. After the desired times, the reaction was quenched in 0.3 M EDTA and the intact DNA primer substrate was quantified on a DNA sequencing gel and the data were fit to a single exponential decay equation: $(\%\,\text{intact D24}-\text{mer}) = A\exp(-k_{exo}t)$, where A is the amplitude, k_{exo} is the rate of excision, and t is the time in seconds.

2.6. Toxicity index

Toxicity index was calculated using the equation: $\text{toxicity index} = 1+(k_{cat}/k_{exo})\times([\text{Ana}-\text{TP}]/[\text{dNTP}])/4D$, where k_{cat} is the maximum rate of incorporation of the natural dNTP, k_{exo} is the rate of exonuclease removal of the chain-terminated drug, [Ana-TP] is the concentration of analog triphosphate, [dNTP] is the concentration of the natural nucleoside triphosphate, and D is the discrimination factor which is a ratio of the efficiency for natural dNTP incorporation to the efficiency of analog triphosphate incorporation, as described previously (Johnson et al., 2001). The ratio [Ana-TP]/[dNTP] was assumed to be 1.

3. Results

3.1. Incorporation of D- and L-D4FC-MP

Pre-steady-state kinetic analyses were employed to study incorporation of D- and L-D4FC-MP. These studies provide two key kinetic parameters: the maximum rate of incorporation (k_{pol}) and equilibrium dissociation constant (K_d). Based on these two parameters, efficiency of incorporation can be calculated (k_{pol}/K_d). Since the efficiency of incorporation is quantitatively expressed as a numerical value, it can easily be compared among different compounds and provide an assessment on how well the enzyme utilizes each of the nucleotides.

A pre-steady-state burst experiment was performed for D-D4FC-MP incorporation in the presence of 80 nM Pol γ, 300 nM D23/D45, 2.5 mM $MgCl_2$, and varying concentrations of D-D4FC-TP. A typical burst kinetic trace is shown in Fig. 3A. The presence of burst in single nucleotide incorporation is a characteristic of most DNA polymerases. The rapid burst phase represents incorporation of the nucleotide into the primer/template substrate which is pre-bound to the enzyme. The slow linear phase represents the rate-limiting elongated product release.

When a pre-steady-state burst experiment was performed for L-D4FC-MP incorporation, *no* burst of the elongated product formation was observed (Fig. 3B). This indicates that the rate-limiting step has changed from product release to a step prior to it. Therefore, in order to specifically focus on elongation steps, L-D4FC-MP incorporation was studied under single-turnover conditions in which enzyme concentration is in excess over the primer/template substrate (Fig. 3C). The experiments were performed in the presence of 200 nM Pol γ, 50 nM D23/D45, 2.5 mM $MgCl_2$, and varying concentrations of L-D4FC-TP. The reaction kinetic time courses were fit to a single exponential equation.

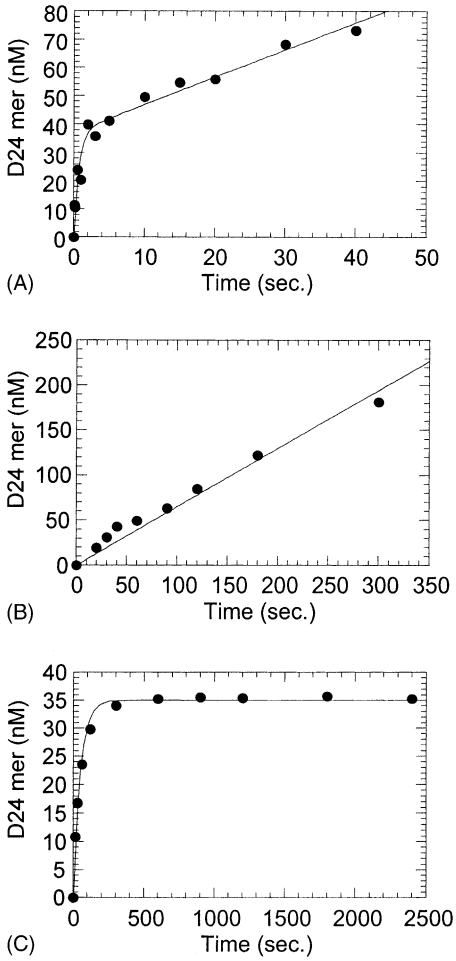

Fig. 3. Incorporation of D- and L-D4FC-MP. (A) Pre-steady-state burst kinetics of incorporation of D-D4FC-MP into D23/D45 primer-template. The presence of a pre-steady-state burst indicates that the rate-limiting step is the release of the product. (B) Incorporation of L-D4FC-MP under the same conditions as (A). The rate of incorporation was slow so that no pre-steady-state burst was observed, indicating that the rate-limiting step altered from product release to substrate incorporation. (C) The rate of incorporation for L-D4FC-MP was determined under single-turnover conditions in which the concentration of the enzyme was in excess of the substrate concentration.

Dependence of the observed burst rate on D-D4FC-TP concentration is shown in Fig. 4A. Based on the hyperbolic fit, parameters k_{pol} and K_d were determined to be $1.27 \pm 0.06\,s^{-1}$, and 0.82 ± 0.2 μM, respectively. Efficiency of incorporation (k_{pol}/K_d) was calculated to be $1.55\,\mu M^{-1}\,s^{-1}$. The single exponential rates were plotted against L-D4FC-TP concentrations and the parameters k_{pol}, K_d, and efficiency of incorporation were determined to be $0.080 \pm 0.010\,s^{-1}$, 167 ± 39 μM, and $0.0005\,\mu M^{-1}\,s^{-1}$, respectively (Fig. 4B).

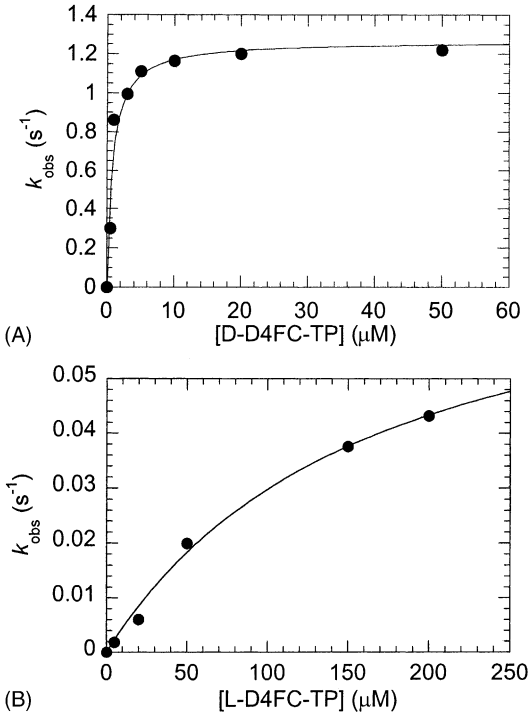

Fig. 4. Dependence of observed incorporation rates on D/L-D4FC-TP concentration. (A) Observed burst rates were plotted against D-D4FC-TP concentrations and the data were fit to a burst equation (see Section 2). (B) Observed single-turnover rates were plotted against L-D4FC-TP concentrations and the solid line indicates the single exponential fit. Parameters are summarized in Table 1.

The parameters are summarized in Table 1 including the previously determined numbers for the natural substrate, dCTP. Both D- and L-D4FC-MP were incorporated much less efficiently than the natural nucleotide, dCMP. When the efficiency was compared between the two enantiomers, D-D4FC-MP was a 3000-fold better substrate than L-D4FC-MP.

3.2. Removal of D- and L-D4FC-MP

In order to evaluate how much mitochondrial toxicity the analogs might show, it is important to consider the exonuclease removal of the analog from the chain-terminated primer end. Removal of D- and L-D4FC-MP from the 3′-end of the primer by DNA excision activity of Pol γ was studied under single-turnover conditions where the concentrations of D24/D45 and Pol γ holoenzyme are 1 and 2 μM, respectively. The intact substrate was quantified from a 20% polyacrylamide gel and the rates were calculated by fitting into an exponential decay equation. As shown in Fig. 5, the rates between D- and L-D4FC-MP removal by Pol γ were almost identical with $0.00064 \pm 0.00004\,s^{-1}$ and $0.00087 \pm 0.00004\,s^{-1}$, respectively.

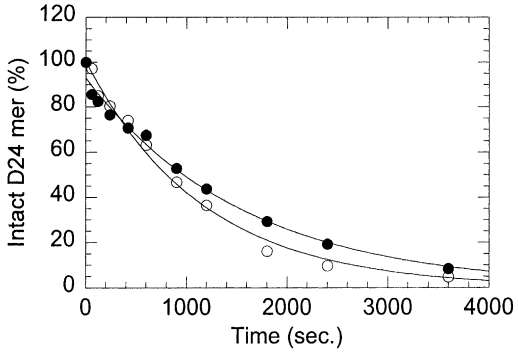

Fig. 5. Removal of D- and L-D4FC-MP. Intact D24-mer substrate concentration (%) was plotted against time. Closed and open circles show D- and L-D4FC-MP removal with rates of 0.00064 and $0.00087\,s^{-1}$, respectively.

Table 2
In vitro kinetic toxicity index

Analog	Toxicity index[a]
ddC-TP	160,000
ddA-TP	18,500
D4T-TP	3,120
D-D4FC-TP	**662**
ddI-TP	7
(+)-3TC-TP[b]	4
PMPApp	2.1
(−)-3TC-TP[c]	1.2
L-D4FC-TP	**1.15**
AZT-TP	1.05
CBV-TP	1.007

[a] Numbers other than D- and L-D4FC-TP were taken from Johnson et al. (2001).
[b] (+)-3TC is also commonly referred to as BCH-189.
[c] (−)-3TC is generally referred to as 3TC.

3.3. Toxicity index

Toxicity index is a kinetically derived parameter that attempts to predict the mitochondrial toxicity of a nucleotide analog. This index was calculated for D- and L-D4FC-TP using the equation shown in the experimental section. A higher number predicts a more toxic analog. The values for D- and L-D4FC-TP are shown in Table 2 in bold characters together

Table 1
Kinetic parameters for dCTP and D/L-D4FC-TP

	k_{pol} (s^{-1})	K_d (μM)	Efficiency ($\mu M^{-1}\,s^{-1}$)	Discrimination
dCTP	44 ± 2[a]	1.1 ± 0.1[a]	40[a]	—
D-D4FC-TP	1.27 ± 0.06	0.82 ± 0.2	1.55 ± 0.39	26
L-D4FC-TP	0.080 ± 0.010	167 ± 39	0.0005 ± 0.0001	83,500

[a] Numbers were taken from Feng et al. (2001).

with the numbers for other analogs determined previously (Johnson et al., 2001). D-D4FC-TP showed similar toxicity index as D4T-TP which contains the same 2′,3′-unsaturated ribose ring. L-D4FC-TP presented much lower toxicity and the value was similar to that of (−)-3TC-TP which contains an oxathiolane ring with an unnatural L-configuration.

4. Discussion

In order to estimate possible mitochondrial toxicity of an enantiomeric pair of nucleoside analogs, D- and L-D4FC, we have studied effects of phosphorylated forms of these analogs on two catalytic activities (DNA polymerization and 3′ → 5′ DNA excision) by human mitochondrial DNA polymerase (Pol γ). Both D- and L-D4FC-MP were incorporated by Pol γ with less efficiency compared to the natural nucleotide, dCMP. Remarkably, the L-D4FC-TP was highly discriminated against relative to dCTP, as its incorporation efficiency was 83,500-fold less. When the two enantiomers were compared, the incorporation efficiency for D-D4FC-MP was 3000-fold higher than for L-D4FC-MP. This observation that Pol γ incorporates the unnatural L-isomer at lower efficiency agrees with the previous results with two enantiomeric pairs of (+)/(−)-3TC-TP, and (+)/(−)-FTC-TP where the stereoselectivity (efficiency of natural isomer/efficiency of unnatural isomer) was 16- and 7900-fold, respectively (Feng et al., 2001, 2004). Thus, the trend that unnatural L-isomers are incorporated by Pol γ less efficiently is not restricted to analogs with an oxathiolane ring but also extends to 2′,3′-unsaturated ribose rings. In order to further generalize this observation, it is important to test other L-analogs with different structural modifications in the ribose ring.

Pol γ possesses 3′ → 5′ exonuclease proofreading activity. This activity has been shown to be higher in excising a mismatched nucleotide than correctly matched one at the 3′-end (Johnson and Johnson, 2001a). Although nucleotide analogs are different from natural substrates, they are still able to make correct base pairs as most of the modifications are in the ribose ring. The rates of removal of D- and L-D4FC-MP from the chain-terminated primers were slow as expected and they were very similar (0.00064 and 0.00087 s^{-1}, respectively). These results indicate that Pol γ may be unable to distinguish between the two enantiomers at the exonuclease active site. This observation is also supported by previous studies with (+)/(−)-3TC-MP and (+)/(−)-FTC-MP, where the rates of removal were similar for the two enantiomeric pairs (Feng et al., 2001, 2004).

Incorporation and removal of other nucleoside analog monophosphates have been studied and taking into account discrimination factors between the analogs and the corresponding natural nucleotide, toxicity indexes for the analogs have been calculated (Johnson et al., 2001). The kinetic toxicity indexes for D- and L-D4FC-TP are shown in Table 2 in bold together with the numbers for other analogs determined previously. Since D-D4FC-MP was 3000-fold more efficiently incorporated by Pol γ than L-D4FC-MP, and the removal rates were almost the same between the two, a higher kinetic toxicity index was obtained for the D-isomer. This suggests that the D-D4FC might present higher mitochondrial toxicity than L-D4FC, although in vitro kinetic results do not always correlate with cell culture data. The kinetic toxicity index for D-D4FC-TP is slightly lower than for D4T-TP and relatively high among all the analogs studied, suggesting that the analogs containing 2′,3′-unsaturation in the ribose ring with the natural configuration present higher toxicity indexes. The number for (−)-3TC-TP which contains an oxathiolane ring with unnatural configuration is low and similar to that for L-D4FC-TP, suggesting that analogs with unnatural configuration may present lower toxicity indexes. Since previous studies have shown that (−)-FTC-MP was 100-fold less efficiently incorporated than (−)-3TC-MP by Pol γ, this trend should also be consistent for (−)-FTC-TP (Feng et al., 2004).

Previously, we have reported studies on the incorporation of both D- and L-D4FC-MP by wild-type and 3TC resistant (M184V) HIV-RT (Ray et al., 2002). The D-D4FC-MP was incorporated as efficiently as the corresponding natural substrate dCMP with the wild-type and the M184V mutant RT, suggesting that RT may have difficulty distinguishing between the analog and the natural nucleotide. This may be the reason why D-D4FC was found to develop only one mutation (K65R) with minor resistance (5- to 8-fold) in cell culture studies (Geleziunas et al., 2003). On the other hand, L-D4FC-MP was incorporated 20- to 30-fold less efficiently than dCMP by the wild-type RT and it was highly selected against by the M184V mutant RT where a three order of magnitude drop incorporation efficiency relative to dCMP was observed. Cell culture studies have shown that L-D4FC-MP elicits K65R in addition to M184V (Schinazi et al., 2001). Therefore, D-D4FC is less prone to lead to resistance mutation development than the L-isomer.

In summary, we have found that D-D4FC-MP was incorporated into a DNA primer approximately 3000-fold more efficiently than L-D4FC-MP by Pol γ and that the rates of removal of both D- and L-D4FC-MP by exonuclease activity of Pol γ were almost identical. The combined results suggest that D-D4FC may cause higher mitochondrial toxicity than L-D4FC in cell-free systems. On the other hand, our previous study has shown that HIV-1 RT incorporates D-D4FC-MP approximately 10-fold more efficiently than L-D4FC-MP, suggesting that D-D4FC may present higher effectiveness against wild-type and M184V mutant forms of HIV than the L-isomer (Ray et al., 2002). Since the effectiveness of NRTIs is a balance between efficiency of incorporation by wild-type and drug resistant mutant forms of HIV-1 RT and mitochondrial toxicity, which for certain nucleotides correlate with efficiency of incorporation and removal by Pol γ, our enzyme kinetic results suggest both enantiomers show promise as antiviral agents.

While inhibition of DNA Pol γ is a potential indicator in predicting mitochondrial toxicity, overall mitochondrial toxicity is clearly a composite of many factors. These are mitochondrial transport and activation, the relative ratios of natural dNTPs and analog-TPs inside the mitochondria, and toxicity unrelated to inhibition of DNA Pol γ. For instance, it was demonstrated that D-D4FC was less toxic than L-D4FC in various cell culture systems (Shi et al., 1999). Furthermore, similar results showing low toxicity of D-D4FC were reported in a cell-based HepG2 assay for mitochondrial toxicity (Schinazi et al., 2002; Lin et al., 1996). Although higher mitochondrial toxicity was observed for L-D4FC by lactic acid assay, the mitochondrial DNA synthesis was not inhibited in the presence of 10 μM of either D- or L-D4FC (Schinazi R.F., unpublished data). Taken together, these data indicate that the toxicity of L-D4FC may not be related to incorporation of the drug into DNA by Pol γ. The cellular data, however, suggest that L-D4FC may exhibit mitochondrial toxicity by a mechanism *unrelated* to an effect on the mitochondrial DNA Pol γ.

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