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Mechanism of action of a novel viral mutagenic covert nucleotide: molecular interactions with HIV-1 reverse transcriptase and host cell DNA polymerases

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Abstract

A novel non-chain terminating nucleoside analog anti-HIV inhibitor, KP-1212 has been designed to form base pairs with multiple bases that may lead to mutagenesis in the HIV-1 viral genome. After multiple replication cycles, the accumulation of mutations surpasses a crucial threshold beyond which the virus can no longer replicate. HIV-1 reverse transcriptase (RT) incorporates the KP-1212 monophosphate into the genome during viral replication after metabolic activation of the KP-1212 nucleoside to the triphosphate. The propensity for forming alternate base pairs with the KP-1212 nucleotide leads to mismatched nucleotides and the subsequent misincorporation is the basis for the inhibitory activity. The results showed that HIV-1 RT and human mitochondrial DNA polymerase (Pol γ) incorporated KP-1212-TP with a significant level of efficiency, whereas mouse DNA polymerase β (Pol β) did not. Misincorporation studies suggest that both HIV-1 RT and Pol γ may cause mutations at significantly high rates. These in vitro data confirm the mechanistic basis of KP-1212 as a viral mutagen but suggest that there may be a potential for toxicity to the mitochondria.

Keywords: KP-1212; Mutagen; HIV-1; Reverse transcriptase; Nucleoside analog

1. Introduction

Nucleoside (analog) reverse transcriptase inhibitors (NRTIs) are the most common class of compounds used in the current anti-HIV chemotherapy. The seven NRTIs approved by the FDA lack a 3′-hydroxyl group in the sugar moiety and the eighth is actually an acyclic nucleotide analog. In each case, once the nucleoside (or nucleotide) is phosphorylated to the active form and incorporated into an elongating DNA strand by HIV-1 reverse transcriptase (RT), the DNA synthesis is terminated. Due to their mechanism of action, these N(t)RTIs are often referred to as "chain terminators". However, many of these drugs may lead to development of resistant viruses due to HIV's exceptionally high mutation rate. Thus, for the virus, the high mutation rate serves as a self-defense system. In contrast to all the chain terminators, covert nucleosides contain an unmodified 2′-deoxyribose sugar with slight modifications in the base, which could further increase the high mutation rate of HIV (Daifuku, 2003; Loeb et al., 1999; Loeb and Mullins, 2000; Anderson et al., 2004). The increased mutation rate could exceed the error threshold for viability and the virus may no longer replicate, a process known as "lethal mutagenesis". Because polymerases from riboviruses and retroviruses show high mutation rates, covert nucleosides are currently being screened for activity against retroviruses and riboviruses including HIV and hepatitis C virus (Anderson et al., 2004). They may also have activity against hepatitis B virus since the virus requires RNA-dependent DNA synthesis during replication processes (Anderson et al., 2004). KP-1212 (5-aza-5,6-dihydro-2′-deoxycytidine) is one of the covert nucleosides which has been modified in the cytosine ring (Fig. 1A). This analog has demonstrated antiviral activity in the nanomolar range and an acceptable toxicity profile (Harris et al., submitted for publication). In addition, KP-1212 has been shown to be active against NRTI-resistant

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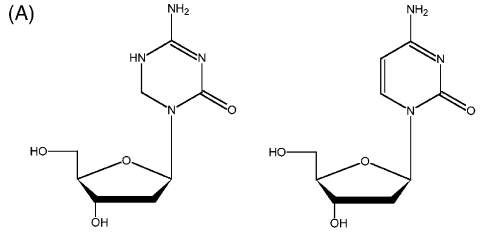

(B)

D20/D36	5′-*TCAGGTCCCTGTTCGGGCGC-3′ 3′-CGAAAGTCCAGGGACAAGCCCGCGGTGACGATCTCT-5′
D21/D36	5′-*TCAGGTCCCTGTTCGGGCGCC-3′ 3′-CGAAAGTCCAGGGACAAGCCCGCGGTGACGATCTCT-5′
D21KP/D36	5′-*TCAGGTCCCTGTTCGGGCGCX-3′ (X:K-P1212-MP) 3′-CGAAAGTCCAGGGACAAGCCCGCGGTGACGATCTCT-5′
D20/D36KP	5′-*TCAGGTCCCTGTTCGGGCGC-3′ (X:KP-1212-MP) 3′-CGAAAGTCCAGGGACAAGCCCGCGXTGACGATCTCT-5′
D21G/D36KP	5′-*TCAGGTCCCTGTTCGGGCGCG-3′ (X:KP-1212-MP) 3′-CGAAAGTCCAGGGACAAGCCCGCGXTGACGATCTCT-5′
D21A/D36KP	5′-*TCAGGTCCCTGTTCGGGCGCA-3′ (X:KP-1212-MP) 3′-CGAAAGTCCAGGGACAAGCCCGCGXTGACGATCTCT-5′

Fig. 1. (A) Structure of KP-1212 and 2′-deoxycytidine, and (B) sequence of primer/templates used in this study.

HIV-1 reverse transcriptase mutants, which is perhaps not surprising as the covert nucleosides are not chain terminators and not physically inhibiting reverse transcriptase but rather halt viral replication by increasing the mutation rate of the enzyme (Harris et al., submitted for publication).

Until now, detailed studies on the mechanism of action of covert nucleosides have not been conducted. We have performed mechanistic studies to address how efficiently HIV-1 RT incorporates KP-1212-MP using purified enzyme and the triphosphate form of KP-1212. A low toxicity profile for KP-1212 has been observed in cell-based assays. It is important to examine whether or not host cell DNA polymerases might incorporate KP-1212-MP and accrue mutations, if, for instance, substantial levels accumulated in the mitochondria. A somewhat similar non-chain terminating NRTI, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU) has shown severe mitochondrial toxicity probably due to mutagenesis in the mitochondrial DNA genome (Lewis and Dalakas, 1995; Lewis et al., 1994; Johnson et al., 2001). Thus, we also have studied the effects of KP-1212-TP on mouse DNA polymerase β (Pol β) and human mitochondrial DNA polymerase (Pol γ), because the fidelity for Pol β has been shown to be as low as that for HIV-1 RT (Johnson and Johnson, 2001b), and Pol γ has been mechanistically shown to be associated with mitochondrial toxicity of NRTIs (Johnson et al., 2001; Lewis et al., 2003; White, 2001). In order to understand the mechanism of action of KP-1212, we have specifically addressed the following questions: (1) What are the steady-state inhibition constants for KP-1212-TP for each enzyme? (2) How efficiently is KP-1212-MP incorporated under pre-steady-state conditions by HIV-1 RT and Pol γ? (3) How fast is KP-1212-MP removed by the exonuclease activity of Pol γ? (4) How is incorporation of the next correct nucleotide affected when KP-1212 is at the 3′-end of the primer? (5) How frequently are mutations generated? (6) How fast are mispaired natural nucleotides removed by the proofreading exonuclease activity of Pol γ?

2. Experimental procedures

2.1. Materials

The KP-1212-TP was synthesized by Koronis Pharmaceuticals (structure of KP-1212 nucleoside is shown in Fig. 1A). Oligonucleotides (D20, D21, D21G, D21A, and D36) were synthesized at Keck facility (Yale University) and purified by 20% polyacrylamide gel electrophoresis. The KP-1212 containing oligonucleotide (D36KP) was synthesized by Koronis Pharmaceuticals and Integrated DNA Technologies, Inc. (Redmond, WA and Coralville IA, respectively). Oligonucleotides were radiolabeled using [γ-^{32}P]ATP (Amersham) and polynucleotide kinase (New England Biolabs) as described previously (Kati et al., 1992). The KP-1212-terminated primer (D21KP) was synthesized using HIV-1 RT as described previously (Johnson et al., 2001). All the sequences for the DNA primer/template substrates are shown in Fig. 1B. The unlabeled and α-phosphate labeled dCTP and γ-phosphate labeled ATP were purchased from Amersham Biosciences. HIV-1 RT was overexpressed in *Escherichia coli* and purified as described previously (Kati et al., 1992). The wild-type and exonuclease-deficient mutant catalytic subunits of Pol γ were expressed in Sf-9 insect cells using baculovirus expression system and the accessory subunit was expressed in *E. coli* and both subunits were purified as described previously (Graves et al., 1998; Johnson et al., 2000). Pol γ holoenzyme was reconstituted by mixing the catalytic and accessory subunits at a 1:5 ratio and incubating for 5 min before the reaction was initiated. Mouse Pol β was kindly provided by Prof. Joanne Sweasy at Yale University. The mouse and human enzymes are almost identical with 96% sequence identity.

2.2. Steady-state competition assays

An unlabeled D20-mer primer was annealed to a 5′-^{32}P-labeled D36-mer template. The steady-state reaction was performed by adding 10 nM enzyme pre-equilibrated with 1 μM primer-template into a solution containing 0–100 μM [α-^{32}P]dCTP, 0–673 μM KP-1212-TP, and 2.5 mM $MgCl_2$ (all the concentrations are after mixing). The reaction was quenched after 10 min for HIV-1 RT, 20 min for Pol γ (exonuclease-deficient mutant form, exo minus), and 2 min for Pol β by adding EDTA to the final concentration of 0.3 M.

The reaction time was determined from the product formation profile in the absence of KP-1212-TP. Since the reaction time was chosen where the product formation is still in a linear dependence, the amount of the products directly correlates with the steady-state rate of product formation. The formation of radiolabeled D21-mer product was separated by 20% polyacrylamide gel electrophoresis, analyzed by a BioRad Molecular Imager FX System and quantified by BioRad Quantity One software. In order to eliminate gel-loading errors, the amount (radioactivity) of the product was normalized against the amount of the radiolabeled D36-mer template. All the experimental data points were globally fit to a competitive inhibition equation to obtain the inhibition constant, and, in order to clearly show competitive inhibition, Lineweaver–Burk plots were generated based on the parameters from the non-linear regression.

2.3. Pre-steady-state KP-1212-MP incorporation

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 (Kati et al., 1992; Kerr and Anderson, 1997). All concentrations of reactants are reported as the final concentrations after mixing.

Incorporation of KP-1212-MP into D20/D36 and D20/R36 by HIV-1 RT and into D20/D36 by Pol γ was studied under burst conditions in which a slight excess of primer/template substrate is mixed with the enzyme. In this experiment, exo minus Pol γ was used in order to avoid complication due to exonuclease removal. The reaction for KP-1212-MP incorporation with HIV-1 RT was initiated by mixing a reactant solution containing varying concentrations of KP-1212-TP, 10 mM $MgCl_2$ in 50 mM Tris–HCl/50 mM NaCl buffer pH 7.8 and another reactant solution containing 300 nM primer/template, 100 nM HIV-1 RT. For Pol γ reactions, a mixture containing KP-1212-TP and 2.5 mM $MgCl_2$ was mixed with 300 nM D20/D36 and 80 nM exo minus Pol γ holoenzyme. The reaction was rapidly quenched using 0.3 M EDTA at the desired times. Products were separated on a 20% polyacrylamide gel containing 8 M urea and analyzed on a BioRad Molecular Imager FX System. For data analyses, time-dependent product formation was fitted to an equation: $[\text{product}] = A(1 - \exp(-k_{obs}t)) + Ak_{ss}t$, where A is the amplitude, k_{obs} the observed burst rate, t the time, and k_{ss} is observed steady-state rate. The program, Kaleidagraph (Synergy Software, Reading, PA) was used for the data analyses.

2.4. Next correct nucleotide incorporation

Incorporation of dAMP into D21KP/D36, whose 3′-end nucleotide of the primer is KP-1212, was studied under burst conditions as described above. For comparison, dAMP incorporation into D21/D36 was also studied. The reaction was initiated by mixing a reactant solution containing varying concentrations of dATP, 10 mM $MgCl_2$ in 50 mM Tris–HCl/50 mM NaCl buffer pH 7.8 and another reactant solution containing 300 nM primer/template (D21/D36 or D21KP/D36) was pre-incubated with 100 nM HIV-1 RT in the same buffer. The same experiments were performed for Pol γ except that the reaction mixture contained 2.5 mM $MgCl_2$, and 100 mM NaCl. The reaction was quenched in 0.3 M EDTA and the products were quantified and analyzed as described above.

2.5. Excision removal of KP-1212-MP by Pol γ

Exonuclease removal studies were performed as described previously (Johnson et al., 2001; Feng et al., 2001; Murakami et al., 2004). The reaction mixture contained 150 nM D21KP/D36, 300 nM wild-type Pol γ, and 2.5 mM $MgCl_2$. After the desired times, the reaction was quenched in 0.3 M EDTA and the shortened primer product was quantified on a DNA sequencing gel and the data were fit to a single exponential equation: $(\text{excision products}) = A(1 - \exp(-k_{exo}t))$, where A is the amplitude, k_{exo} the rate of excision, and t is the time in seconds.

2.6. Misincorporation assays

Incorporation of all four dNTPs (dGTP, dATP, dCTP, and dTTP) into a D20/D36KP primer/template by HIV-1 RT and Pol γ was studied using pre-steady-state kinetics. This D36KP template contains KP-1212 opposite of the next incoming dNTP site (Fig. 1B). In the case of dGTP and dATP incorporation, pre-steady-state burst experiments were performed as described above. For dCTP and dTTP incorporation, single-turnover experiments were performed since the rates of catalysis were so slow that no burst kinetic behavior was observed.

2.7. Exonuclease removal of mismatched nucleotide

Reactions were performed the same as shown above in Section 2.5 except that the primer/template substrates used in this case are D21G/D36KP and D21A/D36KP.

3. Results

3.1. Steady-state competition study

In order to determine K_i values for KP-1212-MP, Michaelis–Menten steady-state kinetic studies for HIV-1 RT, Pol γ, and Pol β were conducted in the presence of varying amounts of KP-1212-TP. (It is important to note that even if KP-1212-MP is incorporated, the RT and other host polymerase activities may not be inhibited.) Double reciprocal (Lineweaver–Burk) plots are shown in Fig. 2 and the values are summarized in Table 1. As shown in Table 1, the K_i value for Pol γ (28 μM) was lower than that for HIV-1 RT

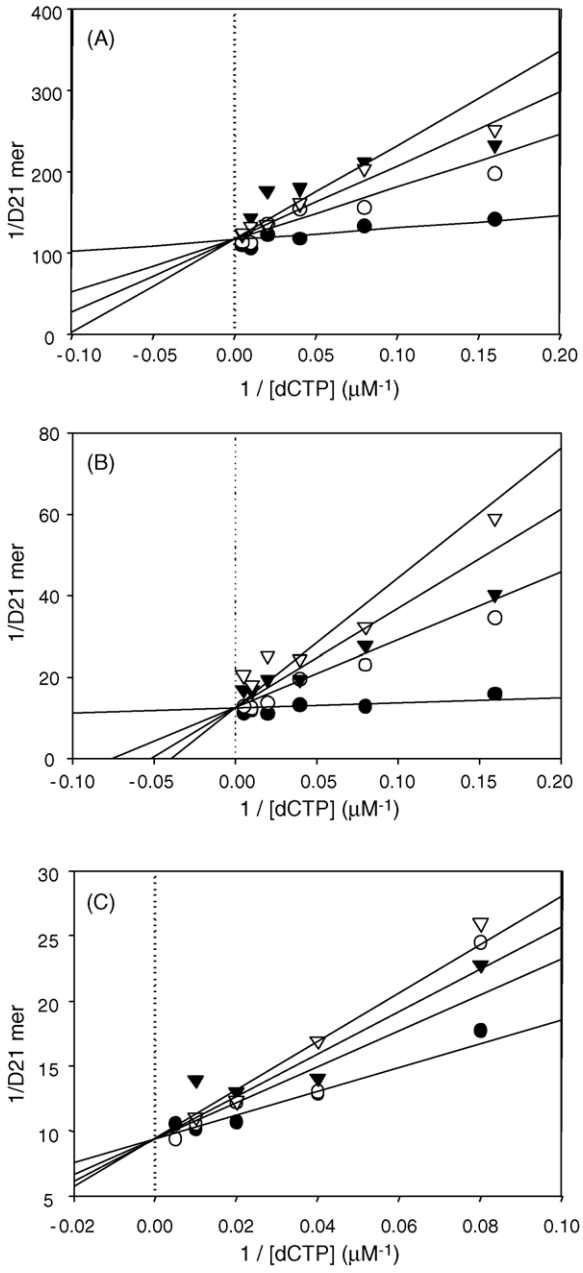

Fig. 2. Double reciprocal plots showing competitive inhibition. Experiments were performed using HIV-1 RT (A), Pol γ (B), and Pol β (C) in the presence of 0 μM (closed circles), 336 μM (open circles), 505 μM (closed triangles), and 673 μM (open triangles) of KP-1212-TP.

(95 μM), whereas the value for Pol β (650 μM) was much higher. Based upon the relative K_i values of KP-1212-TP for each polymerase, further mechanistic studies were warranted for HIV-1 RT and Pol γ.

Table 1
Steady-state inhibition parameters

	HIV-1 RT	Pol γ	Pol β
K_m (μM) for dCTP	1.22 ± 0.66	1.02 ± 0.97	9.66 ± 1.67
K_i (μM) for KP-1212-TP	95.2 ± 57.5	28.0 ± 26.3	650 ± 231

3.2. Pre-steady-state incorporation studies

Pre-steady-state single nucleotide incorporation kinetics is an often-used technique to elucidate the mechanism of polymerase reactions. Typically, DNA polymerases present a rapid "burst" incorporation of natural nucleotide substrates as well as some nucleotide analogs containing modifications. The presence of a burst indicates that the overall rate-limiting step is release of an elongated DNA product. Since KP-1212-TP was not efficiently utilized by Pol β based on the steady-state experiments above, the pre-steady-state experiments were performed with HIV-1 RT and Pol γ. Since HIV-1 RT catalyzes both DNA- and RNA-directed DNA synthesis, incorporation of KP-1212-MP into both D20/D36 and D20/R36 was studied. Although Pol γ is known to catalyze RNA-directed DNA synthesis (Murakami et al., 2003), the physiological role of the activity is not fully understood; thus, effects of KP-1212-TP were studied only on incorporation into D20/D36. In all cases, a burst phase was observed indicating that the mechanism of incorporation should be similar to that of natural nucleotide incorporation (Fig. 3). The equilibrium dissociation constant (K_d) for KP-1212-TP and the maximum rate of incorporation (k_{pol}) were determined by plotting the observed pre-steady-state burst rate against

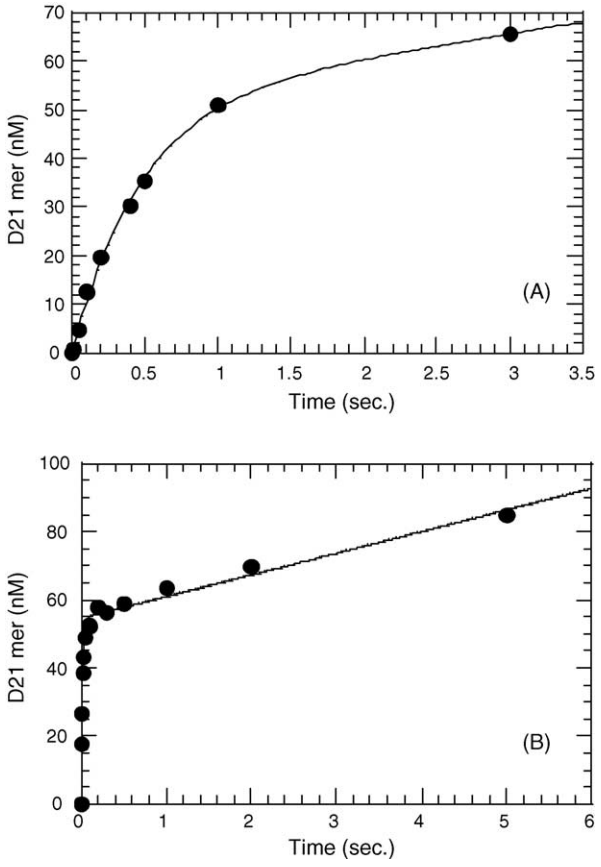

Fig. 3. Pre-steady-state incorporation of KP-1212-MP. Incorporation reactions catalyzed by HIV-1 RT in the presence of 135 μM KP-1212-TP (A) and by Pol γ with 336 μM KP-1212-TP (B).

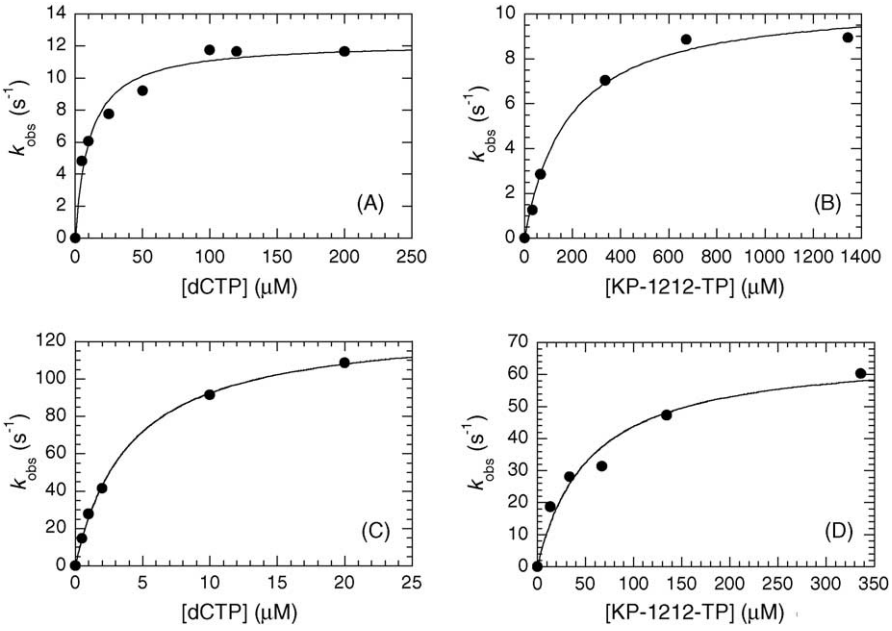

Fig. 4. Dependence of burst incorporation rates on dCTP and KP-1212-TP concentrations using HIV-1 RT (A and B, respectively) and Pol γ (C and D, respectively).

KP-1212-TP concentrations (Fig. 4). As shown in Table 2, the K_d and k_{pol} for KP-1212-TP with HIV-RT are 181 μM and 10.6 s^{-1} for D20/D36 and 506 μM and 83.2 s^{-1} for D20/R36, respectively. When these values were compared with natural dCMP incorporation, the k_{pol} values were very similar within two-fold, whereas the K_d value for KP-1212-TP was approximately 16–18-fold larger than that for dCTP. Similar trends were observed with Pol γ where K_d and k_{pol} were determined to be 53.8 μM and 67.3 s^{-1}, and compared to the values for dCTP, the k_{pol} was within a 2-fold difference and K_d was approximately 13-fold greater. These results indicate that the binding of KP-1212-TP was significantly weaker than that of the natural nucleotide substrate, dCTP.

3.3. Next correct nucleotide incorporation

Since the binding of KP-1212-TP was much weaker than that of dCTP (as shown in Table 2), the incorporation of the next correct nucleotide after KP-1212-MP incorporation may be affected. To address this question, dAMP incorporation into D21-mer and D21KP-mer was studied (see Fig. 1B, for the sequence) using both HIV-1 RT and Pol γ under pre-steady-state burst conditions. The reaction kinetics for both sets of primer/templates showed burst product formation (data not shown). Dependence of the burst rate on KP-1212-TP concentration is shown in Fig. 5. As shown in Table 3, the efficiency of dAMP incorporation into the KP-1212 terminated primer (D21KP) was 19- and 5.5-fold lower than the D21-mer for HIV-1 RT and Pol γ respectively, indicating a higher level of discrimination for the mitochondrial polymerase. Interestingly, the lower incorporation efficiency was due to the rate of incorporation (k_{pol}) rather than binding (K_d), indicating that the incorporation was influenced by the presence of KP-1212 at the primer end.

3.4. Excision removal by Pol γ

Only Pol γ out of the three polymerases studied here possesses 3′ → 5′ exonuclease proofreading activity and this may play an important role in mitochondrial toxicity of nucleoside analogs. The excision removal of KP-1212-MP was studied under single-turnover conditions where the enzyme concentration was in excess over the DNA substrate D21KP/D36 concentration (Fig. 6). The shortened primer products were quantified and the rate of removal was determined to be 0.0269 ± 0.0011 s^{-1}. The rate of KP-1212-MP removal was

Table 2
Pre-steady-state kinetic parameters for KP-1212-MP incorporation

	HIV-1 RT						Pol γ		
	DNA/DNA			DNA/RNA			DNA/DNA		
	K_d (μM)	k_{pol} (s^{-1})	Efficiency ($\mu M^{-1} s^{-1}$)	K_d (μM)	k_{pol} (s^{-1})	Efficiency ($\mu M^{-1} s^{-1}$)	K_d (μM)	k_{pol} (s^{-1})	Efficiency ($\mu M^{-1} s^{-1}$)
dCTP	10.5 ± 2.0	12.2 ± 0.5	0.86	32.1 ± 5.3	61.0 ± 3.3	1.9	4.0 ± 0.2	130 ± 2	32.5
KP-1212-TP	181 ± 32	10.6 ± 0.5	0.06	506 ± 183	83.2 ± 14.5	0.16	53.8 ± 15.3	67.3 ± 6.4	1.25

Table 3
Pre-steady-state parameters for the next correct dAMP incorporation by HIV-1 RT and Pol γ

	D21KP/D36			D21/D36		
	K_d (μM)	k_{pol} (s^{-1})	Efficiency ($\mu M^{-1} s^{-1}$)	K_d (μM)	k_{pol} (s^{-1})	Efficiency ($\mu M^{-1} s^{-1}$)
HIV-1 RT	6.7 ± 1.2	1.73 ± 0.07	0.26	3.0 ± 0.4	14.8 ± 0.4	4.9
Pol γ	5.1 ± 1.0	38.1 ± 2.8	7.44	3.1 ± 0.9	128 ± 12	41

very similar to that of previously reported natural dNMP removal (0.02–0.05 s^{-1}) (Johnson and Johnson, 2001a).

3.5. Misincorporation studies

In order to study the frequency of generation of mutations, incorporation of all four natural nucleotides into a D20/D36KP primer/template containing KP-1212 in the template strand at the site opposite to incoming nucleotide was tested with both HIV-1 RT and Pol γ. As shown in Table 4, both enzymes incorporated dGTP very efficiently, since KP-1212 is considered as a cytidine analog and predicted to basepair with guanine. The efficiency of dGTP incorporation was 0.37 $\mu M^{-1} s^{-1}$ for HIV-1 RT and 1.79 $\mu M^{-1} s^{-1}$ for Pol γ. The other purine nucleotide, dATP was also incorporated at significantly high efficiency of 0.004 $\mu M^{-1} s^{-1}$ for HIV-1 RT and 0.32 $\mu M^{-1} s^{-1}$ for Pol γ, considering dTTP and dCTP showed very poor incorporation with efficiencies of 2.1×10^{-5} and 7.5×10^{-6} $\mu M^{-1} s^{-1}$ for HIV-1 RT and 5.6×10^{-4} and 1.6×10^{-4} $\mu M^{-1} s^{-1}$ for Pol γ, respectively.

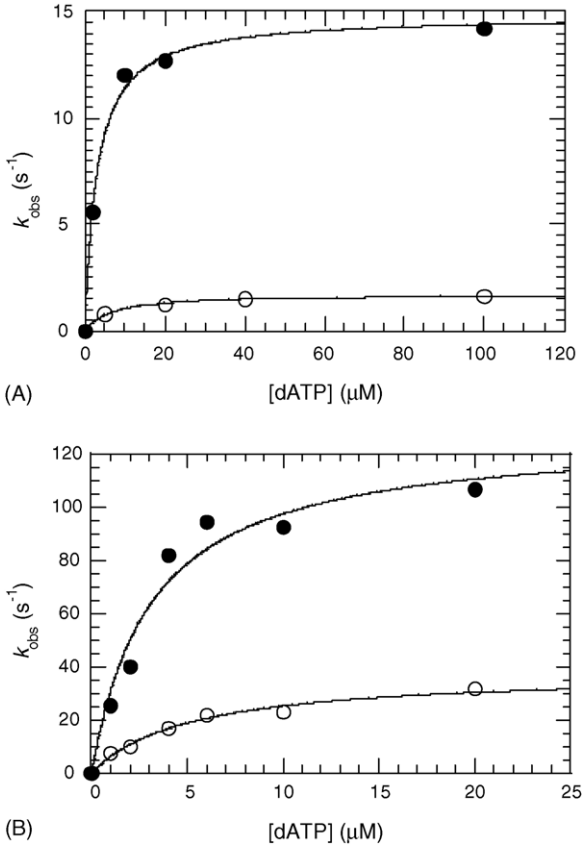

Fig. 5. Dependence of dAMP incorporation rate on dATP concentration with HIV-1 RT (A) and Pol γ (B). The experiments were performed using both D21/D36 (closed circles) and D21KP/D36 (open circles).

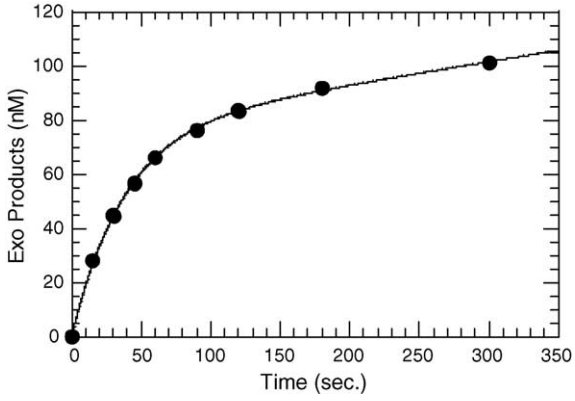

Fig. 6. Exonuclease removal of KP-1212-MP by Pol γ.

3.6. Removal of mispaired nucleotide

Although dATP is incorporated efficiently opposite KP-1212 relative to dGTP, if either of the nucleotides is removed by Pol γ's exonuclease activity effectively, the likelihood of generating mutations at a high rate may decrease significantly. Thus, removal of the natural substrate, dGMP and a mispaired end terminating with dAMP were studied using D21G/D36KP and D21A/D36KP, respectively (data not shown). The rate for the mispaired-end containing dAMP (0.090 ± 0.008 s^{-1}) was slightly faster than for the natural base pair, dGMP (0.065 ± 0.007 s^{-1}).

4. Discussion

The KP-1212 is a nucleoside analog that is modified from cytidine and anticipated to be activated to triphosphate form by cellular kinases and incorporated by HIV-1 RT. In this case, KP-1212-TP would serve as a competitive substrate against dCTP. Thus, we have performed steady-state competition assays using HIV-1 RT and other mammalian polymerases (Pol γ and Pol β) which may be associated with toxicity of this compound since an NRTI containing a 3′-OH, FIAU, has been shown to present high level of mitochondrial toxicity (Lewis and Dalakas, 1995; Lewis et al., 1994). Although we have not tested inhibition of replicative DNA polymerases such as DNA polymerase α, those polymerases exhibit higher fidelity and the inhibition might be expected to be weaker than for

Table 4
Misincorporation studies with HIV-1 RT and Pol γ

	HIV-1 RT				Pol γ			
	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d ($\mu M^{-1}\,s^{-1}$)	Fidelity[a]	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d ($\mu M^{-1}\,s^{-1}$)	Fidelity[a]
dGTP	28.0 ± 1.5	76.5 ± 11.8	0.37	–	92.7 ± 6.8	51.7 ± 11.9	1.79	–
dATP	4.5 ± 0.3	1121 ± 166	0.004	94	23.9 ± 1.2	74.2 ± 14.2	0.32	6.6
dCTP	$(4.1 \pm 0.3) \times 10^{-3}$	538 ± 93	7.5×10^{-6}	49000	0.054 ± 0.002	340 ± 33	1.6×10^{-4}	11200
dTTP	0.11 ± 0.02	5300 ± 1500	2.1×10^{-5}	18000	0.066 ± 0.003	118 ± 17	5.6×10^{-4}	3200

[a] Calculated as $[(k_{pol}/K_d)_{incorrect} + (k_{pol}/K_d)_{correct}]/(k_{pol}/K_d)_{incorrect}$.

Pol β and Pol γ. As shown in Table 1, the K_i for KP-1212-TP with Pol β was the highest among the three polymerases. The K_i value for HIV-1 RT, 95 μM, was seven-fold lower than that for Pol β, suggesting that perhaps Pol β may discriminate against KP-1212 better than both HIV-1 RT and Pol γ. The other host polymerase enzyme, Pol γ, presented about a three-fold lower K_i than HIV-RT, suggesting that if the triphosphate of KP-1212 were in sufficient amounts in the mitochondria, it may be incorporated into mitochondrial DNA and cause mitochondrial toxicity.

Incorporation of KP-1212-TP by HIV-RT and Pol γ was further studied using a pre-steady-state kinetic analysis. As shown in Table 2, the efficiency of KP-1212-MP incorporation for HIV-1 RT and Pol γ was, respectively, 11- and 26-fold lower than dCMP incorporation. This indicates that even though the KP-1212 incorporation efficiency for Pol γ is higher than that of HIV-1 RT, Pol γ is more selective than HIV-1 RT. In both cases, the lower KP-1212 incorporation efficiency was due to weaker binding (higher K_d) compared to dCTP. Since KP-1212 contains a modified cytosine ring and the base is no longer planar, the base pairing with a guanine in the template strand may be altered, hence influencing the binding of the nucleotide.

Based upon the results described above, the K_d for KP-1212-TP rather than k_{pol} was significantly affected, and accordingly, may be due to perturbed basepairing. Consequently, this may cause a shift in the positioning of the incorporated KP-1212 that in turn may affect the next nucleotide incorporation. Therefore, the next correct nucleotide incorporation was studied. As shown in Fig. 5 and Table 3, when the primer end is KP-1212, the efficiency of dAMP incorporation was approximately 20- and 6-fold lower compared to the regular primer with HIV-1 RT and Pol γ, respectively. Interestingly, with both HIV-1 RT and Pol γ, the decreased incorporation efficiencies of dAMP into a primer containing KP-1212 at the 3′-end were due to changes in the rate of incorporation (k_{pol}), but not nucleotide binding (K_d). Since the DNA elongation reaction involves a nucleophilic attack of the 3′-OH group at the α-phosphate of the incoming nucleoside triphosphate, the position of the 3′-OH of the KP-1212 at the primer-end may be suboptimal due to less favorable basepairing, therefore resulting in a decrease in the incorporation rate.

Unlike HIV-1 RT or Pol β, Pol γ possesses 3′ → 5′ exonuclease proofreading activity. When the mitochondrial toxicity of nucleoside analog reverse transcriptase inhibitors is evaluated, this activity plays a significant role (Johnson and Johnson, 2001a,b). One can easily imagine that if a drug is removed quickly, the drug is less toxic. Our earlier studies demonstrating the failure of exonuclease removal of ddC chain-terminated DNA by Pol γ suggested this may play a major role in the greater toxicity observed for ddC (Zalcitabine) (Feng et al., 2001). In contrast, the rate of removal for KP-1212-MP ($0.027\,s^{-1}$) by the exonuclease activity of Pol γ was as fast as natural nucleotide removal ($0.02–0.05\,s^{-1}$). Thus, it is possible that the removal of KP-1212 may play a role in modulating toxicity.

On the other hand, it has been previously shown that the exonuclease removal of a mismatched nucleotide is very fast compared to the correctly matched nucleotide (Johnson and Johnson, 2001a), indicating that the exonuclease active site may to some extent recognize the perturbed basepair between KP-1212 and guanine ring as a correct basepair (Harris et al., submitted for publication).

While mechanistic studies examining incorporation by Pol γ suggest KP-1212 may possibly have the potential to present mitochondrial toxicity, the KP-1212-MP can be efficiently excised by exonuclease removal and cell culture studies show no indication of mitochondrial toxicity based upon an examination of both lactic acid levels and mitochondrial DNA synthesis (Harris et al., submitted for publication). Unlike all other FDA approved nucleoside analog RT inhibitors, KP-1212 contains an unmodified ribose ring with a 3′-OH group that allows polymerases to further elongate the DNA. Our studies to examine the molecular mechanism suggest that both the viral and host polymerases recognize the KP-1212 as a correct nucleoside (cytidine) and thereby incorporates dGMP when the KP-1212 is in the template.

In order to predict the nature and frequency of misincorporations, a series of experiments were performed in which the incorporation of all four natural dNMPs was examined using a D20/D36KP primer/template contained a KP-1212 at the position opposite to the next incoming dNTP in the template strand. Both HIV-1 RT and Pol γ incorporated the natural substrate, dGMP and the purine mismatch, dAMP, efficiently, yet incorporation of pyrimidine nucleotides (dCTP and dTMP) was very poor (Table 4). These results are in accordance with cell culture data discussed below and lend support for suggestion that the *purine-to-purine* mutation rate may increase based upon predictions that as KP-1212 can

undergo tautomerization between imino and amino forms that could basepair with adenine and guanine, respectively (Harris et al., submitted for publication).

Cell-based mutation rate studies have been studied by sequencing *pol* and *env* genes after 11 serial passages in the presence or absence of KP-1212 (Harris et al., submitted for publication). The results showed increased mutation rate in the presence of KP-1212 and type of mutation was either purine-to-purine or pyrimidine-to-pyrimidine which is consistent with our in vitro data. It was somewhat surprising that fidelity for dATP incorporation against KP-1212 by Pol γ was low. The exonuclease removal rates are slightly higher for dAMP relative to the natural dGMP substrate. These in vitro results suggest that mutations could be introduced into mitochondrial DNA at a significantly high rate, however, the exonuclease activity may play a role in modulating mitochondrial toxicity. In cell culture studies, KP-1212 did not show a significant increase in lactic acid production nor a significant decrease in mitochondrial DNA levels at drug concentrations up to 320 μM, which indicate that KP-1212 is not toxic to mitochondria. Differential results comparing cell-based and in vitro assays may be due to cellular factors such as metabolism or transport into mitochondria and further studies are warranted to clarify this issue.

In summary, we have studied effects of a novel covert nucleoside triphosphate on HIV-1 RT, human Pol γ, and Pol β. The results suggest that HIV-1 RT and Pol γ but not Pol β utilize KP-1212-TP as a substrate with significantly high efficiency. Moreover, the pre-steady-state KP-1212-MP incorporation and the next correct nucleotide incorporation studies coupled with the misincorporation studies provide insight into the molecular interactions that allow KP-1212 to serve as a viral mutagen. As the underlying mechanism, a previously predicted increase of the purine-to-purine mutation rate has been confirmed by studies at the molecular and cellular level.

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