Correction

BIOCHEMISTRY

The authors note that Fig. 5 appeared incorrectly. The corrected figure and its legend appear below.

Fig. 5. Functional and structural differences in RT and Pol γ residues surrounding (+)-FTC-TP reveal that the RT incoming nucleotide binding site (A) is more flexible thus less selective than that of human Pol γ (B), which consists of more residues to enhance nucleotide selectivity.

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Probing the structural and molecular basis of nucleotide selectivity by human mitochondrial DNA polymerase γ

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Abstract

Nucleoside analog reverse transcriptase inhibitors (NRTIs) are the essential components of highly active antiretroviral (HAART) therapy targeting HIV reverse transcriptase (RT). NRTI triphosphates (NRTI-TP), the biologically active forms, act as chain terminators of viral DNA synthesis. Unfortunately, NRTIs also inhibit human mitochondrial DNA polymerase (Pol γ), causing unwanted mitochondrial toxicity. Understanding the structural and mechanistic differences between Pol γ and RT in response to NRTIs will provide invaluable insight to aid in designing more effective drugs with lower toxicity. The NRTIs emtricitabine [(−)-2,3′-dideoxy-5-fluoro-3′-thiacytidine, (−)-FTC] and lamivudine, [(−)-2,3′-dideoxy-3′-thiacytidine, (−)-3TC] are both potent RT inhibitors, but Pol γ discriminates against (−)-FTC-TP by two orders of magnitude greater than (−)-3TC-TP. Furthermore, although (−)-FTC-TP is only slightly more potent against HIV RT than its enantiomer (−)-3TC-TP, it is discriminated by human Pol γ four orders of magnitude more efficiently than (−)-3TC-TP. As a result, (−)-FTC-TP is a much less toxic NRTI. Here, we present the structural and kinetic basis for this striking difference by identifying the discriminator residues of drug selectivity in both viral and human enzymes responsible for substrate selection and inhibitor specificity. For the first time, to our knowledge, this work illuminates the mechanism of (−)-FTC-TP differential selectivity and provides a structural scaffold for development of novel NRTIs with lower toxicity.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 5C51, 5C52, and 5C53).

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Significance

Nucleoside analog reverse transcriptase inhibitors (NRTIs) are the cornerstones of treatment for fighting HIV infection. Unfortunately, they also cause drug toxicity by inhibiting human mitochondrial DNA polymerase (Pol γ). Identification of structural differences between the intended target (RT) and adverse reaction target (Pol γ) will provide critical information for designing more potent drugs with lower toxicity. Here, we reveal structural and mechanistic differences between Pol γ and RT by studying NRTIs that have comparable efficacy on RT but significantly different affinities for Pol γ. We identified critical discriminator residues in Pol γ that are fully responsible for its differential response to emtricitabine. More importantly, the topological equivalent residue in RT is essential for activity, thus identifying this region as a hot-spot for inhibitor design.


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Nucleoside analog reverse transcriptase inhibitors (NRTIs) are the cornerstones of treatment for fighting HIV infection. Unfortunately, they also cause drug toxicity by inhibiting human mitochondrial DNA polymerase (Pol γ). Identification of structural differences between the intended target (RT) and adverse reaction target (Pol γ) will provide critical information for designing more potent drugs with lower toxicity. Here, we reveal structural and mechanistic differences between Pol γ and RT by studying NRTIs that have comparable efficacy on RT but significantly different affinities for Pol γ. We identified critical discriminator residues in Pol γ that are fully responsible for its differential response to emtricitabine. More importantly, the topological equivalent residue in RT is essential for activity, thus identifying this region as a hot-spot for inhibitor design.
is able to distinguish the 5-F Crick ∼ in Pol I family DNA polymerases (18). July 14, 2015 discarniates (-)-FTC-TP activities in the holoenzyme (17).

Thus, the holoenzyme consists of a 140-kDa "core" subunit responsible for DNA synthesis, as the Pol γ catalytic subunit, Pol γ pol, is further divided into thumb, palm, and fingers subdomains. The palm subdomain harbors the catalytic residues and a spacer.

Our findings reveal the critical structural features of HIV RT and human Pol γ that are essential for their differential drug response, providing a structural and functional basis for drug efficacy and toxicity. The studies also suggest a possibility for using fluorinated compounds to probe differences between intended and adverse reaction targets in designing potent and highly selective NRTIs. New and improved drugs are urgently required to combat resistance of HIV to current antiretroviral therapies.

Results

Structural Determination of the Pol γ Ternary Complex with DNA and (+) or (-)-FTC-TP. Human Pol γ holoenzyme consists of a 140-kDa catalytic subunit, Pol γ A, and a 110 kDa homodimeric accessory subunit, Pol γ B. Pol γ A has three distinct subdomains, including pol for DNA synthesis, exon for 3′-5′ exonuclease proofreading, and a spacer. pol is further divided into thumb, palm, and fingers subdomains. The palm subdomain harbors the catalytic residues D890 and D1135 for phosphotransfer, whereas the fingers constitute the nucleotide binding site; the positively charged residues R943 and K947 on the O-helix form electrostatic interactions with the negatively charged triphosphate. The apo Pol γ holoenzyme undergoes conformational changes in the presence of DNA and substrate. Specifically, the dimeric Pol γB rotates 22° in ternary complexes, increasing the interaction between Pol γA and the distal Pol γB monomer (Fig. 2). This configuration promotes DNA synthesis, as the Pol γA-distal Pol γB contact area allosterically regulates pol and exon activities in the holoenzyme (17).

Pol γ discriminates (+)-FTC-TP ~8,000-fold more efficiently than (+)-FTC-TP. To understand the stereoselectivity of Pol γ, we crystallized a ternary complex containing Pol γ holoenzyme, a 24-28-mer primer/template duplex DNA where the templating base is guanine, and either (+)-FTC-TP or (-)-FTC-TP. The primer strand is terminated by a deoxyribonucleotide, and the complexes therefore correspond to inhibitor-bound states. The structures were determined using molecular replacement using a Pol γ ternary complex containing dCTP (17). The statistics for data collection and structural refinement are presented in Table S1.

Pol γ Distinguishes the Ribose Modification of FTC-TP and Its Chirality. Both chiral FTC-TP derivatives are seen bound in pol; however, they adopt different binding modes (Fig. 1 and Figs. S1 and S2). The stereochemistry of (+)-FTC-TP is that of a normal substrate, but with modified base (5-F) and ribose (oxathiolane) moieties (Fig. 1). The inhibitor is positioned in the substrate site bounded by Pol γ A R853, Q894, E895, and Y951; its 5-F cytosine forms a Watson–Crick base pair with the templating guanosine, and the triphosphate is bound by R943 and K947 on the ‘O-helix’. The oxathiolane assumes a similar position as deoxyribose of dCTP and thus (+)-FTC-TP is in a catalytically competent configuration (Fig. S1), where the α-phosphate coordinates the metal ion poised for phosphodiester bond formation with the primer 3′-end. In a productive substrate complex, Pol γA R853 hydrogen-bonds with the 3′-OH of the incoming nucleotide and is predicted to function in correct nucleotide selection. However, R853 is unable to interact with the oxathiolane group of (+)-FTC-TP; Pol γ thus has lower affinity for (+)-FTC-TP than dCTP, consistent with kinetic analyses (6).

In contrast, (-)-FTC-TP interacts with the templating guanosine with distorted geometry from a canonical Watson–Crick base pair, where the distances between the potential hydrogen bonding pairs are extended by 0.8–1.0 Å (Fig. 1C). The distorted base pair is due to the chirality of (-)-FTC-TP: Although Pol γ A R943 and K947 still coordinate the triphosphate moiety, the rigid aromatic residue Y951 would sterically clash with the modified ribose. As a result, it forces both the nucleoside and the α-phosphate to be misaligned (Fig. S2). A superposition of the two structures is shown in Fig. 1D. The counterpart to Pol γ A Y951 has been termed the “steric-gate” in Pol I family DNA polymerases (18–20) because the highly conserved tyrosine or phenylalanine is important in discrimination against rNTPs. These structures indicate that the Pol γA O-helix and Y951 are also responsible for substrate/inhibitor stereoechemical specificity.

Pol γ, but Not RT, Discriminates Against the 5-Fluorine of (-)-FTC-TP. Although (-)-3TC-TP and (-)-FTC-TP have comparable efficacy against HIV RT, the addition of 5-F in (-)-FTC-TP reduces Pol γ toxicity 100-fold (Fig. 1L4) (6, 21). To understand the molecular basis for this difference, we docked (-)-FTC-TP from the Pol γ ternary complex in the active site of RT after superpositioning the primer termini (PDB ID code 1RTD; ref. 22). Compared with the Pol γ complex (Fig. 3), the RT substrate-binding site has a different configuration. Most notably, R72 of RT in the β-turn-β substrate-binding loop is predicted to form a hydrogen bond with 5-F of (-)-FTC-TP, an interaction resulting from the orientation imposed by the unnatural (-) enantiomer. Fluorine acts as an electron sink, weakening the Watson–Crick hydrogen bonding between the template and the inhibitor (23, 24) thereby...

Fig. 1. (+)-FTC-TP binds WT Pol γ in a more catalytically competent orientation relative to (-)-FTC-TP. (A) Structures of dC and analogs (-)-3TC (lamivudine) and (-)-FTC (entricitabine). X-ray crystal structures of Pol γ are shown in complex with (+)-FTC-TP (B) and (-)-FTC-TP (C), and superposition of the two structures is shown in D. RT; this suggests that only Pol γ is able to distinguish the 5-F addition (6). Cellular studies also suggest that (+)-FTC is more toxic than (-)-FTC (16). Understanding the structural basis for this striking difference would provide insight into the rational design of additional antiviral compounds with lower toxicity and their consequent widened therapeutic window.

Our findings reveal the critical structural features of HIV RT and human Pol γ that are essential for their differential drug response, providing a structural and functional basis for drug efficacy and toxicity. The studies also suggest a possibility for using fluorinated compounds to probe differences between intended and adverse reaction targets in designing potent and highly selective NRTIs. New and improved drugs are urgently required to combat resistance of HIV to current antiretroviral therapies.

Fig. 2. The ternary complex of Pol γ-DNA with an inhibitor adopts a conformation similar to the substrate complex. It undergoes conformational changes that strengthen subunit interaction and DNA binding.
reducing its affinity. We hypothesize that the R72:5-F hydrogen bond compensates for this reduction and allows RT to use (-)-FTC-TP efficiently. Because (-)-3TC-TP has a 5-H, Watson–Crick base pairing with 5-F precludes interaction with (-)-FTC-TP. Adding 5-F to (-)-3TC-TP therefore should decrease the affinity for human Pol γ, resulting in an improved toxicity profile for (-)-FTC-TP.

Residue I948 in the O-helix of Pol γ occupies the same position in pol as RT R72. Although arginine can support hydrogen bonds, the hydrophobic nature of isoleucine and its 5.6-Å distance from the 5-F precludes interaction with (-)-FTC-TP. Adding 5-F to (-)-3TC-TP therefore should decrease the affinity for human Pol γ, resulting in an improved toxicity profile for (-)-FTC-TP.

To test the structural conclusion that Pol γ A. I948 R is responsible for discrimination against (-)-FTC-TP, we constructed mutants that switch the residues in question in Pol γ and RT, i.e., Pol γ A. I948 R to mimic R72 in the RT active site and R72I in HIV RT. We crystallized and determined the structure of the mutant Pol γ holoenzyme ternary complex to 3.5-Å resolution. Although WT complex crystals only diffracted to moderate resolution, mutant crystals consistently diffracted better, suggesting better packing and/or more ordered complexes (Fig. 4, Fig. S2, and Table S1). The overall structure of the mutant is similar to WT, but several key changes were observed in pol. As predicted, Pol γ A. R948 hydrogen bonds with 5-F of (-)-FTC-TP as in the modeled RT structure (Fig. 3A). Furthermore, although (-)-FTC-TP has distorted base pairing with the template in WT Pol γ, it is closer to canonical Watson–Crick in the I948R mutant. The metal ion is also 1.4 Å closer to the catalytic residues (Fig. 4). These data suggest that Pol γ A. I948 R serves an important discriminator residue against (-)-FTC-TP utilization.

**Discriminator Switching Mutants Swap Pol γ and RT Drug Responses.** We measured the rate of single nucleotide incorporation by Pol γ containing Pol γ A. I948 R (Pol γ A. I948 R) using single-turnover kinetic experiments. A D23/D45 primer/template partial duplex DNA where the templating residue is a guanosine (G) was used for incorporation of either dCTP or (-)-FTC-TP. The maximum rate of polymerization rate ($k_{pol}$) and the dissociation constant for the incoming nucleotide or analog ($K_d$), were determined. Although there is a twofold decrease in $K_{pol}$ of Pol γ A. I948 R using dCTP as a substrate, an improvement in affinity ($K_d$) by nearly the same magnitude results in only a 0.8-fold change in overall incorporation efficiency relative to a normal substrate. Thus, the I948R substitution does not substantially affect natural substrate recognition or catalysis (Table 1).

In contrast, Pol γ A. I948 R shows markedly reduced discrimination against (-)-FTC-TP. Compared with WT, which incorporates the inhibitor at almost negligible efficiency, Pol γ A. I948 R has a 70-fold increase in affinity for (-)-FTC-TP (Table 1), suggesting that the arginine significantly enhances inhibitor binding. When combined with a threefold increase in $k_{pol}$, Pol γ A. I948 R discrimination against (-)-FTC-TP is decreased more than 200-fold relative to WT. In fact, Pol γ A. I948 R incorporates (-)-FTC-TP 10-fold more efficiently than RT (Table 1 and ref. 6). Thus, this single substitution completely abolishes Pol γ’s superior selectivity against (-)-FTC-TP relative to RT. However, the R72I substitution in RT significantly reduces enzyme activity; the mutant protein is unable to incorporate dCTP at physiologically relevant rates as no incorporation of (-)-FTC-TP was observed even after a 5-h incubation.

Following the logic of our conclusions, the Pol γ A. I948 R substitution is predicted to have a smaller effect on (-)-3TC-TP discrimination, as (-)-3TC-TP lacks 5-F. To test this idea, we measured incorporation of (-)-3TC-TP by WT and I948R Pol γ. Although the $K_d$ for (-)-FTC-TP is increased 70-fold for the mutant Pol γ, it is increased only 3.8-fold for (-)-3TC-TP (Table 1), suggesting that the loss of interaction between R948 and 5-F of the cytosine drastically reduces affinity. Furthermore, the change in $k_{pol}$ values for (-)-3TC-TP and (-)-FTC-TP between mutant and WT enzymes are comparable, indicating that differences in incorporation efficiency of the inhibitors are primarily due to differences in binding affinity. Relative to wild

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**Fig. 3.** Active site interactions of RT and Pol γ with (-)-FTC-TP. The active site pockets for the model of RT (A) and ternary structure of Pol γ (B) are shown in complex with DNA and (-)-FTC-TP. Hydrogen bond interactions are detailed below the structures (the 5-F-arginine hydrogen bond distance shown in dashed red is 2.7 Å).

**Fig. 4.** Mutating residue 948 from an isoleucine to an arginine in Pol γ allows the formation of a hydrogen bond with (-)-FTC-TP. The ternary crystal structure of DNA and (-)-FTC-TP in complex with WT Pol γ (A) and I948R Pol γ (B). Replacing an isoleucine with an arginine at this position allows optimal Watson–Crick base pairing, ideal positioning of the catalytic magnesium ion, and a new hydrogen bond between the arginine and 5-F of (-)-FTC-TP. The superposition is shown in C.
type, the change in $K_d$ by the mutant I948R Pol for 3TC (3.8-fold) is comparable to dCTP (1.6-fold), substantiating the idea that the R948 interaction with the 5-F of (-)-FTC-TP is the major determinant for enhanced incorporation efficiency. Conversely and more importantly, the inability of I948 in wild-type Pol γ to interact with the 5-F is the primary reason for (-)-FTC being a less toxic NRTI than (-)-3TC.

**Discussion**

NRTIs present a unique opportunity for a rationalization of drug efficacy and toxicity because the structures of both the intended target HIV RT and the adverse reaction target human Pol γ are known. The two enzymes are thus excellent case studies for structure-based design of inhibitors that exert opposite effects on the structurally homologous viral and human enzymes. Cytosine-based NRTIs, ddc, (-)-3TC, and (-)-FTC, are structurally similar, and have similar drug efficacy against HIV RT, but their toxicity, mediated through Pol γ, spans more than five orders of magnitude. HIV RT is also insensitive to the chirality of the inhibitors, in contrast to human Pol γ. We have presented detailed structural evidence that explains the differential effects of a drug on the two enzymes and identifies the mechanism for how a NRTI exploits differences between the intended and adverse reaction targets to widen the therapeutic index. Although possessing common functions as DNA polymerases, Pol γ and RT differ in their active site and DNA-binding channel configuration, as well as active site flexibility. Determining structural differences between RT and Pol γ is a critical first step toward design of selective potent inhibitors against the HIV polymerase.

**Differences in Architecture Around the Substrate/NRTI Binding Site.** Using criteria used in assessing lead compound interaction with atoms of the target protein (25, 26), we examined residues within 4 Å distance from the NRTI, and compared them between Pol γ and RT. The substrate binding site of RT consists of the positively charged K65 and R72 on the “outside” of the substrate, and Y125, Q151 and M184 on the “inside” (Fig. 5A). Human Pol γ contains additional amino acids: the corresponding outside residues are the positively charged residues R925, R943, and K947, and two additional aromatic residues Y951 and Y955; the inside residues are R853, A1105, and Q1102 (Fig. 5B). Together, these amino acids form a more confined substrate-binding site in Pol γ than in RT.

**Different Mechanisms for Pol γ and RT Substrate Specificity and Fidelity.** The greater number of Pol γ residues forming the active site enables them to have dedicated roles (Fig. 5). For example, Y951, which is important for ribose recognition, is also, in part, a source of drug toxicity conferred by dideoxynucleoside-based NRTIs (20). Y955 facilitates precise base pairing and translocation. Residues on the inside of the incoming nucleotide play important roles in replication fidelity. The substitution A1105T promotes slipped mispairing, leading to increased insertion/deletion mutations, and R853W/Q substitutions result in increased mtDNA mutations (27, 28). The positively charged residues on the O-helix fingers domain are exclusively involved in triphosphate binding. In contrast, as fewer residues surround the RT active site, some perform multiple functions. Q151 serves as the steric gate, but Y183 and M184 on the YMDD loop appear to play complicated roles. Y183 contributes to both dNTP affinity and processivity (29), whereas M184V exhibits an antimutator phenotype (30). Importantly, R72, located on the fingers domain, contributes to both electrostatic interactions with the triphosphate moiety and catalysis and fidelity of RT. Remarkably, mutations conferring drug resistance have been found to affect all active site residues in RT, with the notable exception of R72.

**Differences in NRTI Interactions with Pol γ and RT.** In (-)-3TC, the deoxyribose is substituted with the larger oxathiolane moiety and has the nonnatural (-) chirality, compared with the normal substrate dCTP. Chirality has little impact on RT, as both (-) and (+)-3TC-TP isomers are incorporated with nearly the same efficiency. However, (-)-3TC-TP is two orders of magnitude less toxic, indicating Pol γ/A shows superior discrimination among sugar conformations. More significantly, the addition of 5-F to (-)-3TC-TP to yield (-)-FTC-TP boosts discrimination by another two orders of magnitude, again without substantially affecting drug efficacy for RT. In this study, we revealed that the structural basis for discrimination lies with the single I948 residue on the substrate-binding region of the Pol γ O-helix. Of major importance in drug design, I948 has no role in substrate incorporation, but is the primary cause for reducing the toxicity of (-)-FTC-TP. Interestingly, the residue that occupies the same topographical position in HIV RT is R72. Although the Pol γ I948R substitution, mimicking the RT residue, completely abolishes discrimination against (-)-FTC-TP, the RT R72I substitution yields an inactive protein. In fact, any substitution of R72 severely depresses viral fitness and viability (31–33), providing an explanation for the absence of R72 drug resistant mutants.

![Fig. 5. Functional and structural differences in RT and Pol γ residues surrounding (-)-FTC-TP reveal that the RT incoming nucleotide binding site (A) is more flexible thus less selective than that of human Pol γ (B), which consists of more residues to enhance nucleotide selectivity.](image-url)
Flexibility of the Active Site. The active site of RT is more flexible than that of Pol γ, as bound substrate or inhibitors can induce multiple conformational changes in the β-sheet fingers domain, resulting in different ground state inhibitor complexes. It has been proposed this could be a mechanism for the (-)-3TC resistance of M184V/V mutant enzymes (24). Additionally, even bound non-nucleoside reverse transcriptase inhibitors (NNRTIs) can alter the conformations of the active site or nucleic acid binding channel (35–37). On the contrary, the substrate-binding site of Pol γ undergoes more precise structural changes, evidenced by an unchanged O-helix configuration in normal substrate and inhibitor structures. Even though residues located in the immediate vicinity are not the sole determinants of fidelity, the stringent selection mechanism and confined substrate-binding site of human Pol γ should be able to discriminate against additional substrate analogs. Even bulkier inhibitors that can be incorporated by RT may be discriminated against by Pol γ, which would lower drug toxicity and widen the therapeutic window.

The lessons from our studies that are beneficial for rational drug design include: (i) revealing the structural differences between a drug target (HIV RT) and its adverse target (human Pol γ), and the differential structural flexibility of the active sites; (ii) understanding the different functional roles of active site residues; and (iii) identifying inhibitors that can exploit the structural differences. To provide more robust structural functional correlations, structural studies are needed.

In summary, human Pol γ and HIV RT use different mechanisms for substrate and inhibitor incorporation. A single residue, Pol γ A498, which has no significant role in substrate incorporation, is essential for (-)-FTC-TP selectivity, whereas RT R72 is important for both inhibitor and substrate binding. These conclusions are of paramount importance in drug design: we have shown that the S-F modification in (-)-FTC-TP interacts with a residue vital for activity in the target enzyme but is repelled by the equivalent residue in the adverse reaction target.

Experimental Procedures
Reagents. dCTP was purchased from GE Healthcare, and (-)-FTC-TP was purchased from Toronto Research Chemicals and was purified before use (SI Experimental Procedures). DNA oligonucleotides used for kinetic studies, D2 (5′-GCCTGGCAGCGCTCAACAACTC) and D45 (5′-CGGAGGTCGTTGGAGTGGTGGCAGGCAGGAGGAGTCGTTGGAGTGGTGGCAGGCAGGAGGTATGGCACTGGCCGTCGT), were purchased from Integrated DNA Technologies (Coralville, IA), and were purified using a 20% polyacrylamide denaturing gel (8 M urea). To generate the D23/D45 primer/template, T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (PerkinElmer) was used to phosphorylate oligonucleotides, which was then annealed to the D45 oligonucleotide at 95 °C (5 min), 55 °C (15 min), and 37 °C (10 min). Synthetic oligonucleotides used for crystallography were obtained from Midland Certified and IDT. The nucleic acid scaffolds were generated by mixing equimolar D28 template (5′-CCAGGTATGCGTCTGCGCTGGTTTCTGCGT) and D24 primer (5′-CGAAGACAACGCGACCAGCTGCTGACACAGCGC) at 1 mM primer/template. The DNA oligos were then annealed by heating at 95 °C followed by cooling slowly to room temperature.

Protein Expression, Purification, and Crystallization. To preserve the primer/template DNA during crystallization, Pol γ was made exonuclease-deficient by substituting the catalytic residues with alanines (D198A and E200A). Both the wild type (WT) and exonuclease-deficient (exo-), D198A/E200A (exo-)) Pol γ lack mitochondrial localization sequence (residues 1-29) and the glutamine stretch (polyQ) (residues 43-52). The genes were cloned in pBacPak9 vector (Clontech). Pol γ A498R was constructed in the Pol γ exo- clone by site-specific mutagenesis using QuikChange (Agilent). The WT or mutant C-terminal His-tagged proteins were made in Sf9 insect cells (Invitrogen), grown at 27 °C in SF900-II SFM growth medium and infected at density 1×10^6 cells per mL. The cells were harvested 72 h postinfection based on a modified procedure (39). Pol γ B was used in crystallization as a deletion mutant (Pol γ B-Δ14), in which two residues (137–138) was replaced by the dipeptide Gly-Gly (40, 41). WT or the mutant Pol γ B were cloned into a pET22b (+) vector as a C-Terminal His-tagged construct in E. coli Rosetta (DE3) cells (Novagen). Proteins were purified as described previously (42). The activities of the modified subunits possess near wild-type DNA synthesis activities (39, 43). The RT R72I substitution was introduced to both monomers of the recombinant RT p66/p51 heterodimer using QuikChange. The C-terminal His-tagged R72I RT was made in E. coli and purified as described previously (42, 44). Purity of all proteins was estimated to be >95% after SDS-polyacrylamide gel electrophoresis. For crystallization, the WT or I948R Pol γ holoenzyme (~20 mg/mL) was incubated with 1 mM (-)-FTC-TP or (-)-FTC-TP, and D24/D28 primer/template. Crystals were obtained from sitting-drop method by mixing a 1:1 ratio of the complex with well solution containing 3% (wt/vol) PEG 8000, 150 mM NaCl, 10 mM CaCl_2, 100 mM Mes pH 6.2, 2 mM 2-mercaptoethanol, 0.8 M SD52-201, 1–6% (wt/vol) sucrose, and 2% (vol/vol) Jefaffine M-600. The crystals grew to a maximum size of 0.4 × 0.2 × 0.2 mm within 2–4 d. Before flash freezing, crystals were cryo-protected by introducing a stabilization solution containing glycerol and MPD (up to 20%) and the inhibitor into the crystallization drop. X-ray diffraction data were collected at 100 K using synchrotron sources at Advanced Photon Source and Advanced Light Sources and processed with HKL (45). The structures were determined by molecular replacement method using the previously determined Pol γ-DNA-dNTP ternary complex structure, and refined using the program Phenix (17, 18). The experimental maps around the (-)-FTC-TP or (-)-FTC-TP binding site are illustrated in Figs. S1 and S2.

Single Nucleotide Incorporation Assays. Single nucleotide incorporation assays for were performed in pre-steady-state time frames using a Row-3 bioluminescent quench flow apparatus (Kintek). All experiments were performed at 37 °C in 50 mM Tris (pH 7.8 at 37 °C) and 100 mM NaCl (I948R Pol γ) or 50 mM NaCl (RT21I R72I). The active site concentration for the I948R Pol γ holoenzyme was determined using a burst assay: 50 nM Pol γ I948R was incubated with 250 nM Pol γ b 5 min on ice. The mixture was then incubated with 300 nM of D23/D45 primer/template for 15 min on ice, and followed by equilibration at 37 °C for 5 min. The enzyme-DNA mixture was then mixed with MgCl_2 (5 mM) and dCTP (25 μM) and incubated at 37 °C for various times. After quenching the reaction with 0.3 M EDTA (pH 8.0), the product D24 was separated from the substrate D23 on a 20% denaturing polyacrylamide sequencing gel (8 M urea), and quantified by phosphorimaging (Bio-Rad Molecular Imager FX). A plot of D24 concentration versus time was fit to a burst equation to obtain the active site concentration: [product] = [A]_1 × [exp(-kt)] + [K_a × [dNTP]]/[K_a + [dNTP]], where [A]_1 is observed burst amplitude, K_a is observed steady-state rate, and K_a is dissociation constant of the nucleotide for the enzyme + DNA complex. [dNTP] is the concentration of the natural nucleotide or analog. For RT21I R72I, longer times were required for nucleotide incorporation (up to 5 h), so reactions were mixed manually rather than using a rapid chemical quench. Single nucleotide and analog incorporation experiments were performed under single turnover conditions, in which RT21I R72I (500 nM, total protein concentration) was preincubated with 1.25 μM Pol γ b, mixed with D23/D45 primer/template (50 nM), incubated for 15 min on ice followed by 5 min at 37 °C. Reactions were initiated by rapidly mixing (1:1) 50 μM dNTP (20 μM MgCl_2) and varying concentrations of dCTP (0.1–0.5 μM) and dGTP (0.1–0.5 μM). Similar conditions were used for examining (-)-FTC-TP and (-)-3TC-TP (46) (SI Experimental Procedures). The reaction was quenched with EDTA (0.3 M, pH 8), and the product was separated and quantified. The amount of product formed versus time was fit to a single exponential equation for each nucleotide concentration: [product] = [A]_1 × [exp(-kt)] + [K_a × [dNTP]/(K_a + [dNTP])], to obtain K_a, the maximum rate of nucleotide incorporation, and K_a, the dissociation constant of the nucleotide for the enzyme + DNA complex. [dNTP] is the concentration of the natural nucleotide or analog. For RT21I R72I, longer times were required for nucleotide incorporation (up to 5 h), so reactions were mixed manually rather than using a rapid chemical quench. Single nucleotide and analog incorporation experiments were performed under single turnover conditions, in which RT21I R72I (500 nM, total protein concentration) was preincubated with D23/D45 primer/template (50 nM) on ice for 15 min. After pre-equilibration at 37 °C, the solution was then mixed with concentrations of dCTP (100 μM or 200 μM), or (-)-FTC-TP (25 μM or 6 μM) and MgCl_2 (5 mM). Following quenching, gel separation, quantitation, and fitting to a single exponential equation as described, a K_a value was determined in the case of dCTP [no incorporation of (-)-FTC-TP was observed after 5 h at either concentration]. The reaction rate was too slow to be physiologically meaningful, so the rates of incorporation at additional dCTP concentrations were not measured. However, K_a versus nucleotide concentration was plotted and fit to a hyperbolic equation, and the resulting K_a and K_a were used to give an estimate of efficiency, because limited concentrations of dCTP were used.

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