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Structural insight on processivity, human disease and antiviral drug toxicity

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Abstract

DNA polymerase gamma (Pol γ) is a nuclear encoded, mitochondrially located replicase that conducts all DNA synthesis in the organelle. Structurally, human Pol γ closely resembles bacteriophage T7 DNA polymerase. Perhaps due to this prokaryotic-like feature, Pol γ is highly susceptible to inhibition by drugs designed against HIV reverse transcriptase and HCV RNA polymerase. In this review, I summarize recent structural and biochemical studies towards understanding Pol γ -mediated antiviral drug toxicity.

Unlike the maintenance of nuclear DNA that involves multiple DNA polymerases, a single enzyme, Pol γ , performs all DNA synthesis in mitochondria [1]. Mitochondrial DNA (mtDNA) codes for tRNAs and rRNAs, necessary for protein synthesis in the organelle, but only encodes a subset of the genes for the oxidative phosphorylation electron transfer chain. If mitochondria did evolve from α -protobacteria, most of the bacterial genes, including genes for Pol γ and the other components of oxidative phosphorylation, have migrated to the nucleus. Mutations affecting Pol γ manifest as human diseases involving multisystems with a varied onset time (for reviews see [2-4]). This creates a challenge to correlate genotype with phenotype. A better understanding of the molecular basis for how Pol γ mutations translate to clinical disorders is urgently needed. Pol γ has also been linked to the toxicity of antiviral reagents designed to inhibit viral replication [5-8]. Most notably, these include nucleoside analogs designed to inhibit HIV reverse transcriptase (NRTIs) and hepatitis C virus RNA polymerase. Prolonged usage of NRTIs during AIDS treatment causes severe adverse reactions with clear indications of mitochondrial dysfunction [9].

Human Pol γ holoenzyme is comprised of a catalytic subunit Pol γ A and an accessory dimeric subunit Pol γ B. Pol γ contains three enzymatic activities, DNA polymerase (*pol*), 3'->5' proofreading exonuclease (*exo*), and a 5'-deoxyribosephosphate (5'-dRP) lyase activity [10-12]. Despite all three active sites being located on Pol γ A, Pol γ B alters all activities upon formation of holoenzyme. In comparison to Pol γ A alone, holoenzyme has elevated *pol* and 5'-dRP lyase activities but decreased *exo* activity [10,13-15]. Holoenzyme-catalyzed synthesis is also much more processive.

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A crystal structure of human Pol γ holoenzyme has been recently determined [16]. Successful crystallization of Pol γ required modification of both subunits. An N-terminal poly-glutamine were deleted from Pol γ A, and a non-essential four-helical bundle was removed from Pol γ B. This deletion of Pol γ B is critical for crystallization because a crystal contact is formed at this newly created surface. The holoenzyme crystals were initially assigned to P3₂21 space group, but later found to be twinned and belong to space group P3₂. The structure was solved using the detwinning function in the program cns [54].

The Pol γ A *exo* domain is N-terminal located while the C-terminal polymerase (*pol*) domain is split by an important and unusually large spacer domain (Fig. 1). The *pol* domain presents a typical polymerase right-hand fold with palm, thumb and fingers subdomains. Despite the evidence for a bacterial origin of mitochondria, Pol γ A closely resembles bacteriophage T7 DNAP structurally. The spacer domain can be subdivided into IP (intrinsic processivity) and AID (accessory interacting) subdomain. The IP subdomain presents a unique fold absent in other Pol A family enzymes, whereas the AID subdomain forms the major interacting surface with the accessory subunit Pol γ B in a manner reminiscent of the way T7 gp5 binds to its accessory protein, thioredoxin, in forming T7 DNAP. The two catalytic palm domains superimpose so well (with a rmsd 2.3 Å) that a model of the Pol γ -DNA complex can be constructed by docking the primer-template DNA from the T7 DNAP-DNA structure [17] (Fig. 2).

Mammalian Pol γ B is a structural homolog of certain class II aminoacyl-tRNA synthetases (aaRS), particularly of threonyl-tRNA and glycyl-tRNA synthetases [18], but no longer possesses aaRS activity. However, the tRNA-binding patch appears to be conserved, and Pol γ B is predicted to bind to some tRNAs, although this has not been demonstrated directly. Interestingly, mammalian Pol γ B is a homodimer, but its counterparts in insects (e.g., *Drosophila* and mosquito) are monomeric, and fungal Pol γ seems to have no requirement for an accessory subunit.

Processivity of Pol γ

In humans, Pol γ A interacts with the homodimeric Pol γ B asymmetrically: an extensive interaction exists between Pol γ A and the monomer of Pol γ B proximal to the catalytic subunit, but makes very limited interactions with the distal monomer (Fig. 3). Such an arrangement suggests that a monomeric Pol γ B should be functional. A monomeric human Pol γ B was generated by changing the human protein so that it more closely resembles its insect counterpart. Deletion of a 4-helical bundle in Pol γ B that constitutes 40% of the dimer interface but has no counterpart in *Drosophila*, and removal of two inter-subunit salt bridges, makes human Pol γ B monomeric. This construct enabled comparative studies of the monomeric and dimeric forms.

Processivity is defined as the length of DNA synthesized per binding event, it is determined by at least two factors: the rate of polymerization (k_{pol}), and the duration of DNA-binding (defined by the off rate, k_{off}). Dimeric Pol γ B increases the affinity of Pol γ A for DNA and accelerates the polymerization rate. The two factors contribute synergistically to the increased processivity of holoenzyme and are neatly divided between the two Pol γ B

monomers: the proximal monomer enhances DNA affinity while the distal monomer accelerates the polymerization rate [19]. These results suggest that, in the absence of compensating changes in the catalytic subunit Pol γ A, the mtDNAP of organisms with a monomeric Pol γ B should exhibit lower processivity. This idea has not yet been tested under identical reaction conditions using mtDNAPs isolated from different sources. Of particular note is that human Pol γ B represents a rare example of identical proteins having clearly distinct biological or biochemical functions.

The structure of holoenzyme also sheds light on the mechanism of processivity enhancement by Pol γ B. The interaction between Pol γ A and Pol γ B in holoenzyme is largely maintained by the hydrophobic interaction between the Pol γ A AID subdomain and the proximal Pol γ B monomer. The AID subdomain is predicted to be disordered in the catalytic subunit, but association with Pol γ B stabilizes the other surface of the amphipathic AID subdomain, which contains a high density of positively charged residues (⁴⁹⁶KQKKAKKVKK⁵⁰⁵, the K-tract) that can bind DNA [16]. The K-tract interaction doubles the DNA-contact length of Pol γ A from 10 bp to ~20 bp, clearly enhancing the affinity of holoenzyme for DNA.

The holoenzyme structure suggests that processivity enhancement by Pol γ B is entirely indirect, even though the accessory protein possesses an intrinsic DNA-binding property. Pol γ B DNA-binding site mutants still function as a processivity factor and there is little or no effect on holoenzyme activity on primed single-stranded DNA [20-22]. Nonetheless, the region is important in replication, as the same mutants inhibit synthesis by the intact replisome on duplex DNA [21]. This result suggests that Pol γ B may stabilize the replisome by interacting with the mitochondrial helicase and/or SSB, or perhaps with forked DNA during replication. The Pol γ A AID subdomain may also contribute to replisome formation as the corresponding subdomain in T7 gp5 directly interacts with T7 helicase [23].

Pol γ B coordinates *pol* and *exo* activities

Intriguingly, Pol γ B regulates *pol* and *exo* activities in opposite directions. While Pol γ B increases the rate of DNA synthesis and the processivity of holoenzyme, it simultaneously reduces exonuclease activity. When an incorrect nucleotide is incorporated, it is shuttled to the *exo* site for excision. The two active sites, which are separated by 40 Å, alternate in function, as a primer strand can only be bound to one active site at any given time. Altering the binding equilibrium between the two sites therefore affects each activity in opposite directions.

The mechanism of simultaneous regulation by Pol γ B of both polymerase and exonuclease activities of Pol γ A activities was partially revealed during an investigation of certain Pol γ A mutants. Substitutions of Pol γ A R²³² have been implicated in severe neurological and muscular disorders. The mutant Pol γ A displays almost normal *pol* and *exo* activities – an expected result because R²³² is located outside of either active site. However, the mutant holoenzyme shows almost no response to the presence of Pol γ B even though the two subunits appear to interact normally [24]. The studies suggest that Pol γ B biases the equilibrium of wild-type holoenzyme towards positioning the primer terminus in *pol*, thereby increasing the polymerization rate and reducing exonuclease activity. Substitution of

Pol γ A R²³² appears to block the signal from Pol γ B, possibly by preventing the necessary structural changes associated with shuttling the primer terminus between *exo* and *pol*. Mutant holoenzymes containing Pol γ A R²³² variants therefore exhibit elevated nucleolytic activity and reduced DNA synthesis. These data explain the low DNA content (3-5%) in patients harboring R²³² mutants [25-28], and also suggests how the nucleolytic activity of Pol γ A can be regulated by factors beyond the immediate vicinity of the exonuclease active site.

The third function of Pol γ , 5'-dRP lyase activity, is used during base-excision repair [29-31]. In mitochondria, the high concentration of reactive oxygen species generated in redox reactions is highly damaging to DNA, mtDNA experiences a 10-fold higher rate of oxidative damage than nuclear DNA [32,33]. Historically, DNA repair in mitochondria was thought to be minimal and damaged DNA was simply degraded. However, good evidence of robust repair machinery for base-excision repair, mismatch repair and double-strand break has now been found [10,34-36]. The 5'-dRP lyase activity of Pol γ positions it as an integral part of base-excision repair where, after removal of a damaged base by specific glycosylases, the resulting abasic site is eliminated by the lyase activity. Relative to the prototypical eukaryotic repair DNA polymerase, Pol β , Pol γ exhibits comparable 5'-dRP activity [10]. In a 5'-dRP lyase reaction, an electrophilic nitrogen, typically either an N-terminal group or an internal lysine, makes a nucleophilic attack on the C1' of the 5'-dRP and forms a Schiff's base. Subsequently, through a β -elimination reaction, the 5'-dRP is excised. Evidence for the involvement of Pol γ in removal of 5'-dRP includes trapping a covalently linked Pol γ -DNA intermediate [13,34]. Upon 5'-dRP removal, the polymerase activity of Pol γ fills in the one nucleotide gap, allowing DNA ligase to restore the integrity of the DNA.

Like its regulatory roles in other Pol γ A activities, Pol γ B greatly enhances 5'-dRP lyase activity in holoenzyme [13]. Although the location of the lyase active site on Pol γ A is currently unknown, regions of Pol γ B affecting holoenzyme lyase activity have been identified. These contribute either to formation of Pol γ B dimers or to its intrinsic DNA-binding activity. The nucleophile for Pol γ A 5'-dRP activity is not known but its likely general area can be deduced from the function of Pol γ B. Because Pol γ B increases both *pol* and 5'-dRP lyase activities by biasing the primer terminus towards the *pol* site, the 5'-dRP lyase nucleophile should be located in the vicinity of *pol*.

Pol γ and antiviral drug toxicity

Successful AIDS treatment requires combinatory administration of drugs inhibiting HIV protease, reverse transcriptase and integrase (HAART therapy). The central components of HAART are inhibitors against HIV reverse transcriptase (RT), which is responsible for replicating the viral genome. There are two broad classes of inhibitors against HIV RT: non-nucleoside analogs that allosterically inhibit enzyme activity, and nucleoside analogs (NRTIs) that directly compete with incoming nucleotide triphosphates during synthesis.

After phosphorylation by cellular enzymes, most NRTIs are dNTP analogs where the ribose moiety lacks a 3'-OH group (Fig. 4); they therefore serve as chain terminators. In principle, NRTIs can inhibit the activities of any human DNA polymerase causing drug toxicity, but

studies have revealed that of the human DNA replicases, Pol γ , Pol δ and Pol ϵ , as well as the primase Pol α , Pol γ is inhibited 10^1 - 10^5 -fold more effectively than other polymerases [37]. The high sensitivity of Pol γ to NRTIs inhibition explains, in part, the NRTIs mitochondrial toxicity. Other sources contribute to the mitochondrial adverse reactions are high drug concentrations as a consequence of active transporters and genetic predisposition [38,39]. However, since these inhibitors are effective against HIV-RT, human Pol γ must share some common structural features and/or mechanisms for inhibitor incorporation with the viral enzyme.

Selective inhibition of Pol γ indicates that it is either less discriminating between an NRTI and the substrate nucleotide or less efficient in excision of NRTI-containing primers. The rate of NRTI incorporation does not necessarily correlate with its rate of removal, suggesting that the *pol* and *exo* active sites exhibit independent discrimination criteria. For example, ddCTP, (-)3TC and (+)3TC are all cytidine derivatives (Fig. 4), but Pol γ incorporates ddCTP most effectively but removes it the least. Conversely, Pol γ incorporates (-)3TC least effectively but removes it the fastest [40,41]. Consequently, ddC is among the most toxic of inhibitors. These results indicate the most useful drugs should stimulate high nucleolytic activity and/or low incorporation for human Pol γ , but still be a good substrate for HIV RT.

Selective inhibition of Pol γ

To understand the basis for Pol γ 's susceptibility to NRTIs, it is useful to compare the Pol γ structure to other DNA replicases to try and identify unique features of Pol γ that make it a target for NRTIs. It is also helpful to compare structures of Pol γ with HIV-RT to spot similarities and differences, and to try and exploit differences in the design of new antiviral drugs.

The high susceptibility of Pol γ to NRTIs may lie in structural features around the *pol* and *exo* domains. Pol γ belongs to the Pol A family, whereas other human replicases Pol α , Pol δ and Pol ϵ are Pol B family members. Regardless, the structures of both (and other) families are related: the palm subdomain is the most conserved, but large variations have been observed in the thumb and fingers subdomains [42-45], which form the DNA template and dNTP substrate-binding site, respectively.

Both A-family and B-family polymerases use a positively charged helix in the fingers domain, the 'O-helix', to bind the negatively charged phosphates of the incoming dNTP. To date, the most complete structural characterization of B-family polymerases is from bacteriophage RB69, where the structures of apo, and both synthesis and editing complexes have been determined [44,46,47]. Human Pol α and Pol δ share high sequence homology with RB69, especially around the *exo* and *pol* active sites. In an attempt to rationalize the varying toxicity of NRTIs towards human DNA polymerases, structures of RB69 DNAP and the composite Pol γ -DNA complex (Fig. 5-6) can be therefore used in an initial attempt to rationalize the varying toxicity of NRTIs towards human DNA polymerases.

Several structural features may contribute to Pol γ 's relatively low discrimination against NRTIs. In this context, discussions of polymerase fidelity should focus on Watson-Crick

(W-C) base-pairing as well as the correct structure of the ribose, because Pol γ exhibits varied sensitivity to NRTIs containing different deoxyribose derivatives. Most replicating polymerases use conformational changes in the fingers domain after binding to dNTP to further increase the affinity of the correct nucleotide beyond W-C base-pairing interaction. The B-family RB69 DNAP apo enzyme undergoes a $\sim 60^\circ$ rotation after binding to DNA and a dNTP, a value much larger than Pol γ can exhibit because its apo structure is more closed than other DNA polymerases. The extreme case is seen in the Y-family lesion-bypassing polymerases that conduct error-prone DNA synthesis, where the enzymes barely show any conformational changes in forming the equivalent ternary complex [48-50]. Pol A and Pol B members also use different amino acids to monitor W-C complementarity of an incoming nucleotide (Fig. 5), which allows for differences in the how the enzymes discriminate between the correct and incorrect substrate. A misincorporated nucleotide can be excised either before or after enzyme translocation. Again due to the structural difference in their fingers domain, Pol A and Pol B members accomplish mismatch discrimination using different residues.

To begin to understand Pol γ *exo* activity, we composed a Pol γ editing complex by superimposing the *exo* domain of RB69 DNAP editing complex to Pol γ and then docked the strand-separated primer-template onto Pol γ . Although obviously not rigorous, this exercise is not unreasonable because the *exo* domain does not undergo conformational changes upon DNA-binding for Pol A or Pol B polymerases [46,47,51], and the conformation of *exo* in apo Pol γ is likely very similar to that in its editing mode. The composite structure suggests that the Pol γ *exo* domain mainly interacts with the primer strand; significantly it predicts a specific interaction between an aspartate, D³⁷², with the 3'-OH of the primer (Fig. 6), and thus a ddNMP-terminated primer may only bind weakly to the *exo* site. Weak binding leads to reduced hydrolysis [40,52].

The basis for the toxicity of NRTIs towards Pol γ holoenzyme can be illustrated by comparing the structures of an NRTI bound to HIV RT with a modeled Pol γ -DNA complex. Similarities do exist between the two enzymes, including the arrangement of the palm catalytic subdomain, and these may indeed be the basis for toxicity. Significant differences are also found surrounding the incoming nucleotide-binding site (Fig. 7): Both human Pol γ and HIV RT utilize positively charged residues on the fingers domain to bind the negatively charged triphosphate of an incoming dNTP. However, the interaction of the two enzymes with the nucleoside moiety is different. In Pol γ A, the fingers subdomain is α -helical whereas in HIV RT the fingers domain is composed of β sheets. Obviously, more explicitly useful comparisons have to wait for structures of Pol γ -DNA complexed to NRTIs. However, the predicted variations strongly suggest that significant differences exist between the substrate-binding sites of the two enzymes that could be exploited when designing higher potency antiviral drugs that exhibit lower toxicity. However, a series of structures of Pol γ , captured at all stages of inhibitor incorporation as well as in the editing mode, would be invaluable in understanding antiviral drug toxicity.

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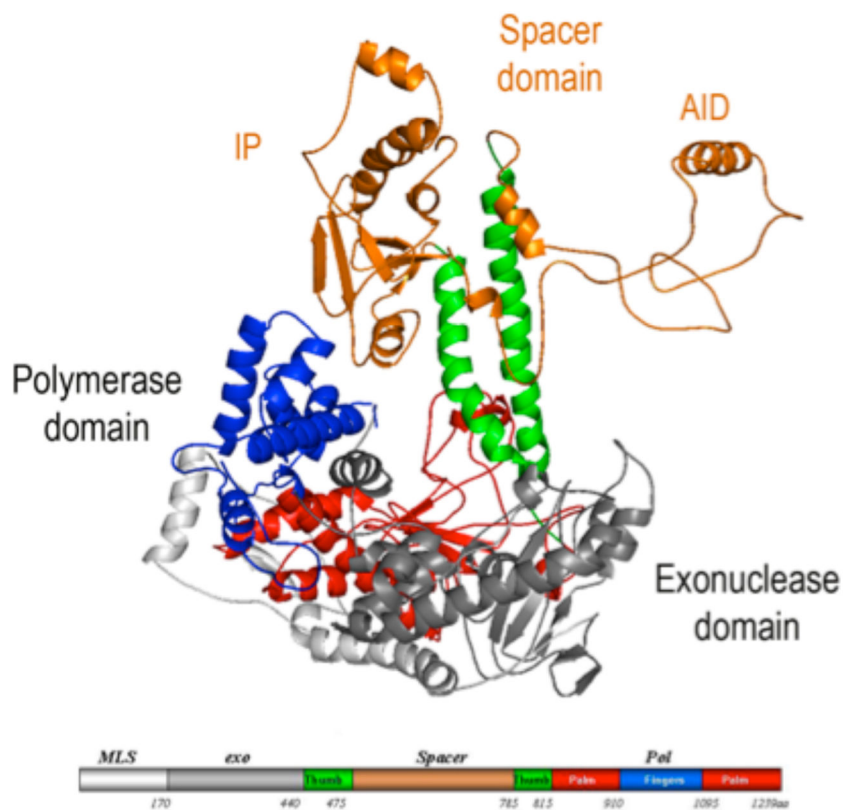


Figure 1. Structure of Pol γ A. The *pol* domain shows a canonical ‘right-hand’ configuration with thumb (green), palm (red) and fingers (blue) subdomains, and the *exo* domain (grey). The spacer domain (orange) presents a unique structure and is divided into two subdomains. Domains are shown in a linear form where the N-terminal domain contains residues 1-170; *exo*: 171-440; spacer: 476-785; *pol*: 441-475 and 786-1239.

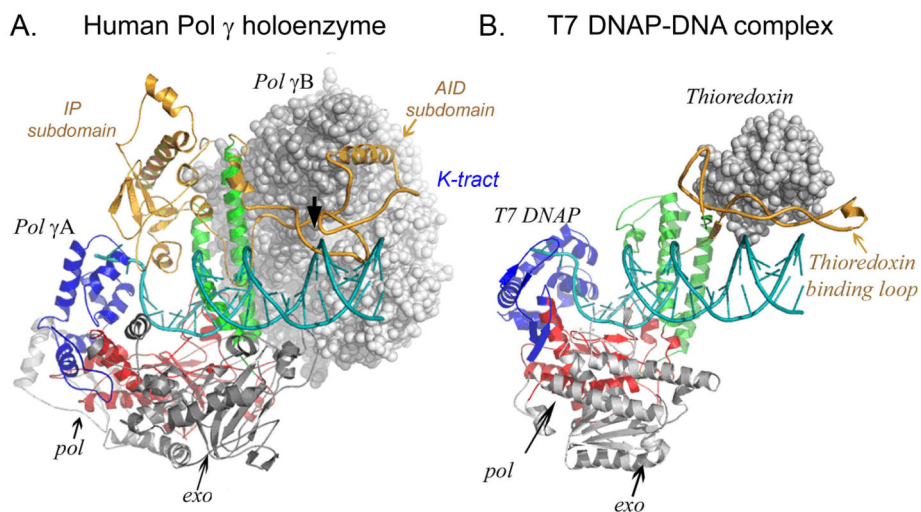


Figure 2. Similarities between human Pol γ and T7 DNA polymerase. The catalytic subunits (ribbon diagram) of the two polymerases are oriented by superpositioning palm (red) subdomains. Pol γ -DNA complex is constructed with a docked DNA from a T7 DNAP-DNA complex structure. The accessory subunits are presented in space-filling models.

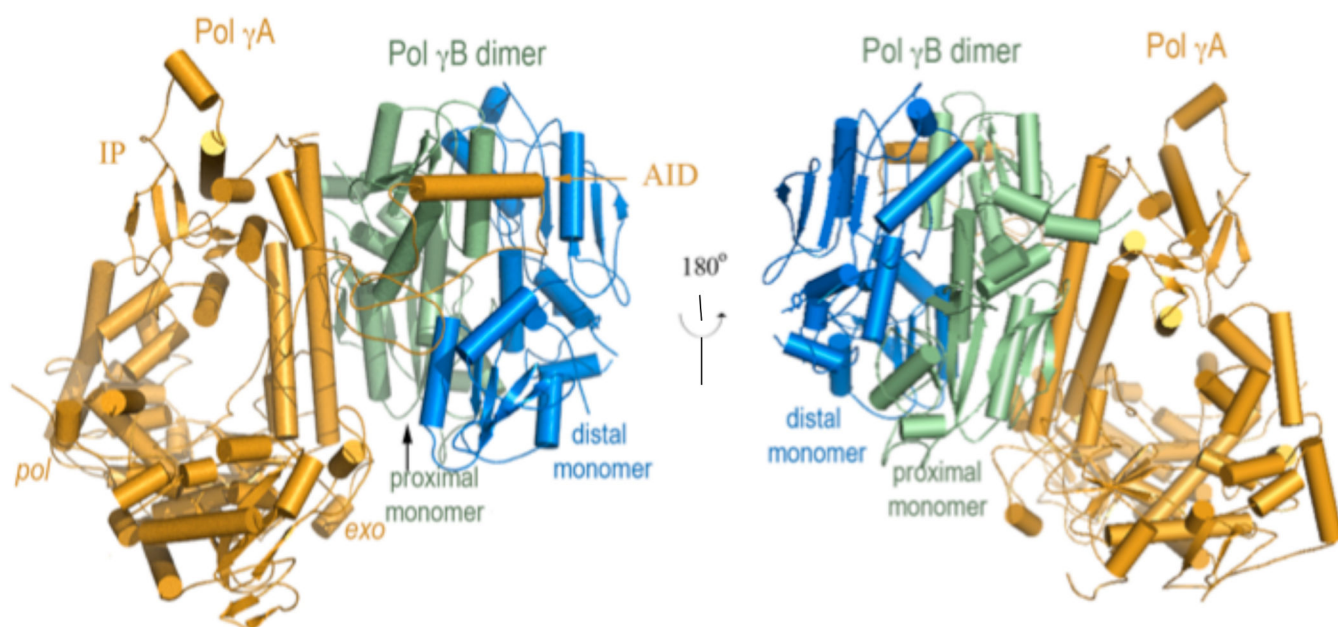


Figure 3. Structure of the heterotrimeric Pol γ holoenzyme containing one catalytic subunit Pol γ A (orange) and the proximal (green) and distal (blue) monomers of Pol γ B. Pol γ A primarily interacts with the proximal monomer of the dimeric Pol γ B.

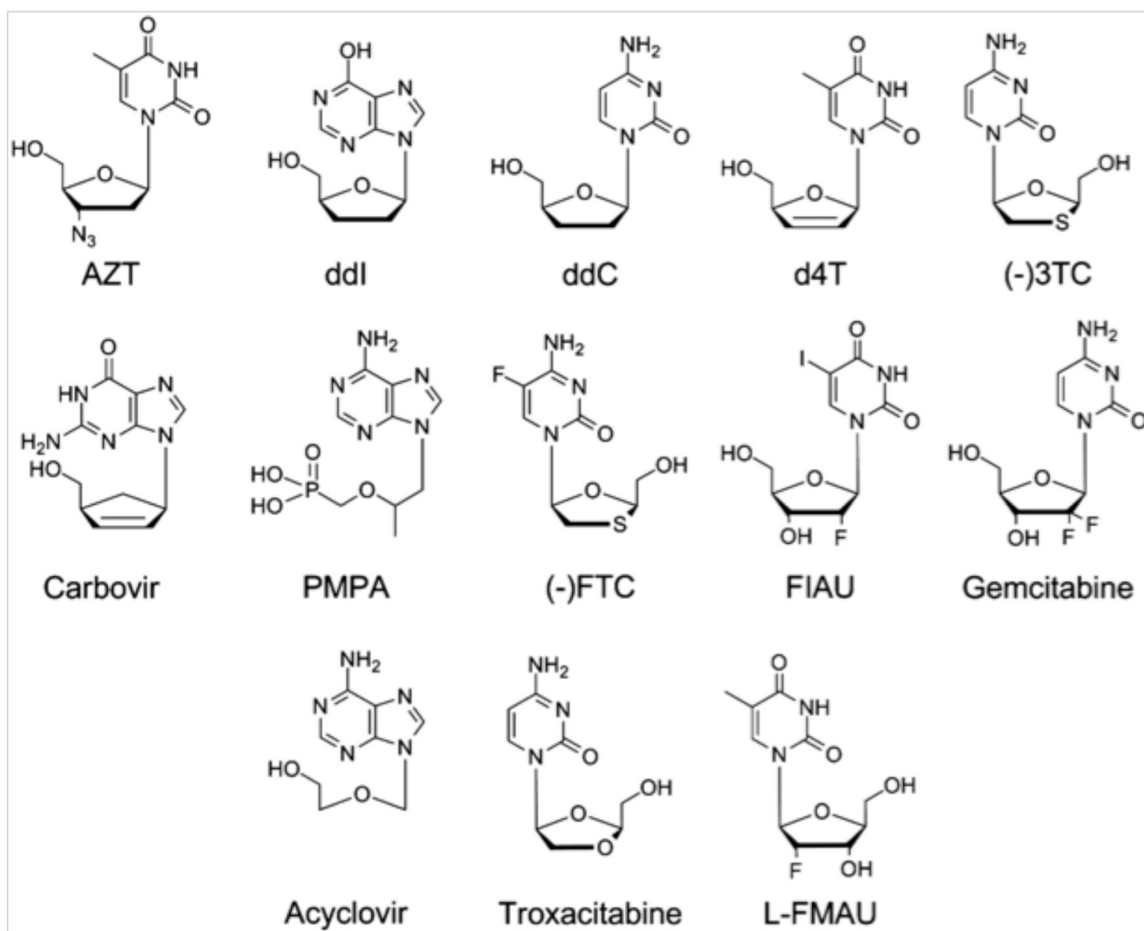


Figure 4.
Structures of selected nucleoside HIV reverse transcriptase analog inhibitors.

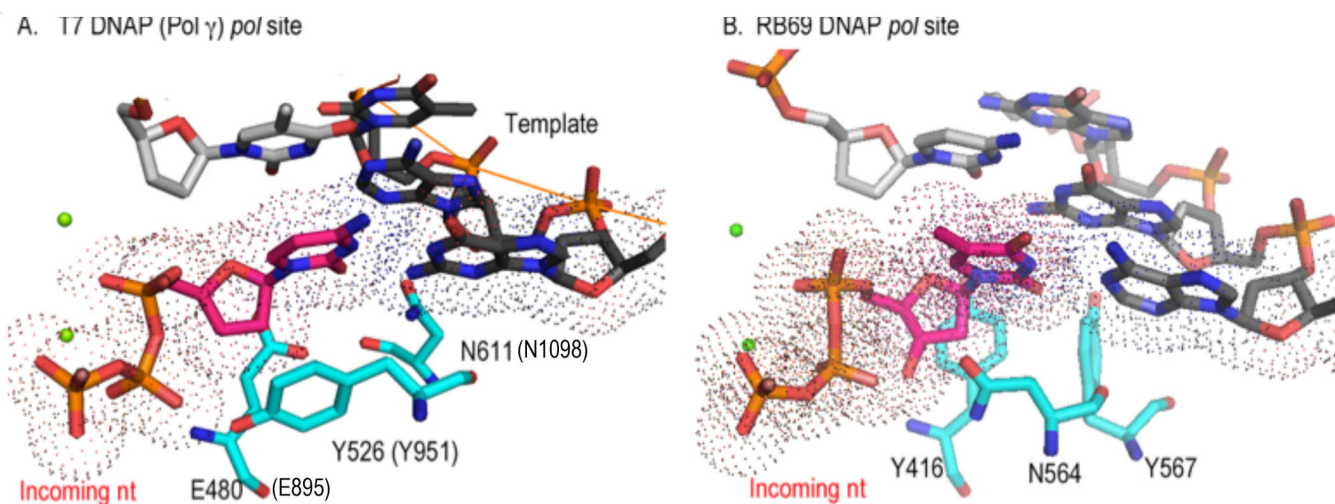


Figure 5.

Comparison of Pol A family and Pol B family in the *pol* site Pol A and Pol B members enforce W-C complementarity between the templating nucleotide and incoming nucleotide differently. In Pol γ (and T7 RNAP), they are accomplished by two sets of check-point residues in the minor groove: E895 and N1098 (E480 and N611) before translocation and R853 and Q1102 (R429 and Q615) after translocation, respectively. In RB69, both bps can be ensured by three tyrosines (Y416, Y564 and Y391) that are conserved in Pol α and Pol δ . The latter arrangement may be the more efficient and rigorous. Deoxyribose selection is conferred in part by Y⁹⁵¹ on the O-helix of the fingers domain. The Y951F substitution in Pol γ A reduces ddCTP incorporation by 5000 fold [53].

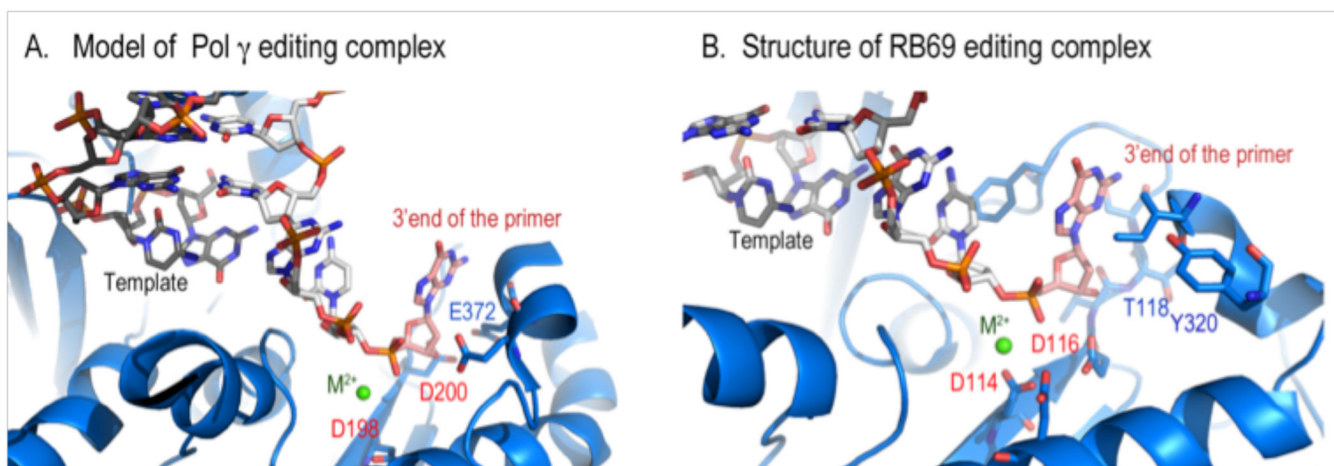


Figure 6. Comparison of Pol A and Pol B editing complexes. A composed Pol γ editing complex with a unwound primer-template from RB69 showing possible specific interaction between *exo* residue E374 with the 3'OH of the primer.

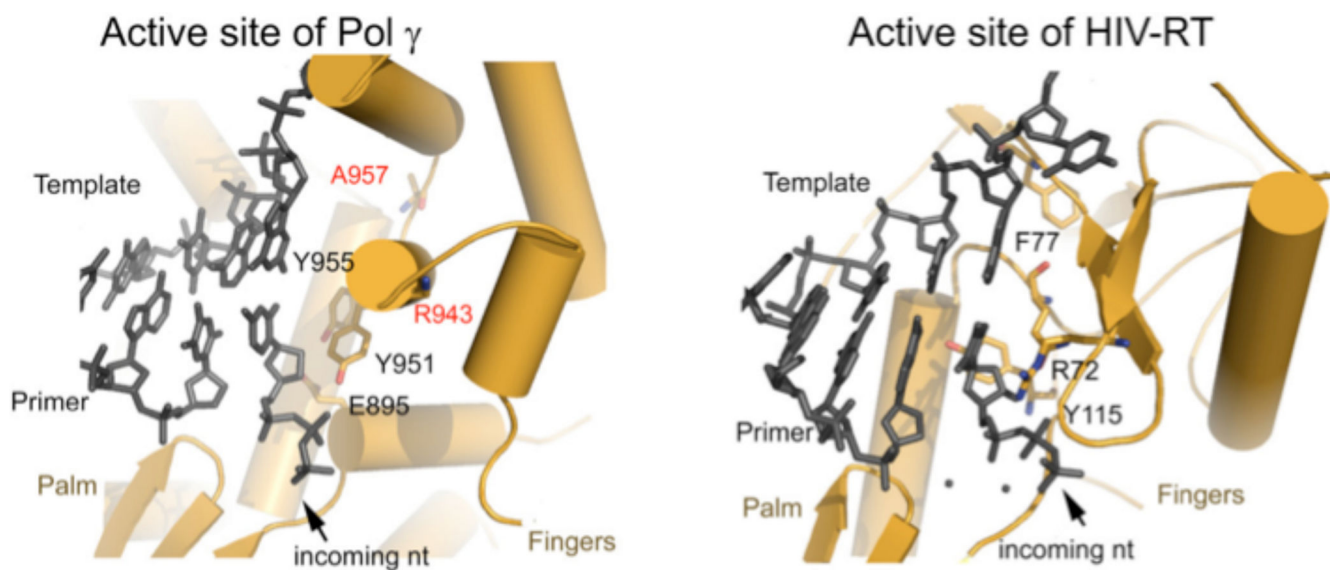


Figure 7. Active sites of human Pol γ (left) and HIV-RT (right). The nucleotide binding site of the two enzymes are different: In Pol γ : it is likely bounded by E⁸⁹⁵, Y⁹⁵¹, Y⁹⁵⁵, and Q¹¹⁰², whereas in HIV RT the binding site is bounded by R⁷², F⁷⁷, Y¹¹⁵, and Q¹⁵¹. The difference provides exploitable space for more potent and specific antiviral drug design.