HIV-1 reverse transcriptase complex with DNA and nevirapine reveals non-nucleoside inhibition mechanism

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Combinations of nucleoside and non-nucleoside inhibitors (NNRTIs) of HIV-1 reverse transcriptase (RT) are widely used in anti-AIDS therapies. Five NNRTIs, including nevirapine, are clinical drugs; however, the molecular mechanism of inhibition by NNRTIs is not clear. We determined the crystal structures of RT–DNA–nevirapine, RT–DNA, and RT–DNA–AZT-triphosphate complexes at 2.85-, 2.70- and 2.80-Å resolution, respectively. The RT–DNA complex in the crystal could bind nevirapine or AZT-triphosphate but not both. Binding of nevirapine led to opening of the NNRTI-binding pocket. The pocket formation caused shifting of the 3’ end of the DNA primer by ~5.5 Å away from its polymerase active site position. Nucleic acid interactions with fingers and palm subdomains were reduced, the dNTP-binding pocket was distorted and the thumb opened up. The structures elucidate complementary roles of nucleoside and non-nucleoside inhibitors in inhibiting RT.

The enzyme RT of HIV-1 is responsible for copying the viral single-stranded RNA genome to double-stranded DNA (dsDNA) in the cytoplasm of infected cells. This 117-kDa heterodimeric (p66 and p51) protein performs three catalytic steps: (i) RNA-dependent DNA polymerization to synthesize a (−) strand DNA complementing the viral (−) strand RNA genome, (ii) RNase H cleavage of the RNA strand, and (iii) DNA-dependent DNA polymerization to synthesize dsDNA using the (−) strand DNA as the template. The dsDNA is transported into the nucleus as a preintegration complex and integrated into the chromosome of the infected cell. HIV-1 infection is chronic and requires lifelong treatment. Emergence of drug-resistant HIV-1 strains and side effects impede the long-term use of drugs; therefore, new drugs against existing and new targets are required and are constantly being developed. HIV-1 infection, in general, is treated with combinations of three or more antiviral agents. Twenty-six individual drugs are approved, thirteen of which inhibit RT (http://www.fda.gov/ForConsumers/byAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm). RT drugs are either (i) nucleoside or nucleotide inhibitors (NRTIs) that are incorporated into the growing DNA strand and act as chain terminators, because NRTIs lack a 3’-OH group, or (ii) NNRTIs (also referred to below as non-nucleosides) that are allosteric inhibitors of DNA polymerization. Several anti-retroviral therapy regimens use non-nucleosides in combination with NRTIs; nevirapine, delavirdine, efavirenz, etravirine and rilpivirine (also known as TMC278 or Edurant) are non-nucleoside drugs.

Structures of RT have been known since almost two decades ago, when binary complexes of RT with nevirapine1 and with DNA2 were reported. An innovative protein–nucleic acid cross-linking technique helped obtain an RT–DNA–dTTP ternary complex structure3. Subsequently, a large number of RT structures have been studied that help in understanding the enzymatic activities, inhibition and mechanisms of drug resistance4,5, and these studies have aided the design of new drugs6. RT has a hand-like structure7 (Fig. 1). The palm contains the polymerase active site and the non-nucleoside-binding pocket, which are separated by ~10 Å. The major conformational changes in RT8 characterized by structural studies are (i) the thumb lifts up to bind nucleic acid9,10, (ii) the fingers fold down to capture dNTP substrates in the presence of nucleic acid11, and (iii) non-nucleoside binding leads to thumb hyperextension. Pre-steady-state and steady-state kinetics data suggest that the binding of a non-nucleoside inhibits the chemical step of DNA polymerization11,12; however, the precise effects of this binding on nucleic acid and dNTP are unclear13, and RT–non-nucleoside association and dissociation are complex processes14 that are not yet conclusively explained by kinetics experiments. Binding of a non-nucleoside can enhance p66 and p51 dimerization15. Recent single-molecule FRET studies16,17 revealed that RT frequently flips and slides over nucleic acid substrates in the process of copying the viral RNA into dsDNA. An RT–nucleic acid complex is stabilized in a polymerization-competent conformation when dNTP is present. By contrast, nevirapine has a destabilizing effect that has been interpreted as the consequence of loss of thumb and fingers interactions with nucleic acid15. Binding of an incoming dNTP at the polymerase active site decreased the efficiency of cross-linking, whereas NNRTI binding increased cross-linking18; site-directed photo-cross-linking of the fingers subdomain of HIV-1 RT to an extended template was carried out using photolinkers of different length to monitor changes in the distance between particular positions on the surface of the protein and a nucleic acid substrate. Pre-steady state kinetics analyses11,12,19 reported no decrease in binding of DNA or dNTP upon binding of an NNRTI; in fact, dNTP-binding was enhanced at saturating concentrations.

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The postulated mechanisms of inhibition by non-nucleosides include (i) restriction of thumb mobility\(^1\), (ii) distortion of the catalytic triad\(^{20}\), (iii) repositioning of the primer grip\(^{21}\), and (iv) loosening of the thumb and fingers clamp\(^{17}\).

Non-nucleosides indirectly interfere with DNA polymerization. Therefore, structures of RT–nucleic acid–NNRTI (±dNTP or analog) complexes are essential for understanding inhibition of polymerization and excision\(^{22,23}\) by a non-nucleoside and to visualize how both types of RT drugs synergistically inhibit DNA polymerization\(^{24}\). Here we report an RT–DNA binary crystal form in which RT–DNA could undergo conformational changes to accommodate 3′-azido-3′-deoxythymidine\(^{25}\) (known as AZT or zidovudine) triphosphate (AZTTP) at the dNTP-binding site, or nevirapine at the non-nucleoside-binding pocket. The differences between the structures directly reflect the impact of nevirapine binding on the RT–DNA complex and help illustrate the structural basis of non-nucleoside inhibition.

RESULTS

Polymerase competent state of RT–DNA binary complex

In completing synthesis of a dsDNA from the viral RNA genome, RT makes several jumps and binds different nucleic acid sequences\(^{26}\). Recent single-molecule studies\(^{16,17}\) revealed that RT binds a nucleic acid in two distinct modes: a polymerase-competent mode (mode-1), analogous to that observed in crystal structures, and a switched mode (mode-2). Binding of dNTP stabilizes mode-1 for RT–nucleic acid complexes, and only the mode-1 complex can bind dNTP and incorporate nucleotide. Previous structures of RT in complex with non-cross-linked nucleic acids\(^{21,27}\) were naturally formed by mixing dsDNA or RNA-DNA with RT in solution; however, RT cross-linking to DNA\(^3\) stabilized the complex for studying the polymerase-relevant states of the enzyme. In our current study, RT–DNA cross-linking was used to obtain a stable polymerase-relevant high-resolution structure in the presence of a non-nucleoside drug, which pertains to the fact that nevirapine binding increases the dynamics of an RT–nucleic acid complex, as revealed by the single-molecule FRET study\(^{17}\).

AZTTP or nevirapine binding to RT–DNA complex in a crystal

Recent successful experiments were designed on the basis of the following facts: (i) a typical protein crystal has >50% disordered solvent, which may permit repositioning of a flexible domain if the domain is not constrained by interactions with neighboring symmetry-related molecules in the crystal; (ii) in an earlier experiment, we used soaking to replace tenofovir diphosphate with its natural counterpart dATP in a crystal\(^{28}\); and (iii) dNTP analogs have been successfully soaked into RT–DNA crystals\(^{29}\). Therefore, a crystal form of the RT–DNA complex in which the thumb and fingers subdomains have enough room to flex may permit binding of dNTPs and non-nucleosides by soaking. We discovered such a crystal form for the D498N mutant RT cross-linked with DNA containing an AZT-terminated primer (see Online Methods), and we soaked nevirapine and AZTTP as the incoming dTTP analog (Fig. 1). An attempt to crystallize RT cross-linked with a 2′,3′-dideoxymethylene (dT) primer-terminated DNA did not produce diffraction quality crystals; change in DNA sequence and incoming dNTP in the past had led to new crystal forms\(^{30}\) or were unsuccessful in yielding crystals. Our attempts enabled us to obtain the crystal structures of RT–DNA, RT–DNA–AZTTP,

![Figure 1](https://example.com/figure1.png)

Table 1 Data collection and refinement statistics

| Data collection | RT–DNA binary complex | AZTTP-ternary complex | Nevirapine-ternary complex |
|-----------------|-----------------------|-----------------------|---------------------------|
| Space group     | \(P2_1\)               | \(P2_1\)               | \(P2_1\)                  |
| Cell dimensions | \(a, b, c (\AA)\)      | 89.48, 133.17, 139.90 | 89.82, 132.05, 142.73     |
| \(\alpha, \beta, \gamma (\degree)\) | 90, 98.67, 90 | 90, 98.12, 90 | 90, 100.84, 90 |
| Resolution (\AA) | 50.0–2.7 | 50.0–2.8 | 40.0–2.85 |
| \(R_{merge}\) (〈2.7-2.0〉) | 0.089 (0.625) | 0.083 (0.523) | 0.090 (0.618) |
| Completeness (%) | 98.4 (97.2) | 92.8 (85.1) | 99.0 (97.7) |
| Redundancy | 3.9 (3.2) | 3.2 (2.4) | 3.6 (2.9) |
| Refinement | Resolution (\AA) | 44.0–2.7 | 45.0–2.8 | 38.4–2.85 |
| No. reflections | 86,138 | 73,170 | 75,395 |
| \(R_{work} / R_{free}\) | 0.233 / 0.268 | 0.229 / 0.262 | 0.250 / 0.298 |
| No. atoms | Protein + DNA | 17,627 | 17,632 | 17,469 |
| | Ligand/ion | 64 | 64 | 40 |
| | \(B\)-factors (\AA^2) | 80 | 64 | 83 |
| | Protein + DNA | 82 | 82 | 72 |
| Bond lengths (\AA) | 0.009 | 0.012 | 0.010 |
| Bond angles (\degree) | 1.27 | 1.38 | 1.34 |

One crystal was used for each dataset.

Values in parentheses are for highest-resolution shell.
(called AZTTP-ternary below) and RT–DNA–nevirapine (called nevirapine-ternary below) complexes at 2.70, 2.80 and 2.85 Å resolution, respectively (Table 1). The crystals soaked with AZTTP together with nevirapine in the presence or absence of MgCl₂ (or MnCl₂) formed the nevirapine-ternary complex only, despite the fact that this crystal form allows RT–DNA to bind either AZTTP or nevirapine individually. In all three structures, the primer 3′ end AZT was designed to occupy the P site and blocked further incorporation of nucleotides. Incoming AZTTP in the AZTTP-ternary structure occupied the N site (Fig. 2a), and the thymine was paired with the template adenine base.

**Impact of nevirapine or AZTTP binding on the RT–DNA complex**

Binding of AZTTP shrank the unit cell volume of RT–DNA crystals by 1.2%, and binding of nevirapine expanded the volume by 0.67%; however, individual cell parameters changed more upon binding of nevirapine (Table 1). The AZTTP-ternary structure represents an RT–polymerase complex having architecture similar to that in all RT-ternary structures, despite differences in RT or DNA sequences, incoming dNTP or analogs, and crystallization conditions or parameters (Supplementary Fig. 1 and Supplementary Table 1) in different studies; the base-pairing, metal chelation and other interactions of AZTTP with RT resemble those of the dTTP³ substrate and AZTppppA (an ATP-mediated excision product of AZT)⁵,²²,²³ in respective cocrystal ternary structures. AZTTP binding causes the tip of the fingers to close and wrap around the incoming dNTP or analog (Supplementary Fig. 2a). Upon AZTTP binding, the conserved β3–β4 region folds in by ~2 Å, and side chains rearrange to form a closed dNTP-binding pocket in the ternary structure, analogous to that observed upon soaking of GS-9148 (ref. 29). Contrary to what was found in the binary complex structure determined in the presence of a monoclonal antibody³, the fingers subdomain in the current binary structure is not wide open (Supplementary Fig. 2b); however, it is highly flexible. Apart from the fingers rearrangement, AZTTP binding has no major discernible impact on RT–DNA conformation. The 3′-azido group of the primer terminal AZT occupies the position of cofactor metal ion A (Fig. 1).

The mode of binding and interactions of nevirapine in nevirapine-ternary and nevirapine-binary¹,²⁰ structures are similar; however, the binding of nevirapine had a profound effect on RT–DNA conformation compared to the binding of AZTTP. The opening of the non-nucleoside-binding pocket upon nevirapine binding is accompanied by switching of the Tyr181 and Tyr188 rotamer conformations and shearing of the β12–β13-β14 sheet away from the β6–β10–β9 sheet (Fig. 2a–c and Supplementary Movie 1); the β6–β10–β9 sheet contains the polymerase 'catalytic triad' (Asp110, Asp185 and Asp186), and the β12–β13-β14 sheet contains the ‘primer grip’ that positions the primer strand appropriately for nucleotide incorporation. Upon binding of nevirapine, the primer grip is shifted by ~4 Å, the shifted primer grip lifts the DNA primer terminus away from the P site (Fig. 2a), and crucial interactions of the conserved catalytic YMMD motif with the primer terminus are lost. The nucleotide complementary to AZT and the upstream template overhang are

![Figure 2](image-url)
Figure 3 Impact of nevirapine binding on thumb, fingers and DNA. (a) Stereo view of a palm superposition of nevirapine-ternary (yellow), AZTTP-ternary (gray) and nevirapine-binary\(^{20}\) (blue) structures shows that the thumb subdomain is hyperextended upon nevirapine binding; the tip of the thumb is ~7.5 and 11 Å away in nevirapine-ternary and nevirapine-binary\(^{20}\) structures, respectively, from the tip in the AZTTP-ternary structure, based on the described palm superposition. The thumbs of the nevirapine-ternary and the first nevirapine-binary structures\(^{1}\), however, are in close proximity, based on the palm superposition (Supplementary Fig. 3). In the higher resolution nevirapine-binary structure\(^{20}\), the thumb was further extended as a result of crystal contacts. (b) Zoomed stereo view of the polymerase active site region of the superimposed structures in a. The YMDD loop in the nevirapine-ternary (yellow) has an intermediate state between the AZTTP-ternary (gray) and nevirapine-binary\(^{20}\) (blue) structures, indicating active site flexibility even when nevirapine is bound; the C\(\alpha\) atom of Asp185 in the nevirapine-ternary structure is displaced ~1 Å from its counterpart in the other two structures. (c) Repositioning of fingers subdomain, based on described superposition and color scheme in panel a. The thick arrows pointing to the right represent the fingers repositioning to the nevirapine-binary, and the thin arrows pointing up represent the repositioning to the nevirapine-ternary from the AZTTP-ternary structure. (d) Comparison of interactions of the DNA template–primer with the RT in RT–DNA (blue), AZTTP-ternary (red) and nevirapine-ternary (green) structures; the extent of the interactions (y axis) is represented by the number of interatomic distances (Å) between a nucleotide and the RT. The interactions of DNA with fingers and palm are decreased upon nevirapine binding; the interactions with the thumb, connection and RNase H are not substantially altered.

DISCUSSION

Primer repositioning obstructs polymerization and excision

DNA polymerization by RT is carried out by cyclic repetitions of three structurally distinct steps (Fig. 4a): (i) binding of dNTP, (ii) nucleotide incorporation with concomitant release of pyrophosphate and (iii) translocation of the incorporated nucleotide from the N site to the P site. Incorporation of an NRTI stalls the cycle and blocks further elongation. An incorporated AZT can readily shuttle between P and N sites, and a pyrophosphate donor such as ATP, with the assistance of AZT-resistant mutations in RT, excises the AZT molecule from the N site. Excision is carried out by completing one reverse cycle of polymerization, structurally represented by RT–DNA–AZT (P-complex) → RT–DNA–AZT (N-complex)\(^{3}\) → RT–DNA–AZTppppA (excision product complex). The P\(^\prime\)-complex, represented by the nevirapine-ternary structure, is unlikely to accommodate ordered binding of dNTP in a catalytically competent mode. Even if a dNTP were to bind, the primer 3′ end at the P\(^\prime\) site would not allow nucleotide incorporation.
**Figure 4** A molecular mechanism of non-nucleoside inhibition and impact of DNA binding on resistance to non-nucleoside drugs. (a) Scheme representing effect of non-nucleoside inhibitor binding on DNA polymerization by RT. For incorporating a nucleotide, RT completes three structurally distinct steps in a clockwise cycle. Incorporation of an NRTI drug blocks the cyclic process, leading to a P-complex structure lacking 3’-OH, such as the structure of the RT–DNA–AZT-terminated complex. RT removes AZT by following the three steps in a reverse cycle and using a pyrophosphate donor (ATP or PPI). Binding of a non-nucleoside inhibitor shifts the primer end from the P site to the P′ site. This P′-complex is catalytically incompetent, because the 3′ end is positioned away from the polymerase active site. Release of the non-nucleoside inhibitor would shift the P′-complex to the P-complex and restore DNA polymerization by RT. (b) Positions of three structural elements: the β6–β10–β9 sheet (yellow), β12–β13–β14 sheet (gray) and loop 95–103 (cyan) + Glu138 loop (blue) of P51 in the nevirapine-ternary structure. These elements are responsible for, and rearranged upon, the binding of nevirapine (green). Non-nucleoside inhibitor binding locks the primer grip away from the active site, whereas binding of dsDNA requires the primer grip to be positioned near the active site, as in the RT–DNA and AZTTP-ternary structures. Red arrows indicate the structural movements (Supplementary Movie 1) between the polymerase-competent and non-nucleoside-bound states.

**dNTP binding in the presence of nevirapine**

Earlier reported kinetics data suggested that nevirapine improves binding of dNTPs and inhibits the chemical step of nucleotide incorporation11,12 that directly correlates with the proposed catalytic ‘distortion’ model13 of non-nucleoside inhibition. A later pre-steady-state kinetics study19 using elemental effects31 (dTTPαS versus dTTP) revealed that a conformational state and not the chemical step leading to nucleotide incorporation is blocked by a non-nucleoside inhibitor, and this study favored the primer-grip distortion model22 as a possible molecular mechanism for non-nucleoside inhibition. The nevirapine-ternary structure presented in this study defines the impact of nevirapine binding on DNA positioning and RT–DNA interactions. Also, the structure shows that the primer grip repositioning locked the thumb at a hyperextended position1 and results in a loss of inter-atomic interactions between DNA and the polymerase domain of RT17. Although the RT–DNA complex in crystal could bind nevirapine or AZTTP, the complex did not bind both simultaneously and so did not provide direct evidence of dNTP binding. In the nevirapine-ternary structure, the flexible fingers (Fig. 3c), repositioned template–primer and disordered template overhang would reduce ordered dNTP binding at the N site. However, the flexibility of the fingers may allow alternative modes of dNTP binding to RT in presence of nevirapine. Structures of DNA polymerases (such as the Klenow fragment)32,33 have shown that the transition from open to closed fingers is responsible for the binding and incorporation of the correct nucleotides complementing the template overhang, and a recent single-molecule FRET experiment on the Klenow fragment demonstrated that there is a high degree of flexibility of the fingers in the absence of substrates34.

According to pre-steady state kinetics19, dNTPs are expected to bind the nevirapine-ternary complex in a metal-dependent mode but not in a catalytically relevant mode. Structures of DNA polymerases before and after misincorporation of a dATP opposite to an abasic nucleotide have revealed rearrangements of DNA and protein at the polymerase active sites. An open complex of KlenTaq DNA polymerase is formed upon binding of ddATP in a catalytically relevant mode opposite to a template furan35; Tyr671 on the O-helix interacts with the ddATP base and partly compensates for the loss of canonical base-pairing. A bacteriophage RB69 DNA polymerase open complex structure36 (PDB 2P5G) was determined with a dAMP incorporated into the primer opposite to a template furan; apparently, lack of stable interactions (base pairing and stacking) of the terminal dAMP led to multiple conformations of the DNA in the four copies of the complex in the asymmetric unit. HIV-1 RT appears to follow the ‘A-rule’ for misaligned primer extension opposite to an abasic moiety in vitro37, which suggests that the polymerase active site conformation of RT could rearrange to accommodate and incorporate a dNTP without canonical base-pairing at the N site. In contrast to the rearrangements of the DNA polymerases discussed here, a nevirapine-ternary structure of RT is expected to bind dNTP in a polymerase-incompetent mode. Although DNA polymerases share markedly similar domain architecture and chemistry of nucleotide incorporation, characteristics such as rate of nucleotide incorporation, mode and extent of protein–nucleic acid interactions, presence or absence of a proof-reading mechanism, fidelity, and rate-limiting steps that select correct versus incorrect nucleotide incorporation38 are highly enzyme specific.

It is likely that dNTPs enter the N site of the nevirapine-ternary complex (Fig. 2g) and bind in multiple orientations. Alternatively, the primer 3′ end of a nucleic acid duplex may slide past the polymerase active site17, and a rearranged dNTP-binding region with the duplex resting over it may define a ordered yet non-productive binding of dNTP. Further biochemical, mutational and structural studies are needed to clarify if there is ordered dNTP-binding to RT and to map the RT-dNTP interactions in the presence of non-nucleosides and nucleic acid.

**Conformational states of RT in presence of NNRTI and DNA**

Taking the biochemical data, clinical observations and structural states of RT into consideration, it appears that RT with non-nucleosides and nucleic acids in solution would exist in three states: nevirapine-ternary (state I), nevirapine-binary or switched mode-2 complex16 (state II) and RT–DNA (state III). Among the three, state III is active and states I and II are inactive for nucleotide incorporation. The relative proportion of the three states would depend upon the binding characteristics and effective concentrations of nevirapine and nucleic acid16. The fraction of RT in state III would incorporate nucleotides, unless DNA primers are terminated by NRTIs, which may be a reason why (i) a combination of non-nucleoside and NRTI is more effective in decreasing the viral load in treatment-naive people than when they are treated with individual components and (ii) both NRTI and non-nucleoside resistance mutations co-emerge in...
response to a drug combination. For example, a recent phase III trial of a triple combination of two NRTIs (tenofovir-disoproxil-fumarate and emtricitabine) and a non-nucleoside (rilpivirine) reported that M184V (or M184I) and E138K mutations are predominantly associated with treatment failure. An arm of the trial that used efavirenz instead of rilpivirine showed M184V (or M184I) and K103N as the predominant resistance mutations, however at a lower frequency. Also, additive effects of nevirapine and AZT have been observed biochemically and clinically.

NNRTI inhibition and resistance mutations
Excision of AZT is diminished in the presence of a non-nucleoside. AZT at the primer 3' terminus is locked at the P' position (Fig. 4a) in nevirapine-bound RT and not permitted to translocate back to the N site for excision. Additionally, the distorted dNTP-binding site (Fig. 2g) may decrease the binding of the excision substrate ATP (or PPI). Nevirapine blocks both DNA polymerization and NRTI excision.

Binding of nevirapine is accompanied by repositioning of the primer grip away from the active site, whereas binding of DNA in a catalytically competent mode requires the primer grip to be near the active site (Fig. 4b and Supplementary Movie 1). A bound non-nucleoside is surrounded by three walls: (i) the β6–β10–β9 sheet, (ii) the β12–β13–β14 sheet and (iii) the 100–105 loop of the p66+Glu138 loop of p51. Rearrangements of the loops would permit entry and exit of most non-nucleosides. In contrast to the pocket residues that directly interact with an inhibitor, the loop residues Lys103 and Glu138 (of p51) do not have extensive interactions with a bound non-nucleoside. The loop mutations facilitate exit of non-nucleosides from the pocket, permitting the primer grip to position nucleic acid in a catalytically relevant mode and shifting the equilibrium toward state III. The mutation K103N confers relatively uniform resistance to chemically diverse non-nucleosides, and as discussed above, two loop mutations E138K and K103N are predominantly associated with treatment failure of the rilpivirine and efavirenz arms, respectively.

Non-nucleoside resistance mutations affect NRTI susceptibility, implying communication between the two sites even in the absence of a non-nucleoside. The common non-nucleoside resistance mutation Y181C enhances AZT sensitivity in a thymidine analog mutant background. A recent study has shown that the E138K mutation is compensatory for M184I (or V) and vice versa. The individual mutations E138K, M184V or M184I reduce the virus fitness by two to three times; however E138K and M184I (or V) in combination restores the viral fitness equivalent to that of wild-type HIV-1.

Conclusions
The current structures of three RT complexes, the earlier RT structures and the abundant available clinical and biochemical data enabled us to analyze the specific effects of non-nucleosides on RT structure and function. Nevirapine binding has a direct impact on the RT–DNA complex. The primer grip is displaced, and consequently, the primer terminus moves out, the thumb is locked at a hyperextended position, interactions between DNA and the polymerase domain are decreased, the dNTP-binding site is distorted and the RNase H active site is repositioned with respect to the polymerase active site. Enhanced understanding of the mechanistic details of non-nucleoside inhibition may also aid development of allosteric inhibitors that would block the conformational mobility of molecular machines such as hepatitis C NS5B polymerase and bacterial RNA polymerase and other macromolecular assemblies associated with various diseases.
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ONLINE METHODS

RT expression, RT–DNA cross-linking and purification. RT construct RT127A used for the current structural studies was derived from a previously reported construct RT13A. The mutation D498N in the RT127A construct was introduced using the methods described therein. Asp498 is a part of the RNase H active site. The D498N mutant RT blocks RNase H activity; however, the mutant RT has polymerase activity comparable to wild-type RT. The D498N mutant RT was expressed and purified as previously reported. Briefly, the RT was expressed in BL21-CodonPlus–RIL cells, induced with 1 mM IPTG at an A600 of 0.9, followed by expression at 37 °C for 3 h. The cells were sonicated at a power output of ~45 W with a Misonix 3000 sonicator. The samples were purified using a nickel–nitrilotriacetic acid (Ni–NTA) column according to the manufacturer’s recommendations (Qiagen). The final purification step was carried out using a Mono Q column, and the purified RT samples were buffer exchanged into 10 mM Tris, pH 8.0, and 75 mM NaCl.

The 27-mer DNA template (5′-ATGGAAGGCGCCCAACAGGACTG TG-3′) was synthesized by Integrated DNA Technologies. The 20-mer primer (5′-ACAGTTCCCTGTTCGGCGCC-3′) bearing a cross-linkable thioalkyl tether (on G in the primer strand) was custom synthesized by Midland Certified Reagent Company, using phosphoramidite that was custom synthesized by Chemgenes, and the primer was annealed to the template. The 27-20 mer dsDNA was cross-linked to RT127A at the mutated Q258C site of p66, and the cross-linked primer was extended with an AZT at the 3′ end through RT polymerization. The cross-linked RT–DNA (AZT-terminated primer) complex was purified using Ni–NTA and heparin columns in tandem as previously described. The His tag was removed by adding human rhinovirus 14 3C protease at a 1:10 ratio to the RT–DNA complex at 4 °C for 48 h. Ni-NTA beads were added to remove the cleaved His tag and His-tagged protease. An Amicon Ultra-4 Ultracel unit (30-kDa cutoff) was used to exchange the buffer into 75 mM NaCl, 10 mM Tris-HCl, pH 8.0. The protein was concentrated to 15 mg ml−1.

Crystal soaking and freezing. Binary complex: an RT–DNA (AZT-terminated primer) binary complex crystal of size 200 × 200 × 60 µm3 was transferred to 50 mM Bis-Tris propane, pH 6.8–7.2, 100 mM (NH4)2SO4, 5% sucrose (w/v), 5% glycerol (v/v) and 20 mM MgCl2 for 15 min. Finally the crystals were stabilized for one minute in 50 µl of this solution, raised to 20% glycerol (v/v) for cryoprotection. This experiment produced only the nevirapine-ternary complex; no Mg2+ ion or AZTTP binding was detected at the dNTP-binding site when nevirapine was bound to HIV–1 RT in datasets from multiple crystals or when Mn2+ was substituted for Mg2+. Crystals soaked with nevirapine after washing out Mg2+ ions and without adding AZTTP also produced the nevirapine-ternary complex.

X-ray crystallography. Multiple X-ray diffraction datasets were collected from the above-described crystals and duplicate soaks using synchrotron sources: the F1 beamline at CHESS and the X25 beamline at BNL. The data were processed and scaled using HKL2000 (ref. 51) (Table 1). The diffraction data from the nevirapine-ternary complex crystals were highly anisotropic, and observed structure factors were corrected using the University of California, Los Angeles MBI – Diffraction Anisotropy Server. The structures were solved by molecular replacement, using protein atoms in the crystal structure of RT–DNA-tenovir-diphosphate, and the subdomains were positioned by rigid-body refinements in which each RT molecule was divided into thirteen segments. Each crystal structure had two RT complexes per asymmetric unit. The three datasets corresponding to the three RT complexes were non-isomorphous with each other, which allowed us to conduct six-fold real-space averaging among the three crystal forms and two-fold noncrystallographic symmetry within each crystal form. DMMULTI3, as implemented in CCP4, was used for averaging and phase extension. The electron density maps calculated from averaged phases and figures of merit helped position the individual amino acid residues, the DNA, the nevirapine and the AZTTP. The fingers subdomain is the least ordered region in all three structures. The degree of disorder of the fingers varies from higher to lower in the sequence nevirapine-ternary > RT–DNA-binary > AZTTP-ternary; the binding of AZTTP increased the positional stability of the fingers subdomain in the AZTTP-ternary structure. Individual structures were refined using PHENIX, and the model building was carried out using COOT. The figures showing structural information were generated using PyMOL (http://www.pymol.org/).

Crystal soaking and freezing. Binary complex: an RT–DNA (AZT-terminated primer) binary complex crystal of size 200 × 200 × 60 µm3 was transferred to 10 mM Tris, pH 8.0, and 75 mM NaCl. The crystals were then transferred to 50 µl of the stabilization solution plus 2 mM nevirapine for 1 h, then 50 µl with 2 mM each of nevirapine and AZTTP and 20 mM MgCl2 for 15 min. Finally the crystals were stabilized for one minute in 50 µl of this solution, raised to 20% glycerol (v/v) for cryoprotection. This experiment produced only the nevirapine-ternary complex; no Mg2+ ion or AZTTP binding was detected at the dNTP-binding site when nevirapine was bound to HIV–1 RT in datasets from multiple crystals or when Mn2+ was substituted for Mg2+. Crystals soaked with nevirapine after washing out Mg2+ ions and without adding AZTTP also produced the nevirapine-ternary complex.

Crystal soaking and freezing. Binary complex: an RT–DNA (AZT-terminated primer) binary complex crystal of size 180 × 120 × 40 µm3 was transferred to 150 µl of a stabilization solution containing 12% PEG 8000, 10% (v/v) glycerol, and 75 mM NaCl, 10 mM Tris-HCl, pH 8.0. The protein was concentrated to 15 mg ml−1.

Crystal soaking and freezing. Binary complex: an RT–DNA (AZT-terminated primer) binary complex crystal of size 200 × 200 × 60 µm3 was transferred to 50 µl of a stabilization solution containing 12% PEG 8000, 10% (v/v) glycerol, and 75 mM NaCl, 10 mM Tris-HCl, pH 8.0. The protein was concentrated to 15 mg ml−1.

Crystal soaking and freezing. Binary complex: an RT–DNA (AZT-terminated primer) binary complex crystal of size 200 × 200 × 60 µm3 was transferred to 50 µl of a stabilization solution containing 12% PEG 8000, 10% (v/v) glycerol, and 75 mM NaCl, 10 mM Tris-HCl, pH 8.0. The protein was concentrated to 15 mg ml−1.

Crystal soaking and freezing. Binary complex: an RT–DNA (AZT-terminated primer) binary complex crystal of size 200 × 200 × 60 µm3 was transferred to 50 µl of a stabilization solution containing 12% PEG 8000, 10% (v/v) glycerol, and 75 mM NaCl, 10 mM Tris-HCl, pH 8.0. The protein was concentrated to 15 mg ml−1.