Conformational changes in HIV-1 reverse transcriptase induced by nonnucleoside reverse transcriptase inhibitor binding

Nicolas Sluis-Cremer[1][*], N. Alpay Temiz[2] and Ivet Bahar[2]

[1]University of Pittsburgh, Department of Medicine, Division of Infectious Diseases, Pittsburgh, Pennsylvania 15261, USA and [2]Center for Computational Biology and Bioinformatics, and Department of Molecular Genetics and Biochemistry, School of Medicine, University of Pittsburgh, Pennsylvania 15261

*Corresponding author: Nicolas Sluis-Cremer

Address: University of Pittsburgh, Department of Medicine, Division of Infectious Diseases, Scaife Hall S817, 3550 Terrace Street, Pittsburgh, PA 15261, USA

E-mail: CremerN@msx.dept-med.pitt.edu

Tel: (412) 648-8457

Fax: (412) 648-8521

Abstract

Nonnucleoside reverse transcriptase inhibitors (NNRTI) are a group of small hydrophobic compounds with diverse structures that specifically inhibit HIV-1 reverse transcriptase (RT). NNRTIs interact with HIV-1 RT by binding to a single site on the p66 subunit of the p66/p51 heterodimeric enzyme, termed the NNRTI-binding pocket (NNRTI-BP). This binding interaction results in both short-range and long-range distortions of RT structure. In this article, we review the structural, computational and experimental evidence of the NNRTI-induced conformational changes in HIV-1 RT and relate them to the mechanism by which these compounds inhibit HIV-1 reverse transcription.

INTRODUCTION

Reverse transcription of the viral single-stranded (+) RNA genome into double-stranded DNA is an essential step in the human immunodeficiency virus (HIV) life-cycle. Although several viral proteins such as nucleocapsid protein NCp7, matrix protein, integrase, Tat, Nef and Vif have been shown to participate in the regulation and/or efficiency of reverse transcription [1-7], the process of retroviral DNA synthesis is entirely dependent on the enzymatic activities of the retroviral enzyme reverse transcriptase (RT).

HIV-1 RT is a multifunctional enzyme that exhibits two distinct enzymatic activities: (i) a DNA polymerase activity that can use either RNA or DNA as a template; and (ii) an endonucleolytic ribonuclease H (RNase H) activity that specifically degrades the RNA strand of RNA:DNA duplexes. Due to its essential role in the HIV life-cycle, RT is a primary target for anti-HIV drug development and of the 20 antiretroviral agents approved by the U.S. FDA for administration to HIV infected individuals, 11 target the DNA polymerase activity of RT. These 11 inhibitors can be classified into two distinct therapeutic groups:

1. Nucleoside and nucleotide RT inhibitors (NRTIs) which include 3'-azido-2',3'-dideoxythymidine (zidovudine or AZT); 2',3'-didehydro-2',3'-dideoxythymidine (stavudine or D4T); 2',3-dideoxyinosine (didanosine or ddI); 2',3'-dideoxycytosine (zalcitabine or ddC); (-)-β-L-2',3'-dideoxy-3'-thyacytidine (lamivudine or 3TC); 5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (emtricitabine or FTC); (1S,4R)-4-[2-amino-6-(cyclopropyl-amino)-9H-purin-9-yl]-2-cyclopentene-1-methanol succinate (abacavir or ziagen); and (R)-9-(2-phosphonylmethoxy-propyl)adenine (PMPA or tenofovir).

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2. Nonnucleoside RT inhibitors (NNRTIs) which include 11-Cyclopropyl-4-methyl-5,11-dihydro-6H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one (nevirapine; Fig. (**1a**)); 1-[3-[(1-methylethyl)amino]-2-pyridinyl]-4-[[5-[(methylsulfonyl)amino]-1H-indol-2-yl]carbonyl]-piperazine (delavirdine; Fig. (**1b**)); and (4S)-6-chloro-4-cyclopropylethynyl-4-trifluoromethyl-1, 4-dihydro-benzo[d][1, 3]oxazin-2-one (efavirenz; Fig. (**1c**)).

NRTIs are analogs of naturally occurring deoxyribonucleosides which lack a 3'-hydroxyl group on the ribose sugar. NRTIs must be metabolically converted by host-cell enzymes to their corresponding 5'-triphosphates to exhibit antiviral activity [8,9]. In this form, they inhibit HIV RT-mediated reverse transcription by competing with the analogous dNTP substrate for binding and incorporation into the newly synthesized DNA chain [10]. Incorporation of an NRTI into the nascent viral DNA chain results in termination of any further nucleic acid synthesis.

NNRTIs are chemically distinct from nucleosides and unlike the NRTIs, they do not require intracellular metabolism for activity. In general, NNRTIs are a group of small (<600 Da) hydrophobic compounds with diverse structures (Fig. (**1**)) that specifically inhibit HIV-1, but not HIV-2 RT. 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT; Fig. (**1h**)) and tetrahydroimidazobenzodiazepinone (TIBO; (Fig. (**1f**)) were the first NNRTIs identified more than 10 years ago [11,12]. Since then a large number of NNRTI have been identified that can be classified into more than 30 different chemical classes (see [13] for comprehensive review).

THREE-DIMENSIONAL STRUCTURE OF HIV-1 RT

Numerous crystal structures of HIV-1 RT have been solved. These include structures of "free" or unliganded forms of the enzyme [14-16], structures of RT-template/primer (T/P) binary complexes

[17-20], structures of RT-T/P-dNTP ternary complexes [21], as well as structures of NNRTI-bound HIV-1 RT [22-33]. This wealth of structural data has provided considerable insight into HIV-1 RT molecular structure including: (i) the precise location of the DNA polymerase and RNase H active sites; (ii) key amino acid residues involved in substrate binding; (iii) the precise location of the NNRTI binding pocket (NNRTI-BP); (iv) mechanisms of HIV-1 resistance to NRTI and NNRTI; and (v) conformational changes associated with both substrate and NNRTI binding.

HIV-1 RT is an asymmetric heterodimer composed of a 560-amino-acid 66kDa subunit (p66) and a 440-amino-acid 51-kDa subunit (p51) [22]. The p51 polypeptide is derived by HIV-1 protease-mediated cleavage of the C-terminal RNase H domain of p66 polypeptide [35]. The p66/p51 HIV-1 RT heterodimer contains one DNA polymerization active site and one RNase H active site, both of which reside in the p66 subunit at spatially distinct regions (Fig. (**2**)). Although the p51 subunit contains the same amino acid sequence that comprises the DNA polymerase domain of the p66 subunit, the polymerase active site in p51 is not functional.

The p66 subunit shows an overall architectural similarity to the Klenow fragment of *Escherichia coli* DNA polymerase, and consists of the "fingers (residues 1-85 and 118-155), "palm" (residues 86-117 and 156-237), and "thumb" (residues 238-318) subdomains [22]. The polymerase active site, as defined by the three aspartic acid residues D110, D185 and D186, resides in the "palm" subdomain. Additional domains include the "connection" (residues 319-426) and the C-terminal RNase H domain (residues 427-560). The RNase H active site is defined by four conserved acidic amino acids (D443, E478, D498 and D549). The connection domain acts as a tether between the polymerase and RNase H domains, but is also involved in nucleic acid substrate interactions and RT inter-subunit interactions.

The p51 subunit contains the same polymerase and connection domains as the p66 subunit; however, their spatial arrangement differs markedly from those of the p66 subunit. While the p66 subunit adopts an "open" catalytically-competent conformation that can accommodate a nucleic acid template strand, the p51 subunit is in a "closed" conformation and is considered to play a largely structural role [22].

THE NNRTI-BINDING POCKET

Although NNRTI represent, in terms of chemical structures, a heterogeneous class of inhibitors, they all interact with HIV-1 RT by binding to a single site on the p66 subunit of the HIV-1 RT p66/p51 heterodimer termed the NNRTI binding pocket (NNRTI-BP; Fig. (**2**)). The NNRTI-BP is situated between the β6-β10-β9 and β12-β13-β14 sheets in the "palm subdomain" of the p66 subunit, approximately 10Å from the RT DNA polymerase aspartic acid catalytic triad [22]. The NNRTI-BP is predominantly hydrophobic in nature with substantial aromatic character (Y181, Y188, F227, W229, and Y232), but also contains several hydrophilic residues (K101, K103, S105, D192, and E224 of the p66 subunit and E138 of the β7-β8 loop of the p51 subunit). A probable solvent accessible entrance to the NNRTI-BP is located at the p66/p51 heterodimer interface, ringed by residues L100, K101, K103, V179, and Y181 of the p66 subunit and E138 of the p51 subunit [16].

Interestingly, in the absence of ligand, the side chains of Y181 and Y188 of p66 point into the hydrophobic core and, as a consequence, the NNRTI-BP does not exist in the free enzyme [14,16]. Instead, a surface depression is present at a location which is equivalent to the putative entrance of the pocket. NNRTI-binding to HIV-1 RT causes the side chains of both Y181 and Y188 to rotate away from their positions in the hydrophobic core thereby creating a space to accommodate the ligand [16].

The major difference in the location of the secondary structural elements that form the pocket between the structures of HIV-1 RT with and without NNRTI is a differential twisting (about 30˚) of the β12-β13-β14 sheet, which results in an expansion of the NNRTI-BP.

FIRST AND SECOND GENERATION NNRTIs: DIFFERENCES IN MODE OF BINDING TO HIV-1 RT

NNRTIs can be broadly categorized into first- or second-generation compounds [36]. The first generation NNRTIs, such as nevirapine, delavirdine, TIBO, and loviride (α-anilinophenylacetamido (α-APA)), were mainly discovered by random screening and are associated with the rapid development of drug resistance mutations. The second generation NNRTIs, which include efavirenz, the quinoxaline talviraline (HBY-097) and the imidazole capravirine, were developed as a result of comprehensive strategies involving molecular modeling, rationale-based drug synthesis and biological and pharmacokinetic evaluations. Second generation NNRTIs tend to be more potent than the first generation compounds, and in general are more active against a broader spectrum of drug-resistant strains of HIV-1 [36].

Analysis of the available structural data indicates a number of common features of the NNRTI pharmacophores important for interaction with the RT NNRTI-BP [37,38]. These include an aromatic ring capable of π-stacking interactions, NH-C=O or NH-C=S groups able to participate in hydrogen bonding, and one or more hydrocarbon-rich region that participate in hydrophobic contacts. However, some differences in the modes of binding of the first and second-generation NNRTIs to the HIV-1 RT NNRTI-BP have been documented.

Examination of the X-ray crystal structures of HIV-1 RT in complex with first generation NNRTI such as nevirapine, TIBO or loviride (α-APA or R89439) has demonstrated striking similarities in the geometry of the bound inhibitor [24]. In general, the binding of NNRTI in the pocket can be likened to a "butterfly" resting on the β6-β10-β9 sheet and facing toward the putative entrance of the pocket (Fig. (**3a**)). Based on this analogy, the binding site can be divided into two distinct regions, termed wing I and wing II. The wing I region is lined by residues Y181, Y188 and W229. Wing II has fewer hydrophobic interactions compared to wing I and interacts with the side chains of K101, K103, V106, V179, Y318 and possibly the main chain atoms of H235 and P236. The body of the "butterfly" has interactions with the main chain atoms of Y188, Y189 and G190, and with the side chains of V106 and V179. The back of the "butterfly" is flanked with residues L100 and L234, which interact with both wings. The head of the "butterfly" is flanked by the side chain of E138 from the p51 subunit, which partially covers the entrance of the pocket and has potential interactions with both wings.

Although defined as a first generation inhibitor, delavirdine [39] is bulkier than other NNRTIs (volume of delaviridine ~ 380Å^3 as opposed to 230-290 Å^3 for others) and it also exhibits a different binding mode compared to other first generation NNRTIs (Fig. (**3b**)) [26]. Although it binds in the NNRTI-BP, its size and shape cause the molecule to extend beyond the usual pocket and to project into the solvent. Delavirdine also interacts with regions of the NNRTI-BP inaccessible to other NNRTIs. For example, delavirdine's piperazine ring conformation positions the inhibitor very close to V106 and into contact with a set of residues not usually contacted by other NNRTIs [26]. Two of these novel interactions are particularly important for stabilizing the RT-delavirdine complex: (i) hydrogen bonding to the main chain of K103; and (ii) extensive hydrophobic interactions between the indole ring of delavirdine and P236 (Fig. (**3b**)).

In contrast to the first generation NNRTIs, second generation inhibitors tend to be more "flexible" and their binding frequently differs from the commonly seen butterfly-like geometry. As a result, second-generation NNRTI make new contacts with residues in the drug binding pocket or make contacts with main-chain residues that are unlikely to be disrupted by single side chain mutations [36]. To illustrate these differences, the binding interactions between the HIV-1 NNRTI-BP and either HYB097 [29] or capravirine (S1153 [32]) are briefly reviewed.

In HYB097 [29], the quinoxaline ring structure prevents the molecule from bending to adapt to the V-shaped body causing it to form a flatter structure (Fig. (**3c**)). This causes extensive contacts between HYB097 and L100, an interaction that is not observed with inhibitors that assume the butterfly-like shape. Furthermore, the flexibility in the torsion angles of both the iso-propoxycarbonyl and the methylthiomethylene groups permits the HYB 097 molecule to adapt its conformation to accommodate changes in protein-inhibitor interactions [29]. This conformational flexibility may partially explain why HBY 097 retains potency against a number of HIV-1 RT mutations that can confer high-level resistance to other NNRTIs [70]

The binding of capravirine to HIV-1 RT involves extensive main chain hydrogen bonding, a feature that has not been observed in many of the other RT/NNRTI complexes [32]. This network of hydrogen bonds involves the main chains of residues 101, 103 and 236. (Fig. (**3d**)). Important interactions include the formation of two hydrogen bonds between the carbamoyloxymethyl group and the protein main chain of P236, a hydrogen bond from the carbamate nitrogen to the main chain of K103, and a water mediated hydrogen bond between the imidazole nitrogen of capravirine and the main-chain carbonyl of K101 [32]. These novel binding features may also help to explain why capravirine is resilient to many of the resistance mutations associated with NNRTI-resistance.

NNRTI-INDUCED CONFORMATIONAL CHANGES IN HIV-1 RT: STRUCTURAL AND COMPUTATIONAL STUDIES

Comparison of the available structures of HIV-1 RT indicates that the binding of an NNRTI causes both short-range and long-range distortions of the HIV-1 RT structure.

The short-range distortions include the conformational changes of the amino acids and/or structural elements that form the NNRTI-BP, such as the re-orientation of the side chains of Y181 and Y188, and the displacement of the β12-β13-β14 sheet (discussed above).

The long-range distortions involve a hinge-bending movement of the p66 thumb subdomain that results in the displacements of the p66 connection, the RNase H domain, and the p51 subunit relative to the polymerase active site [14-16,24]. If structures are super-imposed on the C^{α} atoms of the β6-β10-β9 sheet, the p66 thumb subdomain of NNRTI bound RT is rotated approximately by 40˚ relative to the p66 fingers subdomain compared with its position in the free enzyme [24]. Associated with the hinge movement of the p66 thumb, the p66 connection and RNase H domains move away relative to the fingers subdomain with 13˚ and 15˚ rotations, respectively, compared with the corresponding positions in the free enzyme [24]. The position of the p66 fingers subdomain in the RT-NNRTI complexes is relatively unchanged. The subdomains in the p51 subunit of the NNRTI-bound RT also move 10˚, 12˚, 15˚ and 17˚ for the fingers, palm, thumb and connection subdomains, respectively [24]. As a result, the inter-subunit interactions at the heterodimer interfaces are maintained in both the free RT and NNRTI-RT complexes [24].

While comparisons of the different crystal forms of enzymes give insights into the relative position and flexibility of different domains (as described above), direct computational assessments provide information with respect to the collective dynamics of an enzyme. In this regard, using the

Gaussian network model (GNM) [40], the collective motions in HIV-1 RT were analyzed [41]. This latter study showed that the thumb and finger subdomains of the p66 subunit undergo correlated motions with respect to each other and anticorrelated motions with respect to the RNase H subdomain of p66 subunit and thumb subdomain of p51. Recently, an extended version of the GNM [40] referred to as an anisotropic network model (ANM) [42] was exploited to compare the dynamics of RT in unliganded and NNRTI-bound forms [43]. This study showed that NNRTI binding does not suppress the mobility of the fingers, palm or thumb subdomains of RT, but directly interferes with the global hinge-bending mechanism that controls the cooperative motions of the p66 fingers and thumb subdomains [43]. In the free enzyme, the p66 thumb and fingers subdomains form a highly unified block that undergoes in-phase oscillations about the p66 palm subdomain, with the latter serving as an anchor. The RNase H domain and the p51 thumb subdomain also perform *en bloc* motions; however these motions are negatively correlated with those of the p66 fingers and thumb (Fig .(**4**)). In NNRTI-bound RT, the p66 fingers and RNase H domain fluctuate in opposite directions (anticorrelated motions), giving rise to open and closed conformations (Fig. (**4**)). The p66 palm and connection serve as a rigid support for these flexible regions. The p66 thumb, on the other hand, is subject to orthogonal, but cooperative, motions with respect to the p66 fingers and RNase H. In other words, the net effect of NNRTI binding to RT is to change the direction of domain movements. Molecular dynamics studies of unliganded RT [44] and RT complexed with double stranded DNA [45] also show the flexibility of p66 thumb and fingers subdomains and the correlated or anti –rorrelated motions of the NNRTI-BP with respect to other subdomains of p66 subunit. A recent molecular dynamics and steered molecular dynamics NNRTI binding (α-APA) study, on the other hand, suggests that binding of NNRTIs restricts the flexibility and mobility of p66 thumb subdomain [46].

NNRTI-INDUCED CONFORMATIONAL CHANGES IN HIV-1 RT: NNRTI BINDING IMPACTS ON THE INTER-SUBUNIT INTERACTIONS OF RT

An interesting aspect of the NNRTI-BP is that it is situated close to the subunit-subunit interface, and the entrance of the pocket is composed of residues from the p66 (L100, K101, K103, V179 and Y181) and p51 (E138) subunits that also form part of the RT dimer interface. Several studies have evaluated the ability of NNRTI to impact on the dimeric structure of HIV-1 RT and surprising results have been obtained in this regard [47-51].

2',5'-Bis-*O*-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3'spiro-5''-(4''-amino-1',2'-oxathiole-2',2'-dioxide)thymine (TSAO-T) is the prototype of an unusual class of NNRTI which have structures and mechanism of actions quite distinct from conventional NNRTI [47,52-54]. The N3-ethyl derivative of TSAO-T, TSAO-e^{3}T has been shown to destabilize heterodimeric p66/p51 HIV-1 RT [47]; the Gibbs free energy of RT dimer dissociation is decreased in the presence of increasing concentrations of TSAOe^{3}T, resulting in loss of dimer stability of 4.0 kcal·mol^{-1}. This loss of energy is not sufficient to induce subunit dissociation in the absence of denaturant. High-level drug resistance to TSAO is mediated by the E138K mutation in the p51 subunit of HIV-1 RT [55]. The introduction of this mutation into RT significantly diminishes the ability of TSAO to bind to and inhibit the enzyme and accordingly TSAO-e^{3}T is unable to destabilize the subunit interactions of the E138K mutant enzyme. Modeling experiments have suggested that TSAO may bind to a site in RT that is overlapping with, but distinct from, the NNRTI binding site where it appears to make significant interactions with the p51 subunit of the enzyme [47,56]. On the basis of this model, the TSAO-induced changes in RT dimer stability likely arise from conformational perturbations that affect the p66/p51 RT interface.

N-(4-tert-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH) is a multitarget inhibitor of HIV-1 RT that binds to both the DNA polymerase and RNase H domains of the enzyme,

and inhibits both enzymatic activities [57,58]. BBNH binding to HIV-1 RT also impacts on the dimeric stability of the heterodimeric enzyme in that BBNH binding to RT decreases the value of the Gibbs free energy of RT dimer dissociation by 3.8 kcal·mol^{-1} [48]. To evaluate whether this loss of Gibbs free energy was mediated by BBNH binding to one or more sites in RT, a variety of BBNH analogs were synthesized and evaluated for their ability to destabilize (or weaken) the protein-protein interactions of the heterodimer [48]. It was found that *N*-acyl hydrazone binding in the DNA polymerase domain alone was sufficient to elicit the observed decrease in Gibbs free energy. In this regard, it has been speculated that BBNH binds to HIV-1 RT in a manner analogous to TSAOe^{3}T.

It has also recently been reported that several NNRTIs exhibit an unexpected capacity to dramatically increase the association of the p66 and p51 RT subunits [49]. Using a yeast two hybrid RT dimerization assay that specifically detects the interaction between the p66 and p51 RT subunits [59] it was shown that several NNRTI, including efavirenz, nevirapine, HBY 097 and α-APA, can significantly increase the β-galactosidase readout in a yeast reporter strain. Interestingly, delavirdine exhibited no capacity to either enhance or destabilize the inter-subunit interactions in RT. Additional studies showed that the NNRTI-induced enhancement effect on RT dimerization requires drug binding to the NNRTI-BP as introduction of the drug resistance mutation Y181C in the NNRTI-BP negates the enhancement effect mediated by nevirapine [49].

Based on these studies, NNRTIs can be classified into three distinct groups: (i) NNRTI that bind to RT and destabilize the inter-subunit interactions (e.g. TSAO-e^{3}T or BBNH); (ii) NNRTI that bind to RT and enhance the inter-subunit interactions (e.g. nevirapine, efavirenz); and (iii) NNRTI that bind to RT and have no effect on the inter-subunit interactions in RT (e.g. delavirdine). At time of writing, the molecular mechanisms by which NNRTI binding to RT can modulate the inter-subunit

interactions of the enzyme, and the impact that this modulation has on RT enzymatic functioning and HIV-1 viral replication are not known.

MECHANISM OF INHIBITION OF HIV-1 REVERSE TRANSCRIPTION BY NNRTI

Based on the structural, computational and biochemical studies described above, several possible mechanisms for the inhibition of HIV-1 RT by NNRTI have been suggested. These include:

(i) The conformational changes in the NNRTI-BP induced by NNRTI binding distort the precise geometry of the DNA polymerase catalytic site, especially the highly conserved Y183 M184 D185 D186 motif [15] and/or NNRTI binding deforms the structural elements that comprise the "primer grip", a region in RT that is involved in the precise positioning of the primer DNA strand in the polymerase active site [16]. Either of these conformational changes would inhibit the DNA polymerization reaction by preventing establishment of a catalytically competent ternary complex.

(ii) The NNRTI-BP may normally function as a hinge between the palm and thumb subdomains and the mobility of the thumb may be important to facilitate template/primer (T/P) translocation during DNA polymerization. It has been suggested that the binding of NNRTIs may restrict the mobility of the thumb subdomain (the "arthritic thumb" model) thus slowing or preventing T/P translocation and thereby inhibiting facile elongation of nascent viral DNA [22,51]. A recent steered molecular dynamics study on NNRTI binding supports this hypothesis [46]. However, the computational studies on the collective dynamics of HIV-1 RT described above (see Fig.4) do not support this model in that NNRTI binding was not found to suppress the mobility of the thumb or other subdomains in RT, but

rather changes their direction of movement [43]. Nevertheless, these changes in the directions of domain fluctuations could elicit a similar effect of hindering the ability of RT to translocate along the T/P nucleic acid substrate.

(iii) The inhibition of DNA polymerization reaction may result as a consequence of the destabilized or enhanced inter-subunit interactions in p66/p51 HIV-1 RT [47-51]. Enzyme structure and function is dependent on a delicate balance between the conflicting demands of molecular flexibility (entropy) and stability (enthalpy), and any modifications that affect this balance will have significant repercussions on molecular functioning. Since HIV-1 RT heterodimer formation is essential for enzymatic functioning [61,62], as is the structural flexibility of the subdomains in the p66 subunit of the enzyme [63,64], modulation of either of these parameters would surely inhibit enzyme activity.

The various mechanisms suggested are not mutually exclusive and NNRTIs may exert multiple inhibitory effects on RT-catalyzed DNA synthesis.

Several kinetic studies have been conducted with the aim of elucidating the mechanism by which NNRTI inhibit RT DNA polymerization. However, most of these studies used steady-state kinetic approaches that cannot elucidate the detailed interactions of these drugs with RT at the polymerase active site. The reason for the limited scope of steady-state experiments is their inability to resolve kinetic steps masked by the rate limiting step of a reaction. This point is particularly important with the reaction mechanism of RT (Fig. (**5**)). HIV-1 RT DNA synthesis follows an ordered 'bi bi' mechanism involving several RT mechanistic species [65-67]. Free RT first binds the template/primer (T/P) to form a tight RT-T/P$_n$ binary complex (Fig. (**5**), Step 1). This is followed by the binding of dNTP, to

form the RT-T/P_n-dNTP ternary complex (Fig. (**5**), Step 2). The binding of dNTP is a two-step process with an initial loose complex followed by a tighter binding complex as the enzyme undergoes a rate-limiting change in protein conformation to form a very tight ternary complex, RT*-T/P_n-dNTP (Fig. (**5**), Step 3) [68]. The formation of this ternary complex allows the critical transition state to be reached, enabling nucleophilic attack by the 3'-OH primer terminus on the α-phosphate of the bound dNTP (Fig. (**5**), Step 4). The rate limiting step for the reaction is the release of the elongated DNA from RT (Fig. (**5**), Step 6). This is the step that is examined during steady-state kinetic analyses.

Pre-steady state kinetic experiments, which allow the direct measurement of the individual reactions occurring at the enzymes active site (Fig. (**5**), Steps 2-5), were used to investigate the mechanism of inhibition of DNA polymerization by three "first generation" NNRTIs (including nevirapine) [69]. This study indicated that NNRTI blocked the chemical reaction (Fig. (**5**), Step 4), but did not interfere with nucleotide binding or the nucleotide-induced conformational change. Rather, in the presence of saturating concentrations of the inhibitors, the nucleoside triphosphate bound tightly (K_d, 100 nM), but nonproductively. Unfortunately, similar kinetic studies have not been conducted using delavirdine, "second-generation" NNRTI (e.g. efavirenz) or other unusual NNRTI (e.g. TSAOe^{3}T), and it is not known if they would elicit the same effect.

CONCLUSIONS

NNRTIs form a group of chemically diverse compounds that specifically inhibit HIV-1 RT by targeting a non-substrate binding site on the enzyme, termed the NNRTI-BP. Structural, computational and biochemical studies have demonstrated that NNRTI binding to HIV-1 RT induces both short-range and long-range conformational changes in enzyme structure. Based on these data, several possible mechanisms for the inhibition of HIV-1 RT by NNRTI have been suggested. These include

conformational changes that distort the precise geometry of the DNA polymerase active site, conformational changes that impact on the domain motions of the enzyme and inhibit DNA translocation, as well as changes in the inter-subunit interactions of the enzyme that ultimately impact on DNA polymerization. Detailed kinetic analyses of the mechanism(s) by which structurally different NNRTIs inhibit reverse transcription are lacking. The elucidation of this information would enable a better mechanistic understanding of how NNRTIs inhibit HIV-1 RT, and could potentially be used to identify and/or develop more potent inhibitors of RT.

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FIGURE LEGENDS

Fig.**(1)**. Chemical structures of NNRTI described in this study. (**a**) 11-Cyclopropyl-4-methyl-5,11-dihydro-6H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one (nevirapine); (**b**) Piperazine, 1-[3-[(1-methylethyl)amino]-2-pyridinyl]-4-[[5-[(methylsulfonyl)amino]-1H-indol-2-yl]carbonyl]-piperazine (delavirdine); (**c**) (4S)-6-Chloro-4-cyclopropylethynyl-4-trifluoromethyl-1, 4-dihydro-benzo[d][1, 3]oxazin-2-one (efavirenz); (**d**) 5-(3,5-Dichlorophenyl)thio-4-isopropyl-1-(4-pyridyl)methyl-1H-imidazol-2-ylmethyl carbamate (capravirine); (**e**) (S)-7-Methoxy-3,4-dihydro-2-[(methylthio)methyl]-3-thioxo-2(1H)-quinoxalinecarboxylic acid, isopropyl ester (HBY-097); (**f**) S-(+)-4,5,6,7-Tetrahydro-5-methyl-6-(3-methyl-2-butenyl)-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-thione (TIBO); (**g**) (+/-)-2,6-Dichloro-.alpha.-[(2-acetyl-5-methylphenyl)amino]benzamide (α-APA) ; (**h**) 1-[(2-Hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT); (**i**) Thymidine, 3-ethyl, [2',5'-bis-O-(tert-butyldimethylsilyl)-.beta.-D-ribofuranosyl]-3'-spiro-5-(4-amino-1,2-oxathiole-2,2-dioxide) (TSAOe^{3}T); (**j**) N-(tert-Butylbenzoyl)-2-hydroxynaphthaldehyde hydrazone (BBNH).

Fig.(**2**). Structure of HIV-1 RT in complex with nevirapine (PDB identifier: 1RTH). The p66 fingers, palm, thumb and connection subdomains and the RNase H domain are colored blue, yellow, red, green and magenta, respectively. Nevirapine is shown in space-fill and is colored purple. The residues that comprise the DNA polymerase active site (in the "palm" subdomain) and RNase H active site are shown in space-fill.
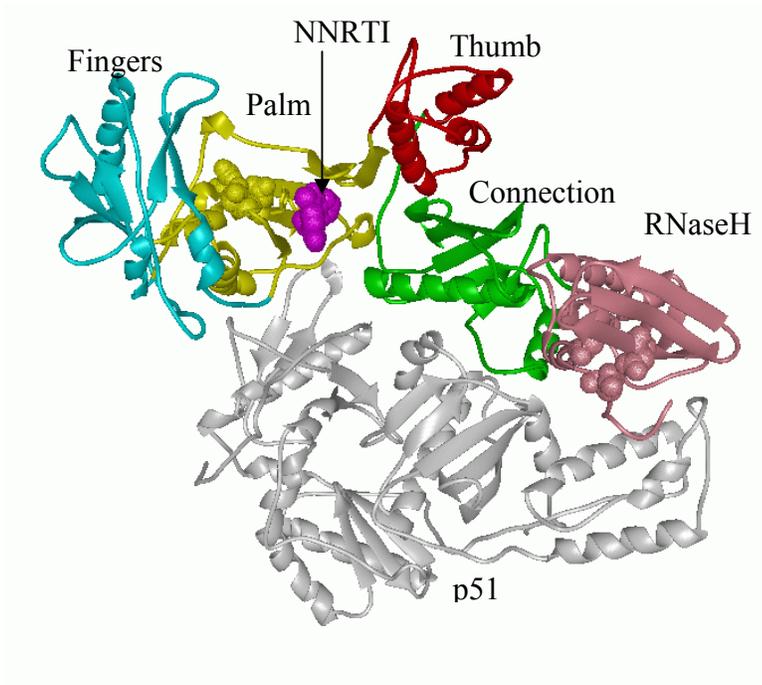
Fig.(**3**). Stereoview of nevirapine (1RTH; **a**), delavirdine (1KLM; **b**), HBY-097 (1BQM; **c**) and capravirine (1EP4, **d**) positioned in the NNRTI binding pocket.

Fig.(**4**). Residue fluctuations along the X-, Y- and Z-directions for unliganded (black line) and nevirapine-bound (blue line) RT. The X axis coincides with the out-of-plane direction; the Y and Z axes lie along the in-plane directions. See the ribbon diagrams on the right. The p66 fingers, thumb, RNase H, and the p51 thumb are colored blue, red, pink, and magenta, respectively.
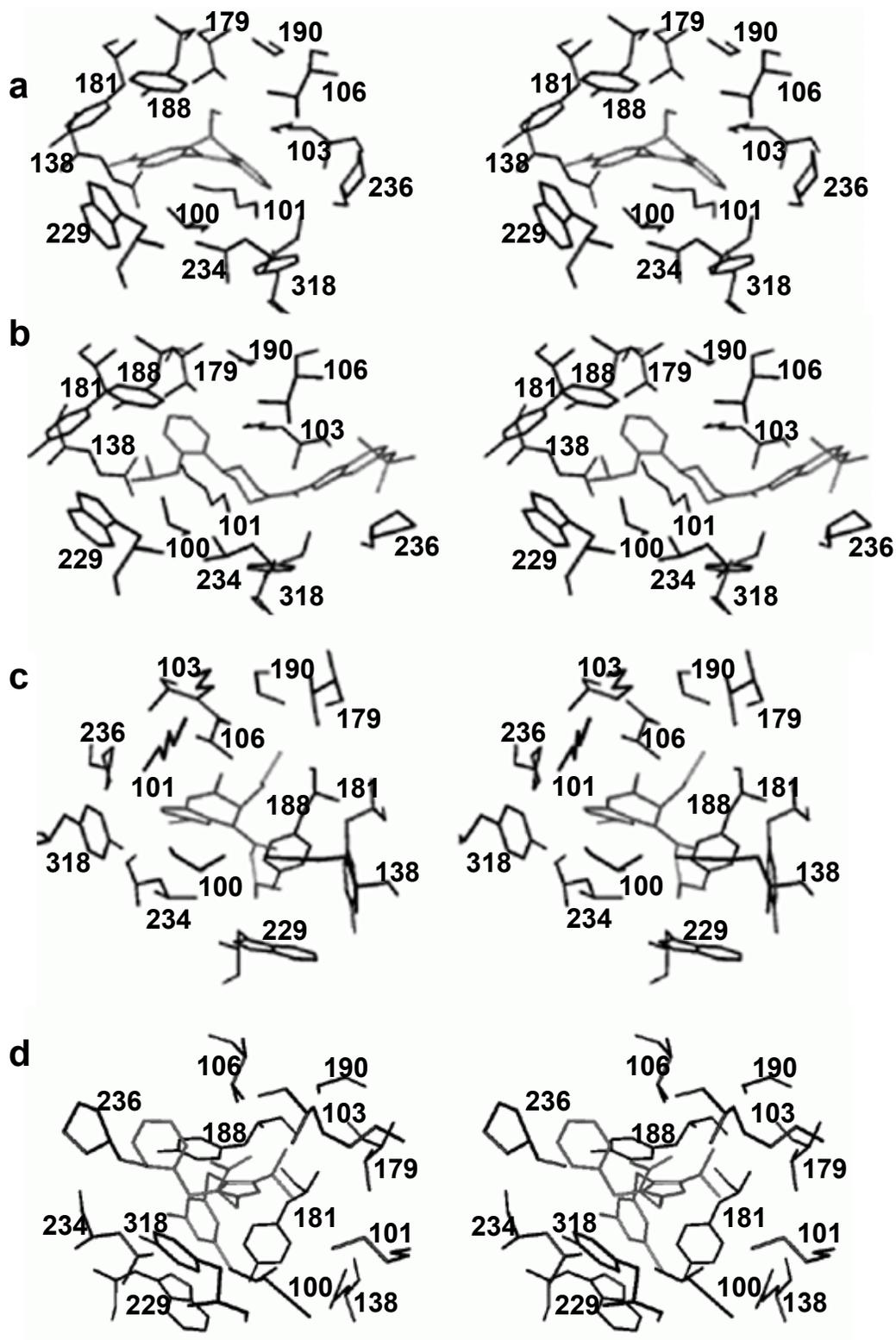
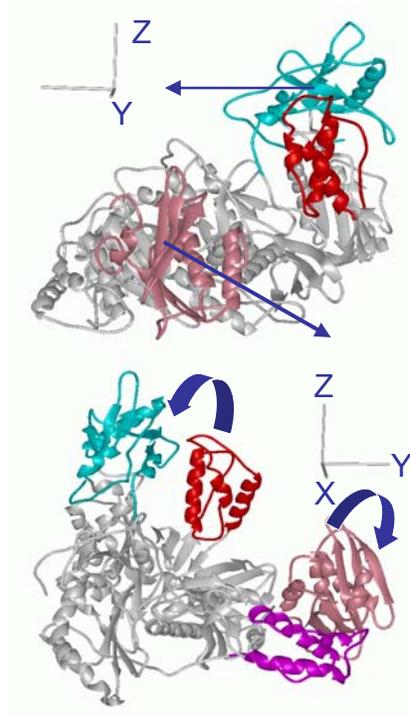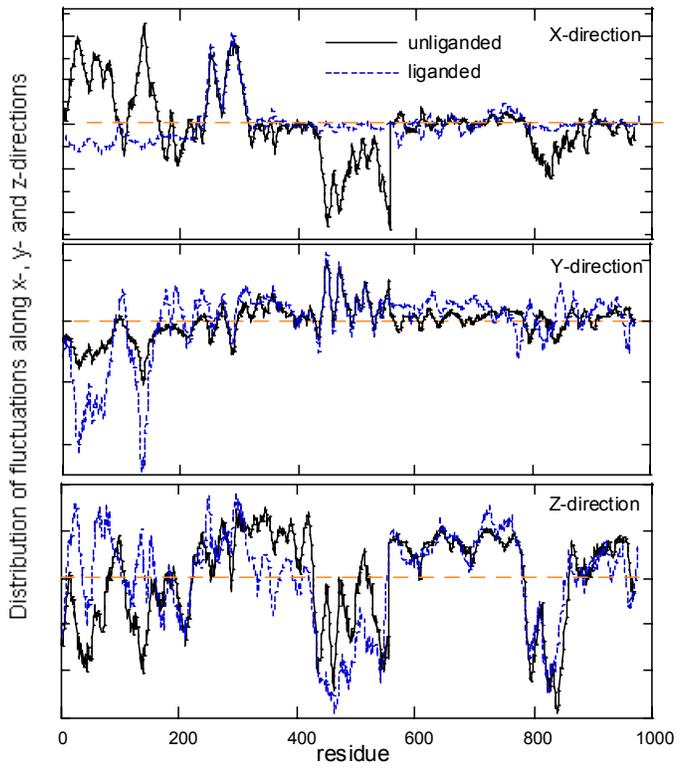Fig.(**5**). Schematic of the ordered kinetic mechanism of RT-catalyzed DNA synthesis
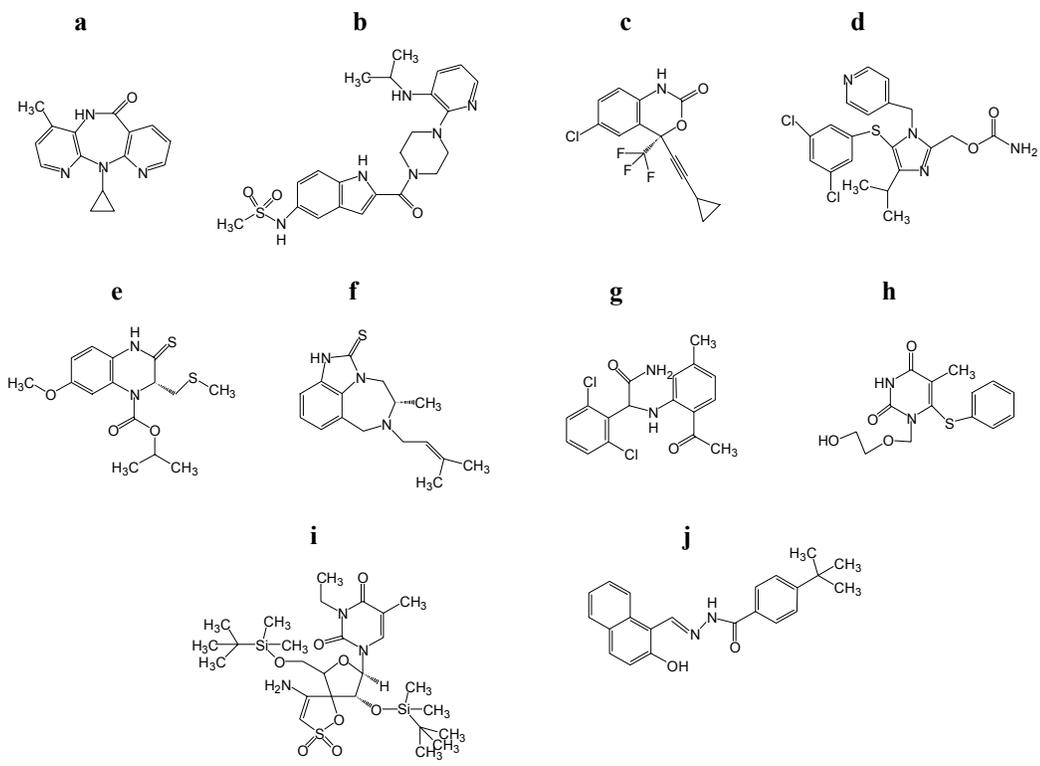
Sluis-Cremer et al., Fig.(**1**).

Sluis-Cremer et al., Fig.(**2**).

Sluis-Cremer et al., Fig.(**3**).

Sluis-Cremer et al., Fig. (**4**)

Step 1

RT + T/P$_n$

RT-T/P$_n$

dNTP ⟶ Step 2

RT-T/P$_n$-dNTP

Step 3
Rate-limiting in
absence of NNRTI

RT*-T/P$_n$-dNTP

Step 4
Rate-limiting in
presence of NNRTI

RT-T/P$_{n+1}$-PPi

PPi ⟶ Step 5

Step 6
RT + T/P$_{n+1}$ Overall rate
limiting step

RT-T/P$_{n+1}$

Sluis-Cremer et al., Fig.(**5**).