Probing nonnucleoside inhibitor-induced active-site distortion in HIV-1 reverse transcriptase by transient kinetic analyses

QING XIA,¹ JESSICA RADZIO,¹ KAREN S. ANDERSON,² AND NICOLAS SLUIS-CREMER¹

¹Division of Infectious Diseases, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, USA

²Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06520, USA

(RECEIVED February 14, 2007; FINAL REVISION May 11, 2007; ACCEPTED May 20, 2007)

Abstract

Nonnucleoside reverse transcriptase inhibitors (NNRTI) are a group of structurally diverse compounds that bind to a single site in HIV-1 reverse transcriptase (RT), termed the NNRTI-binding pocket (NNRTI-BP). NNRTI binding to RT induces conformational changes in the enzyme that affect key elements of the polymerase active site and also the association between the two protein subunits. To determine which conformational changes contribute to the mechanism of inhibition of HIV-1 reverse transcription, we used transient kinetic analyses to probe the catalytic events that occur directly at the enzyme's polymerase active site when the NNRTI-BP was occupied by nevirapine, efavirenz, or delavirdine. Our results demonstrate that all NNRTI-RT-template/primer (NNRTI-RT-T/P) complexes displayed a metal-dependent increase in dNTP binding affinity (K_d) and a metal-independent decrease in the maximum rate of dNTP incorporation (k_{pol}). The magnitude of the decrease in k_{pol} was dependent on the NNRTI used in the assay: Efavirenz caused the largest decrease followed by delavirdine and then nevirapine. Analyses that were designed to probe direct effects on phosphodiester bond formation suggested that the NNRTI mediate their effects on the chemistry step of the DNA polymerization reaction via an indirect manner. Because each of the NNRTI analyzed in this study exerted largely similar phenotypic effects on single nucleotide addition reactions, whereas each of them are known to exert differential effects on RT dimerization, we conclude that the NNRTI effects on subunit association do not directly contribute to the kinetic mechanism of inhibition of DNA polymerization.

Keywords: HIV; reverse transcriptase; nonnucleoside reverse transcriptase inhibitor; presteady-state kinetics; phosphorothioate metal effect

Reprint requests to: Nicolas Sluis-Cremer, Division of Infectious Diseases, University of Pittsburgh School of Medicine, S817 Scaife Hall, 3550 Terrace Street, Pittsburgh, PA 15261, USA; e-mail: cremern@dom.pitt.edu; fax: (412) 648-8521.

Abbreviations: RT, reverse transcriptase; NNRTI, nonnucleoside reverse transcriptase inhibitor; NNRTI-BP, nonnucleoside reverse transcriptase inhibitor-binding pocket; T/P, template/primer; RT-T/P, reverse transcriptase-template/primer binary complex; RT-T/P-dNTP, reverse transcriptase-template/primer-dNTP ternary complex; NNRTI-RT-T/P, nonnucleoside reverse transcriptase inhibitor-reverse transcriptase-template/primer complex; TTP α S, thymidine-5'-O-1-thiotriphosphate; DEL, delavirdine; NEV, nevirapine; EFV, efavirenz.

Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.072829007>.

Reverse transcription of the viral single-stranded (+) RNA genome into double-stranded DNA is entirely dependent on the enzymatic activities of the retroviral enzyme HIV-1 reverse transcriptase (RT). HIV-1 RT is an asymmetric heterodimer composed of a 560 amino acid 66-kDa subunit (p66) and a 440 amino acid 51-kDa subunit (p51) (Kohlstaedt et al. 1992). The p51 polypeptide is derived by HIV-1 protease-mediated cleavage of the C-terminal ribonuclease H (RNase H) domain of the p66 polypeptide. 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. Although the p51 subunit contains the same amino acid sequence that comprises the DNA polymerase domain of the p66 subunit, the polymerase active site is not functional (Kohlstaedt et al. 1992). Due to its essential role in the HIV-1 life cycle, RT is a primary target for anti-HIV drug development and two distinct therapeutic groups of inhibitors have been identified: (1) nucleoside or nucleotide RT inhibitors and (2) nonnucleoside RT inhibitors (NNRTIs).

NNRTI bind to a single site in the p66 subunit of HIV-1 RT, termed the NNRTI binding-pocket (NNRTI-BP), that is situated ~ 10 Å from the enzyme's polymerase active site (Kohlstaedt et al. 1992). Three possible mechanisms of inhibition of HIV-1 reverse transcription by NNRTI have been suggested based on crystal structure analyses (Parniak and Sluis-Cremer 2000). Esnouf et al. (1995) reported that NNRTI binding to RT in the NNRTI-BP distorts the precise geometry of the DNA polymerase catalytic site, especially the highly conserved Y183 M184 D185 D186 motif, and proposed that this class of drugs inhibits DNA polymerization by locking the polymerase active site in an inactive conformation. Hsiou et al. (1996) observed that NNRTI binding deformed 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. This change in primer grip conformation is expected to alter the position and conformation of the template-primer (T/P) substrate, thereby preventing the establishment of a catalytically competent ternary complex (Hsiou et al. 1996). Finally, Kohlstaedt et al. (1992) proposed that the NNRTI-BP may normally function as a hinge between the palm and thumb domains. Since the mobility of the thumb may be important to facilitate template/primer (T/P) translocation during DNA polymerization, the binding of NNRTIs may restrict the mobility of the thumb domain, thus slowing

down or preventing T/P translocation and thereby inhibiting facile elongation of nascent viral DNA.

NNRTI inhibition of DNA polymerization may also result as a consequence of the drugs impacting on the intersubunit interactions of the p66/p51 HIV-1 RT (Sluis-Cremer and Tachedjian 2002; Sluis-Cremer et al. 2004). HIV-1 RT is an obligate heterodimer, and any modifications that effect the intersubunit interactions of RT will have significant repercussions on the enzyme's molecular functioning (Tachedjian et al. 2005b). In this regard, Tachedjian et al. (2001, 2005a) demonstrated that some NNRTIs act as chemical enhancers of RT dimerization. Interestingly, not all NNRTI exhibited the same capacity to enhance RT dimerization. For instance, efavirenz (EFV) was found to exert a potent enhancement effect on RT dimerization, whereas nevirapine (NEV) only exerted a moderate effect (Tachedjian et al. 2001). In contrast, delavirdine (DEL) exerted no effect on the intersubunit interactions of the enzyme (Tachedjian et al. 2001). Although these NNRTI all occupy the same binding site in HIV-1 RT, the intermolecular interactions that stabilize the protein-drug interactions are different for each inhibitor. For example, NEV assumes a "butterfly-like" structure (Ding et al. 1995) in which its "wings" interact with residues K101, K103, V106, V179, Y181, Y188, and W229, while its "body," "back," and "head" interact with the main chain atoms of Y188 and G190 and the side chains of V106 and V179, residues L100 and L234, and E138 from the p51 subunit, respectively (Fig. 1, red molecule). DEL is a bulkier molecule than NEV, which causes it to extend beyond the usual pocket and to project into the solvent (Fig. 1, green molecule; Esnouf et al. 1997). The piperazine ring conformation of DEL also positions the inhibitor very close to V106, which facilitates hydrogen-bonding interactions with the main chain of K103 and extensive

Figure 1. Overlay of the NNRTI-BP of HIV-1 RT in complex with NEV (Kohlstaedt et al. 1992) DEL, (Esnouf et al. 1997), or EFV (Ren et al. 2000). NEV, DEL, and EFV are colored red, green, and blue, respectively.

hydrophobic interactions between the indole ring of DEL and P236 (Esnouf et al. 1997). EFV is a smaller compound which fits more compactly in the NNRTI-BP (Fig. 1, blue molecule), thereby limiting contacts with the readily mutable Y181 and Y188 side chains (Ren et al. 2000). Tachedjian et al. (2001) and Tachedjian and Goff (2003) proposed that these observed differences in the mode of binding of each of these drugs within the NNRTI-BP may account for their observed differential effects on RT dimerization. Detailed kinetic analyses using transient kinetic analyses have previously been applied to elucidate the kinetic mechanism by which NEV and two tetrahydroimidazobenzodiazepinone (TIBO) derivatives (O-TIBO and CI-TIBO) inhibit HIV-1 reverse transcription (Rittinger et al. 1995; Spence et al. 1995). These studies established that the dNTP substrate and NNRTI could simultaneously bind to enzyme, and that there was communication between the active site and the NNRTI binding pocket. These studies also suggested that the NNRTI caused the rate-limiting step of the RT-mediated nucleotide addition reaction to be altered from the conformational change preceding chemistry to the chemistry step itself. However, since these studies were reported in 1995, no additional studies have been conducted on the “next generation NNRTI” such as DEL and EFV. As point of fact, both NEV and CI-TIBO exert similar effects on RT dimerization (Tachedjian et al. 2001), and therefore it is not known if DEL and EFV exhibit similar or different kinetic mechanisms of action in comparison with NEV. Therefore, the specific objectives of this study were twofold. First, we wanted to probe the nature of the communication between the NNRTI-BP and polymerase active site and attempt to relate the kinetic observations with the currently available crystal structure data. Second, we wanted to ascertain if the “next-generation” inhibitors, such as DEL or EFV, exhibited similar or different mechanisms of inhibition of HIV-1 reverse transcription in comparison with NEV.

Results

Most studies detailing the mechanism of action of NNRTI on HIV-1 RT-mediated DNA synthesis reactions have been carried out using steady-state kinetics. Because steady-state experiments are unable to resolve kinetic steps that are masked by the rate-limiting step of a reaction (the release of DNA substrate from RT), this approach cannot elucidate the detailed interactions of the drug with RT at the polymerase active site. Accordingly, we have used a presteady-state kinetic approach to provide detailed mechanistic insights into the catalytic events that occur directly at the enzyme's active site in the absence and presence of NEV, DEL, or EFV. A series of presteady-state kinetic experiments have been described previously for studies investigating the kinetic interaction of NNRTIs with HIV-1 RT (Spence

et al. 1995, 1996; Wang et al. 2004). These experiments have been employed in this study to elucidate the kinetic mechanism of inhibition of NEV, DEL, or EFV on single nucleotide incorporation reactions carried out by HIV-1 RT. To provide a more in-depth analysis of the effects of phosphodiester bond formation and assess whether these effects are direct or indirect, we have also extended the study to evaluate the effect of these NNRTI inhibitors on dTTP incorporation facilitated by MnCl_2 and CoCl_2 , and on thymidine-5'-O-1-thiotriphosphate (dTTP α S) incorporation.

Determination of dissociation constants (K_d) for NEV, DEL, and EFV for the RT-template/primer (T/P) binary complex using presteady-state burst reactions

Presteady-state burst experiments provide (1) a direct measurement of the rate of polymerization at the active site, (2) the burst amplitude which defines the concentration of the reactive RT-T/P complexes, and (3) a steady-state turnover rate which is limited by the dissociation of the product complex (i.e., T/P $_{+1}$) from HIV-1 RT. As described previously (Spence et al. 1995, 1996; Wang et al. 2004), we found that the burst amplitude is reduced monotonically with increasing concentrations of NEV, DEL, or EFV (Fig. 2A). Since the burst amplitude represents the amount of uninhibited RT-T/P complex available for fast nucleotide incorporation in the first turnover, one can determine the K_d for a particular NNRTI for the RT-T/P binary complex by plotting the burst amplitude versus NNRTI concentration and by fitting the data to the appropriate hyperbolic algorithm (Fig. 2B). Using this method, we calculated K_d values of 25.0 ± 3.5 nM, 16.6 ± 4.3 nM, and 2.6 ± 1.3 nM for NEV, DEL, and EFV for the RT-T/P binary complex, respectively. The K_d value calculated for NEV in this study (25 nM) is essentially identical to the K_d value (20 nM) previously reported for NEV for the RT-T/P binary complex (Spence et al. 1995).

Mg²⁺-dTTP incorporation reactions carried out by RT-T/P and NNRTI-RT-T/P complexes

RT-T/P complexes saturated with NEV, DEL, or EFV all exhibited slow but measurable DNA polymerization rates, which enabled us to use single nucleotide turnover conditions to determine the kinetic parameters of nucleotide incorporation facilitated by RT-T/P and NNRTI-RT-T/P complexes (Fig. 3). In these experiments, the RT-T/P and NNRTI-RT-T/P complexes were rapidly mixed with Mg^{2+} -dTTP solutions, and the reactions were stopped after designated times by the addition of 0.5 M EDTA. The data demonstrate that all three inhibitors exert profound effects on both nucleotide affinity and the rate of nucleotide incorporation (Table 1). For each of the

Figure 2. Determination of an equilibrium constant for efavirenz for RT-T/P. (A) Burst reactions carried out in the presence of different of 0 (●), 2 nM (▼), 4 nM (Δ), 7.5 nM (○), 15 nM (■), and 35 nM (□) EFV, respectively. The final concentrations of RT, T/P, TTP, and Mg^{2+} in the assay were 50 nM, 120 nM, 20 μ M, and 10 mM, respectively. (B) The amplitudes of the burst phases (●) were plotted against EFV concentration and fit to a hyperbolic function (solid line) to yield a K_d value of 2.6 ± 1.3 nM. Similar experiments were conducted to determine K_d values for NEV and DEL (data not shown).

NNRTI-RT-T/P complexes, the affinity of the Mg^{2+} -dTTP substrate was increased 130-fold compared with the RT-T/P complex. This effect on K_d appeared to be independent of the NNRTI used in the assay. In contrast, the rate of Mg^{2+} -dTTP incorporation (k_{pol}) was significantly decreased in the NNRTI-RT-T/P complexes. The magnitude of this decrease was dependent on the NNRTI used in the assay; k_{pol} was decreased by each of the NNRTI in the order of EFV > DEL > NEV.

Mn^{2+} -dTTP and Co^{2+} -dTTP incorporation reactions carried out by RT-T/P and NNRTI-RT-T/P complexes

HIV-1 RT, like most polymerase enzymes, can substitute $MgCl_2$ for other divalent metal ion cofactors in nucleotide addition reactions. To determine the ability of different metal ions to substitute for Mg^{2+} in HIV-1 RT-mediated nucleotide incorporation reactions, and also to determine the optimal concentration for each metal ion, single-turnover experiments were carried out in which an RT-T/P

complex was mixed with an equal volume of [metal ion]-dTTP to initiate DNA synthesis. An apparent rate constant (k_{app}) for TTP incorporation was then calculated for different [metal ion]-dTTP (Fig. 4). Both Mn^{2+} -dTTP and Co^{2+} -dTTP can activate HIV-1 RT as well as Mg^{2+} -dTTP. The optimal metal ion concentrations for single-nucleotide incorporation were 10 mM, 2 mM, and 1 mM for Mg^{2+} -, Mn^{2+} -, and Co^{2+} -dTTP, respectively. Additional reactions were also carried out to evaluate whether NNRTI binding to the RT-T/P complex impacted on metal ion recognition (Fig. 4). In this regard, the metal optima determined for the RT-T/P complex were identical to those determined for all NNRTI-RT-T/P complexes. Optimal $MnCl_2$ and $CoCl_2$ concentrations were used in all the experiments described below.

Presteady-state kinetic parameters were determined for Mn^{2+} -dTTP and Co^{2+} -dTTP incorporation reactions facilitated by RT-T/P and NNRTI-RT-T/P complexes (Table 2). As described above, reactions were carried out in which RT-T/P and NNRTI-RT-T/P complexes were rapidly mixed with a Mn^{2+} - or Co^{2+} -TTP solutions and then stopped after designated times by the addition of 0.5 M EDTA. When $MnCl_2$ was used as a metal ion cofactor, the affinity of the nucleotide substrate (K_d) for the NNRTI-RT-T/P complex was increased 20-fold (as opposed to 130-fold for Mg^{2+} -TTP; Table 1). Again, this effect on K_d was independent of the NNRTI used in the assay. Interestingly, when $CoCl_2$ was used as the metal ion cofactor, no differences in the apparent affinity for the TTP substrate were observed between the RT-T/P and NNRTI-RT-T/P complexes. In contrast, each of the NNRTI significantly slowed the rate of nucleotide incorporation. In general, the extent to which k_{pol} was decreased by the NNRTI was independent of the metal ion used in the assay (Table 1).

Incorporation of the Sp-isomer of thymidine-5'-O-1-thiotriphosphate (dTTP α S) by RT-T/P and NNRTI-T/P complexes

Phosphorothioate elemental effects, derived from experiments which compare the rates of incorporation of the natural dNTP substrate versus dNTP α S, are frequently used as a diagnostic for determining whether the chemical step of polymerization reactions is rate-limiting (Kuchta et al. 1987; Patel et al. 1991; Reardon 1992; Hsieh et al. 1993; Zinnen et al. 1994; Woodside and Guengerich 2002; Ray et al. 2003; Choi and Guengerich 2004; Zang et al. 2005). dNTP α S is synthesized as a mixture of two isomers, termed pro-Sp and pro-Rp, both of which can be incorporated by HIV-1 RT (Radzio and Sluis-Cremer 2005). However, RT does exhibit a significant stereo-selective preference for incorporating Sp-dNTP α S over Rp-dNTP α S (Radzio and Sluis-Cremer 2005), and

Figure 3. Mg^{2+} -TTP concentration dependence of the nucleotide incorporation rate in the absence (*left* panel) or presence (*right* panel) of EFV. (A) Experimental conditions as described in Materials and Methods. The concentrations of TTP used were 1 μM ($\bullet$), 5 μM (Δ), 12.5 μM ($\blacksquare$), 20 μM ($\diamond$), and 35 μM ($\blacktriangle$) in the absence of inhibitor (*left* panel) and 0.05 μM ($\bullet$), 0.1 μM (Δ), 0.2 μM ($\blacksquare$), 0.5 μM ($\diamond$), 1 μM ($\square$), and 5 μM in the presence of inhibitor (*right* panel). (B) The single incorporation rates ($\bullet$), as a function of TTP concentration, were fit to hyperbola (solid line) that corresponds to a K_d value of $2.6 \pm 1.1 \mu\text{M}$ and a k_{pol} value of $8.9 \pm 1.77 \text{ sec}^{-1}$ for RT-T/P (*left* panel); and a K_d value of $0.02 \pm 0.006 \mu\text{M}$ and k_{pol} value of $0.0012 \pm 0.0004 \text{ sec}^{-1}$ for EFV-RT-T/P (*right* panel). All data in Tables 1 and 2 were derived using identical analyses as described in this figure.

accordingly, we only report data for the incorporation of Sp-dTTP α S by RT-T/P and NNRTI-RT-T/P using Mg^{2+} , Mn^{2+} , and Co^{2+} as metal ion cofactors (Table 2). Consistent with previous studies, the kinetic parameters (K_d , k_{pol} , k_{pol}/K_d) for the incorporation of Sp-dTTP α S by RT-T/P are very similar to those calculated for the

incorporation of the natural dTTP substrate (Radzio and Sluis-Cremer 2005). Moreover, no phosphorothioate metal effects ($k_{\text{pol}}^{\text{dTTP}}/k_{\text{pol}}^{\text{Sp-dTTP}\alpha\text{S}} > 3\text{--}4$) were calculated for RT-T/P. Interestingly, the kinetic parameters for the incorporation of Sp-dTTP α S by NNRTI-RT-T/P were also essentially identical to those calculated for

Table 1. Presteady-state kinetic parameters determined for incorporation of TTP by RT-T/P and NNRTI-RT-T/P using different metal ion cofactors

	K_d (μM)	k_{pol} (sec^{-1})	k_{pol}/K_d ($\mu\text{M}^{-1} \cdot \text{sec}^{-1}$)	Fold change in ^a :		
				K_d	k_{pol}	k_{pol}/K_d
Mg²⁺						
RT-T/P	2.60 $\pm$ 1.41	8.90 $\pm$ 1.77	1.2	—	—	—
NEV-RT-T/P	0.02 $\pm$ 0.01	0.0060 $\pm$ 0.0009	3.0×10^{-1}	130	1483	4
DEL-RT-T/P	0.02 $\pm$ 0.01	0.0027 $\pm$ 0.0006	1.4×10^{-1}	130	3296	9
EFV-RT-T/P	0.02 $\pm$ 0.006	0.0012 $\pm$ 0.0004	6.0×10^{-2}	130	7417	20
Mn²⁺						
RT-T/P	3.44 $\pm$ 0.60	5.10 $\pm$ 0.79	1.5	—	—	—
NEV-RT-T/P	0.22 $\pm$ 0.07	0.0054 $\pm$ 0.0011	2.5×10^{-2}	16	944	60
DEL-RT-T/P	0.20 $\pm$ 0.05	0.0023 $\pm$ 0.0009	1.0×10^{-2}	17	2217	50
EFV-RT-T/P	0.18 $\pm$ 0.03	0.0009 $\pm$ 0.0001	5.0×10^{-3}	19	5667	300
Co²⁺						
RT-T/P	21.40 $\pm$ 3.44	2.17 $\pm$ 0.52	1.0×10^{-1}	—	—	—
NEV-RT-T/P	20.21 $\pm$ 3.01	0.0019 $\pm$ 0.0005	9.4×10^{-5}	1.1	1142	1063
DEL-RT-T/P	21.77 $\pm$ 2.88	0.0010 $\pm$ 0.0007	4.6×10^{-5}	1.0	2170	2174
EFV-RT-T/P	17.09 $\pm$ 2.55	0.0003 $\pm$ 0.0001	1.8×10^{-5}	1.3	7233	5556

^aFold change in kinetic parameter as determined by: [RT-T/P]/[NNRTI-RT-T/P].

Figure 4. Dependence of HIV-1 RT DNA polymerase activity on Mg²⁺ (A), Mn²⁺ (B), or Co²⁺ (C). RT-T/P (○; left-hand ordinate) or EFV-RT-T/P (●; right-hand ordinate) was mixed with [metal ion]-TTP (200 μM), and the reaction was quenched after an allocated time. The results were fitted to a single-exponential equation to obtain an apparent catalytic rate (k_{app}) for each metal ion concentration. k_{app} was then plotted against metal ion concentration. The optimal metal ion concentrations for single-nucleotide incorporation by both RT-T/P and E-RT-T/P were 10 mM, 2 mM, and 1 mM for the Mg²⁺-, Mn²⁺-, and Co²⁺-TTP, respectively.

dTTP. Phosphorothioate metal effects were also not calculated for these complexes, possibly suggesting that the chemistry step of the reaction is not adversely effected by NNRTI binding.

Discussion

Inhibitor binding in the NNRTI-BP communicates several conformational changes in HIV-1 RT that distort the precise geometry of the catalytic aspartic acid residues in the polymerase active site (Esnouf et al. 1995), the primer grip, a motif that is involved in the precise positioning of the primer DNA strand in the active site (Hsiou et al. 1996), and the enzyme's protein-protein

subunit interactions (Tachedjian et al. 2001, 2005a). In order to delineate the role of these NNRTI-induced conformational changes in blocking dNTP incorporation reactions, we have used the presteady-state kinetic approach to probe the catalytic events that occur directly in the active site of HIV-1 RT.

Kinetic experiments were designed to probe dNTP binding and incorporation, metal ion interactions, and phosphodiester bond formation in the absence and presence of three structurally diverse NNRTI (NEV, DEL, and EFV). The results presented in Tables 1 and 2 clearly demonstrate that all three inhibitors exerted largely similar phenotypic effects on each of the kinetic parameters determined. k_{pol} was the only kinetic parameter differentially effected by each of the inhibitors (k_{pol} was decreased in the order of EFV > DEL > NEV). The order in which EFV, DEL, and NEV decreased k_{pol} is consistent with their observed ability to inhibit HIV-1 replication in cell culture (Young et al. 1995). Taken together, these data suggest that all three inhibitors exhibit similar molecular mechanisms of inhibition of DNA synthesis. However, as described previously, EFV, DEL, and NEV exert differential effects on HIV-1 RT dimerization (Tachedjian et al. 2001, 2005a): EFV acts as a potent enhancer of RT dimerization; NEV exerts only a moderate enhancement effect on RT dimerization; and DEL exerts no effects on p66/p51 RT heterodimer formation. Therefore, we must conclude that the conformational changes induced in the dimer interface of HIV-1 RT by structurally diverse NNRTI do not directly contribute to their ability to inhibit DNA synthesis. Our data, however, do not preclude the fact that NNRTI could impact on other stages of the HIV-1 viral life cycle. In this regard, we recently demonstrated that EFV, but not NEV or DEL, accelerates the proteolytic processing events of the Gag-Pol polyprotein, suggesting that the enhancement effect on RT dimerization may impact on the late stages (post-integration) of viral replication (Tachedjian et al. 2005a; Figueiredo et al. 2006).

It was previously reported that NEV and TIBO binding to an RT-T/P complex increased the affinity (K_d) for the incoming Mg²⁺-dNTP but decreased the rate of its incorporation (k_{pol}) (Spence et al. 1995, 1996). Our studies confirm these findings (Table 1). Spence et al. (1995, 1996) analyzed this finding in terms of a two-step dNTP binding mechanism and proposed that the large increase in Mg²⁺-dNTP affinity was due to a rate-limiting chemistry step which enabled measurement of the overall K_d for dNTP binding and not just the ground-state dNTP binding affinity that is measured in the absence of inhibitor when a conformational change preceding chemistry is rate-limiting. However, the underlying molecular mechanism for the effects of NNRTIs on chemical catalysis and whether these effects are direct or indirect

Table 2. Presteady-state kinetic parameters

	K_d (μM)	k_{pol} (sec^{-1})	k_{pol}/K_d ($\mu\text{M}^{-1}\cdot\text{sec}^{-1}$)	Metal effect ^a
10 mM Mg²⁺				
RT-T/P	2.88 ± 1.21	6.77 ± 0.98	2.4	1.3
NEV-RT-T/P	0.02 ± 0.008	0.0057 ± 0.0011	2.9×10^{-1}	1.1
DEL-RT-T/P	0.02 ± 0.010	0.0030 ± 0.0009	1.5×10^{-1}	0.9
EFV-RT-T/P	0.02 ± 0.009	0.0009 ± 0.0002	4.5×10^{-2}	1.3
2 mM Mn²⁺				
RT-T/P	4.23 ± 1.04	3.22 ± 0.88	7.6×10^{-1}	1.6
NEV-RT-T/P	0.21 ± 0.09	0.0028 ± 0.0009	1.3×10^{-2}	1.9
DEL-RT-T/P	0.20 ± 0.03	0.0017 ± 0.0006	8.5×10^{-3}	1.4
EFV-RT-T/P	0.20 ± 0.06	0.0005 ± 0.0001	2.5×10^{-3}	1.8
1 mM Co²⁺				
RT-T/P	20.21 ± 4.40	2.27 ± 0.74	1.1×10^{-1}	1.0
NEV-RT-T/P	22.53 ± 5.63	0.0013 ± 0.0005	5.8×10^{-5}	1.5
DEL-RT-T/P	21.01 ± 4.74	0.0007 ± 0.0002	3.3×10^{-5}	1.4
EFV-RT-T/P	21.63 ± 3.10	0.0003 ± 0.0001	1.4×10^{-5}	1.0

Determined for incorporation of Sp-TTP α S by RT-T/P and NNRTI-RT-T/P using different metal ion cofactors.

^a Metal effect defined as $k_{\text{pol}}^{\text{TTP}}/k_{\text{pol}}^{\text{Sp-TTP}\alpha\text{S}}$.

on phosphodiester bond formation remain unclear. In the present study, this issue was addressed through the use of alternate metals and dNTP(α S) substrates. In regard to the latter, phosphorothioate elemental effects are frequently used as a diagnostic for determining whether the chemical step of polymerization reactions is rate-limiting, although these assessments may be viewed with caution (Shan and Herschlag 2002). For HIV-1 RT, kinetic studies have demonstrated the absence of a metal effect under normal polymerization conditions, suggesting that the rate-limiting step of the reaction preceded chemical catalysis (Reardon 1992; Hsieh et al. 1993; Ray et al. 2003). However, large metal effects (ranging from 3 to 72) have been detected in HIV-1 RT misincorporation reactions (Zinnen et al. 1994), phosphorolytic excision reactions (Ray et al. 2003), and nucleotide addition reactions opposite modified DNA adducts (Woodside and Guengerich 2002; Choi and Guengerich 2004; Zang et al. 2005). In all of these studies it was proposed that the chemistry step, and not the conformational change preceding chemistry, was rate-limiting.

Our data show that the NNRTI effect on K_d is metal-dependent: The large increase in K_d for Mg²⁺-TTP is ~ 10 -fold less for Mn²⁺-TTP and not apparent for Co²⁺-TTP (Table 1). In contrast, the NNRTI effect on k_{pol} is metal independent, and the rate of incorporation for each metal-dNTP is essentially similar for a given inhibitor (Table 1). Furthermore, we show that no phosphorothioate elemental effects were evident for any of the NNRTI-RT-T/P complexes examined in this study, irrespective of the metal ion used in the assay (Table 2). This suggests that the slow rate of dNTP incorporation observed for NNRTI-RT-T/P complexes may not be due to a direct effect of the inhibitor on phosphodiester bond formation but rather to an indirect effect through alteration/perturbation of the constellation of

amino acids involved in positioning the active site for efficient catalysis. In light of our results, we propose that distinct alterations in the enzyme's DNA polymerase active site contribute to the observed effects of NNRTI binding on metal-dNTP binding and dNTP incorporation. As described by Hsiou et al. (1996), inhibitor binding in the NNRTI-BP causes the primer grip to be shifted upward by ~ 5 Å in comparison with its position in the RT-T/P binary (Fig. 5A) and RT-T/P-dNTP ternary (Fig. 5B) complexes. For chemical catalysis to occur at the active site, the dNTP and T/P substrates need to be correctly aligned. In this regard, the slow rate of dNTP incorporation observed for the NNRTI-RT-T/P complexes might be due to changes in the position and conformation of the primer grip, which significantly slow down the necessary conformational changes that are required to align the substrates and to facilitate phosphodiester bond formation. Inhibitor binding in the NNRTI-BP has also been shown to distort the precise geometry of the DNA polymerase catalytic site, especially the highly conserved Y183 M184 D185 D186 motif (Esnouf et al. 1995). We believe that these conformational changes may influence metal-dNTP recognition and binding. Recently, Zakharova et al. (2004) proposed a "dynamic metal-ion binding site" for the RB69 DNA polymerase in which specific amino acid residues serve as alternative ligands for the metal ions destined to occupy the A and B catalytic sites, prior to the conformational change that produces a competent ternary complex poised for phosphoryl transfer. There is compelling evidence that many other polymerases, including HIV-1 RT, use a similar strategy for catalytic metal ion binding (Zakharova et al. 2004). Therefore, the observed distortion of the YMDD motif may alter the dynamic metal-ion binding site in HIV-1 RT resulting in the differential recognition of metal-dNTP substrates. Detailed kinetic analyses of the roles of

Figure 5. Overlay of the three-dimensional structures of EFV-RT (Ren et al. 2000) with the RT-T/P binary complex (A) (Ding et al. 1998) or the RT-T/P-dNTP ternary complex (B) (Huang et al. 1998). The active site residues (D110, D185, D186), a ribbon representation of the primer grip motif of HIV-1 RT (residues 227–240), the nucleic acid substrates, TTP (B only), and EFV are indicated. The primer grip motif in the EFV-RT structure (red) is shifted ~ 5 Å upward compared with the same motifs in the RT-T/P (A, pink) and RT-T/P-dNTP (B, pink) complexes.

the conserved amino acids in the HIV-1 RT polymerase active site and their contribution to binding different metal ions have not been carried out. Such studies would undoubtedly improve our understanding of metal-dNTP binding interactions at the enzyme's active site, and also the impact that NNRTIs have on this interaction.

While our study implies that all NNRTI that bind to the well-defined NNRTI-BP in RT exhibit similar mechanisms of inhibition of HIV-1 reverse transcription, it does not necessarily imply that all nonnucleoside inhibitors will elicit the same mechanism of action. In this regard, biochemical studies have demonstrated that the inhibitors 2-naphthalenesulfonic acid (4-hydroxy-7-[[[5-hydroxy-6-[(4-cinnamylphenyl)azo]-7-sulfo-2-naphthalenyl]amino]-carbonyl]amino]-3-[(4-cinnamylphenyl)azo], [2',5'-bis-O-(tert-butyl)dimethylsilyl]-beta-D-ribofuranosyl]-3'-spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''-dioxide)thymine derivatives and indolopyridones all exhibit different mechanisms of inhibition in comparison with drugs that bind directly to the NNRTI-BP (Camarasa et al. 2004; Wang et al. 2004; Jochmans et al. 2006). In addition, a crystal structure of HIV-1 RT in complex with CP94707 demonstrates a unique binding interaction between this inhibitor and RT that has not been observed in other NNRTI-RT structures (Pata et al. 2004). Thus, there is compelling evidence that NNRTI that elicit different mechanisms of action can be identified and/or developed.

Materials and Methods

Reagents

Wild-type HIV-1 RT was overexpressed and purified to homogeneity as described previously (Le Grice et al. 1995). Enzyme

concentration was determined spectrophotometrically at 280 nm using an extinction coefficient (ϵ_{280}) of $260\,450\text{ M}^{-1}\text{ cm}^{-1}$. The purified (>99% purity) Sp-isomer of dTTP α S was purchased from BioLog (distributed by Alexis Corporation). dTTP was purchased from Roche Diagnostics. DNA oligonucleotides were synthesized by Integrated DNA Technologies, Inc. NEV and EFV were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH. DEL was purchased from Biomol. All other reagents were of the highest quality available and were used without further purification.

Presteady-state kinetics of single nucleotide incorporation

Presteady-state DNA polymerization reactions were carried out using a 19 nucleotide DNA primer (5'-GTCCCTGTTCGGGCGCCAC-3') annealed to a 45 nucleotide DNA template (5'-TAGTCAGAATGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACA-3'). The DNA primer was 5'-radiolabeled with [γ - ^{32}P]-ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (Fisher Scientific) as described in the manufacturer's protocol, and were then subjected to further purification using denaturing polyacrylamide gel electrophoresis using 7 M urea–14% polyacrylamide gels. The 5'- ^{32}P -labeled DNA primer was then annealed to the DNA template by adding a 1:1.5 molar ratio of primer to template at 90°C and allowing the mixture to slowly cool to ambient room temperature.

Rapid quench experiments were carried out using a Kintek RQF-3 instrument (Kintek Corp.). In all experiments described, RT and the DNA template/primer (T/P) were preincubated in reaction buffer (50 mM Tris-HCl pH 7.5, 50 mM KCl) prior to mixing with an equivalent volume of nucleotide in the same reaction buffer containing a specific concentration of divalent metal ion cofactor. Reactions were terminated at times ranging from 10 msec to 30 min by quenching with 0.5 M EDTA, pH 8.0. In reactions that included NNRTI, the inhibitor (dissolved in DMSO) was preincubated with RT-T/P prior to mixing with nucleotide and divalent metal ion. The final concentration of DMSO in the experiment was <3% and had no effect on the

measured rates. Quenched samples were mixed with an equal volume of gel loading buffer (98% deionized formamide, 10 mM EDTA, and 1 mg/mL each of bromophenol blue and xylene cyanol), denatured at 85°C for 5 min, and the products were separated from the substrates on a 7 M urea–14% polyacrylamide gel. The disappearance of substrate (19 mer) and the formation of product (20 mer) were analyzed using a Bio-Rad GS525 Molecular Imager (Bio-Rad Laboratories).

Data analysis

Data obtained from the kinetic assays were fitted by nonlinear regression using Sigma Plot software (Jandel Scientific) with the appropriate equations. For presteady-state burst experiments, in which the concentration of T/P was at least threefold greater than that of RT, data were fit to a burst equation: $[T/P_{+1}] = A[1 - \exp(-k_1 t) + mt]$, where A represents the burst amplitude, k_1 the burst rate, and m the slope. The steady-state rate is calculated by dividing the slope by the concentration of total active enzyme. For single-turnover experiments which were used to determine the maximum rate of nucleotide incorporation (k_{pol}) and the dissociation constant (K_d) for each nucleotide, the resulting data were fit to a single exponential expression: $[T/P_{+1}] = A(1 - e^{-k_{obs}t})$. K_d and k_{pol} values were calculated by fitting the observed single rate constants (k_{obs}) obtained at different concentrations of dNTP to the hyperbolic expression: $k_{obs} = k_{pol}[dNTP]/(K_d + [dNTP])$, where K_d is the equilibrium dissociation constant for the interaction of dNTP with the RT–T/P complex and k_{pol} is the maximum first-order rate constant for dNMP incorporation.

Determination of equilibrium constants for NNRTI for RT–T/P

Previous studies have shown that the burst rate (k_1) calculated from burst reactions (see above) is independent of the NNRTI concentration used in the assay (Spence et al. 1995, 1996; Wang et al. 2004). However, the amplitude of the burst phase (A) is reduced monotonically with increasing concentrations of inhibitor, and in this regard the amplitude of the burst phase represents the amount of uninhibited RT–T/P complex available for fast nucleotide incorporation in the first turnover. By plotting the amplitude of the burst phase against NNRTI concentration one can determine the dissociation constant (K_d) for the NNRTI–RT–T/P complex by fitting the data to the following hyperbolic relationship: $y = E_0 - 0.5\{(K_d + E_0 + I_0) - [(K_d + E_0 + I_0)^2 - 4E_0I_0]^{1/2}\}$, where y represents the RT–T/P complex, E_0 is the total enzyme concentration, and K_d is the equilibrium dissociation constant for the NNRTI. Based on this approach, we determined a dissociation constant of 25.0 ± 3.5 nM, 16.6 ± 4.3 nM, and 2.6 ± 1.3 nM for NEV, DEL, and EFV for the RT–T/P binary complex, respectively. In all additional experiments, the concentration of NEV, DEL, and EFV was kept at 5 μ M, 3.2 μ M, and 500 nM (~ 200 -fold K_d), which ensured that >99% of the enzyme was NNRTI bound.

Structural analyses of NNRTI–RT complexes

X-ray coordinates of HIV-1 RT complexed with NEV (3HVT; Kohlstaedt et al. 1992), DEL (1KLM; Esnouf et al. 1997), and EFV (1FK9; Ren et al. 2000) were downloaded from the Protein Data Bank. These structures were superimposed with the RT–T/P

binary (2HMI; Ding et al. 1998) and RT–T/P–dNTP ternary (1RTD; Huang et al. 1998) complexes using Molecular Operating Environment (MOE) software (Chemical Computing Group).

Acknowledgments

This research was supported by NIH Grants: GM068406 to N.S.-C. and GM49551 to K.S.A.

References

- Camarasa, M.J., San-Felix, A., Velazquez, S., Perez-Perez, M.J., Gago, F., and Balzarini, J. 2004. TSAO compounds: The comprehensive story of a unique family of HIV-1 specific inhibitors of reverse transcriptase. *Curr. Top. Med. Chem.* **4**: 945–963.
- Choi, J.Y. and Guengerich, F.P. 2004. Analysis of the effect of bulk at N2-alkylguanine DNA adducts on catalytic efficiency and fidelity of the processive DNA polymerases bacteriophage T7 exonuclease- and HIV-1 reverse transcriptase. *J. Biol. Chem.* **279**: 19217–19229.
- Ding, J., Das, K., Moereels, H., Koymans, L., Andries, K., Janssen, P.A., Hughes, S.H., and Arnold, E. 1995. Structure of HIV-1 RT/TIBO R 86183 complex reveals similarity in the binding of diverse nonnucleoside inhibitors. *Nat. Struct. Biol.* **2**: 407–415.
- Ding, J., Das, K., Hsiou, Y., Sarafianos, S.G., Clark Jr., A.D., Jacobo-Molina, A., Tantillo, C., Hughes, S.H., and Arnold, E. 1998. Structure and functional implications of the polymerase active site region in a complex of HIV-1 RT with a double-stranded DNA template-primer and an antibody Fab fragment at 2.8 Å resolution. *J. Mol. Biol.* **284**: 1095–1111.
- Esnouf, R., Ren, J., Ross, C., Jones, Y., Stammers, D., and Stuart, D. 1995. Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. *Nat. Struct. Biol.* **2**: 303–308.
- Esnouf, R.M., Ren, J., Hopkins, A.L., Ross, C.K., Jones, E.Y., Stammers, D.K., and Stuart, D.I. 1997. Unique features in the structure of the complex between HIV-1 reverse transcriptase and the bis(heteroaryl)piperazine (BHAP) U-90152 explain resistance mutations for this nonnucleoside inhibitor. *Proc. Natl. Acad. Sci.* **94**: 3984–3989.
- Figueiredo, A., Moore, K.L., Mak, J., Sluis-Cremer, N., de Bethune, M.P., and Tachedjian, G. 2006. Potent nonnucleoside reverse transcriptase inhibitors target HIV-1 Gag-Pol. *PLoS Pathog.* **2**: e119. doi: 10.1371/journal.ppat.0020119.
- Hsieh, J.C., Zinnen, S., and Modrich, P. 1993. Kinetic mechanism of the DNA-dependent DNA polymerase activity of human immunodeficiency virus reverse transcriptase. *J. Biol. Chem.* **268**: 24607–24613.
- Hsiou, Y., Ding, J., Das, K., Clark Jr., A.D., Hughes, S.H., and Arnold, E. 1996. Structure of unliganded HIV-1 reverse transcriptase at 2.7 Å resolution: Implications of conformational changes for polymerization and inhibition mechanisms. *Structure* **4**: 853–860.
- Huang, H., Chopra, R., Verdine, G.L., and Harrison, S.C. 1998. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: Implications for drug resistance. *Science* **282**: 1669–1675.
- Jochmans, D., Deval, J., Kesteleyn, B., Van Marck, H., Bettens, E., De Baere, I., Dehertogh, P., Ivens, T., Van Ginderen, M., Van Schoubroeck, B., et al. 2006. Indolopyridones inhibit human immunodeficiency virus reverse transcriptase with a novel mechanism of action. *J. Virol.* **80**: 12283–12292.
- Kohlstaedt, L.A., Wang, J., Friedman, J.M., Rice, P.A., and Steitz, T.A. 1992. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**: 1783–1790.
- Kuchta, R.D., Mizrahi, V., Benkovic, P.A., Johnson, K.A., and Benkovic, S.J. 1987. Kinetic mechanism of DNA polymerase I (Klenow). *Biochemistry* **26**: 8410–8417.
- Le Grice, S.F., Cameron, C.E., and Benkovic, S.J. 1995. Purification and characterization of human immunodeficiency virus type 1 reverse transcriptase. *Methods Enzymol.* **262**: 130–144.
- Parniak, M.A. and Sluis-Cremer, N. 2000. Inhibitors of HIV-1 reverse transcriptase. *Adv. Pharmacol.* **49**: 67–109.
- Pata, J.D., Stirtan, W.G., Goldstein, S.W., and Steitz, T.A. 2004. Structure of HIV-1 reverse transcriptase bound to an inhibitor active against mutant reverse transcriptases resistant to other nonnucleoside inhibitors. *Proc. Natl. Acad. Sci.* **101**: 10548–10553.
- Patel, S.S., Wong, I., and Johnson, K.A. 1991. Pre-steady-state kinetic analysis of processive DNA replication including complete characterization of an exonuclease-deficient mutant. *Biochemistry* **30**: 511–525.

- Radzio, J. and Sluis-Cremer, N. 2005. Stereo-selectivity of HIV-1 reverse transcriptase toward isomers of thymidine-5'-O-1-thiotriphosphate. *Protein Sci.* **14**: 1929–1933.
- Ray, A.S., Murakami, E., Basavapathruni, A., Vaccaro, J.A., Ulrich, D., Chu, C.K., Schinazi, R.F., and Anderson, K.S. 2003. Probing the molecular mechanisms of AZT drug resistance mediated by HIV-1 reverse transcriptase using a transient kinetic analysis. *Biochemistry* **42**: 8831–8841.
- Reardon, J.E. 1992. Human immunodeficiency virus reverse transcriptase: Steady-state and pre-steady-state kinetics of nucleotide incorporation. *Biochemistry* **31**: 4473–4479.
- Ren, J., Milton, J., Weaver, K.L., Short, S.A., Stuart, D.I., and Stammers, D.K. 2000. Structural basis for the resilience of efavirenz (DMP-266) to drug resistance mutations in HIV-1 reverse transcriptase. *Struct. Fold. Des.* **8**: 1089–1094.
- Rittinger, K., Divita, G., and Goody, R.S. 1995. Human immunodeficiency virus reverse transcriptase substrate-induced conformational changes and the mechanism of inhibition by nonnucleoside inhibitors. *Proc. Natl. Acad. Sci.* **92**: 8046–8049.
- Shan, S.O. and Herschlag, D. 2002. Dissection of a metal-ion-mediated conformational change in Tetrahymena ribozyme catalysis. *RNA* **8**: 861–872.
- Sluis-Cremer, N. and Tachedjian, G. 2002. Modulation of the oligomeric structures of HIV-1 retroviral enzymes by synthetic peptides and small molecules. *Eur. J. Biochem.* **269**: 5103–5111.
- Sluis-Cremer, N., Temiz, N.A., and Bahar, I. 2004. Conformational changes in HIV-1 reverse transcriptase induced by nonnucleoside reverse transcriptase inhibitor binding. *Curr HIV Res.* **2**: 323–332.
- Spence, R.A., Kati, W.M., Anderson, K.S., and Johnson, K.A. 1995. Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. *Science* **267**: 988–993.
- Spence, R.A., Anderson, K.S., and Johnson, K.A. 1996. HIV-1 reverse transcriptase resistance to nonnucleoside inhibitors. *Biochemistry* **35**: 1054–1063.
- Tachedjian, G. and Goff, S.P. 2003. The effect of NNRTIs on HIV reverse transcriptase dimerization. *Curr. Opin. Investig. Drugs* **4**: 966–973.
- Tachedjian, G., Orlova, M., Sarafianos, S.G., Arnold, E., and Goff, S.P. 2001. Nonnucleoside reverse transcriptase inhibitors are chemical enhancers of dimerization of the HIV type 1 reverse transcriptase. *Proc. Natl. Acad. Sci.* **98**: 7188–7193.
- Tachedjian, G., Moore, K.L., Goff, S.P., and Sluis-Cremer, N. 2005a. Efavirenz enhances the proteolytic processing of an HIV-1 pol polyprotein precursor and reverse transcriptase homodimer formation. *FEBS Lett.* **579**: 379–384.
- Tachedjian, G., Radzio, J., and Sluis-Cremer, N. 2005b. Relationship between enzyme activity and dimeric structure of recombinant HIV-1 reverse transcriptase. *Proteins* **60**: 5–13.
- Wang, L.Z., Kenyon, G.L., and Johnson, K.A. 2004. Novel mechanism of inhibition of HIV-1 reverse transcriptase by a new non-nucleoside analog, KM-1. *J. Biol. Chem.* **279**: 38424–38432.
- Woodside, A.M. and Guengerich, F.P. 2002. Misincorporation and stalling at O(6)-methylguanine and O(6)-benzylguanine: Evidence for inactive polymerase complexes. *Biochemistry* **41**: 1039–1050.
- Young, S.D., Britcher, S.F., Tran, L.O., Payne, L.S., Lumma, W.C., Lyle, T.A., Huff, J.R., Anderson, P.S., Olsen, D.B., Carroll, S.S., et al. 1995. L-743, 726 (DMP-266): A novel, highly potent nonnucleoside inhibitor of the human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* **39**: 2602–2605.
- Zakharova, E., Wang, J., and Konigsberg, W. 2004. The activity of selected RB69 DNA polymerase mutants can be restored by manganese ions: The existence of alternative metal ion ligands used during the polymerization cycle. *Biochemistry* **43**: 6587–6595.
- Zang, H., Harris, T.M., and Guengerich, F.P. 2005. Kinetics of nucleotide incorporation opposite polycyclic aromatic hydrocarbon–DNA adducts by processive bacteriophage T7 DNA polymerase. *Chem. Res. Toxicol.* **18**: 389–400.
- Zinnen, S., Hsieh, J.C., and Modrich, P. 1994. Misincorporation and mispaired primer extension by human immunodeficiency virus reverse transcriptase. *J. Biol. Chem.* **269**: 24195–24202.