HIV-1 Reverse Transcriptase Plus-strand Initiation Exhibits Preferential Sensitivity to Non-nucleoside Reverse Transcriptase Inhibitors in Vitro*

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Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are highly specific and potent allosteric inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. NNRTIs inhibit reverse transcription in a substrate length-dependent manner in biochemical assays and in cell-based HIV-1 replication assays, suggesting a stochastic inhibitory mechanism. Surprisingly, we observed that NNRTIs potently inhibited plus-strand initiation in vitro under conditions in which little or no inhibition of minus-strand DNA synthesis was observed. In assays that recapitulated the initiation of plus-strand DNA synthesis, greater inhibition was observed with an RNA PPT primer than with a DNA primer of corresponding sequence and with wild-type reverse transcriptase but not with NNRTI-resistant enzymes. Structural elements that dictate sensitivity to NNRTIs were revealed using modified plus-strand initiation substrates. The data presented here suggest that specific inhibition of plus-strand initiation may be an important mechanism by which NNRTIs block HIV-1 replication.

Reverse transcription is a highly choreographed, multistep process in which the plus-strand RNA genome of the human immunodeficiency virus type 1 (HIV-1)2 is converted into a double-stranded cDNA (1, 2). Reverse transcription is catalyzed by the multifunctional, virally encoded reverse transcriptase (RT), which carries out both RNA- and DNA-dependent polymerase activities and also contains a ribonuclease H activity (3). RNA-templated minus-strand synthesis initiates at a primer binding site immediately downstream of the left long terminal repeat using a tRNA primer and continues until RT reaches the 3′-end of the RNA genome. RNase H acts both concurrently and subsequently to degrade the RNA template and allow the DNA to anneal to a complementary sequence (right long terminal repeat) located at the 3′-end of the genome in a process called minus-strand transfer. Following minus-strand transfer, minus-strand synthesis is completed. In contrast to the initiation of minus-strand synthesis in which a single exogenous primer is used, plus-strand synthesis initiates from polypurine-rich RNA primers (polypurine tracts or PPTs) derived from the viral genome by the RNase H activity during and after minus-strand synthesis. The site of plus-strand initiation from the 3′-PPT primer ultimately defines the left end of the double-stranded viral DNA. The resulting sequence is required for integration by the viral integrase, and thus failure to generate these correct ends can result in products that cannot be integrated into the host genome.

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are potent and highly specific inhibitors of HIV-1 RT that are widely used in the clinic for the treatment of HIV-1 infection. Despite considerable structural diversity within this class of inhibitors, all NNRTIs bind to RT at a hydrophobic pocket ~10 Å from the polymerase active site in the palm subdomain of p66 (4, 5). NNRTIs are not competitive with either primer-template or nucleoside triphosphates (6). Rather, they allosterically inhibit RT by inducing a conformational change in the enzyme that significantly reduces the rate of nucleotide transfer (7, 8). In addition to inhibiting polymerase activity of RT, NNRTIs allosterically affect both the rate and pattern of RNase H cleavage in vitro (9–11).

In HIV-1 viral replication assays, initiation of RNA-primed minus-strand synthesis is only slightly inhibited by NNRTIs at concentrations that greatly exceed those necessary for complete suppression of viral replication (12). In cell-based replication assays as well as in biochemical assays, the ability of NNRTIs to inhibit polymerization of full-length products has been shown to correlate with the length of the template (12, 13). Thus, the generation of shorter replication products is not as effectively inhibited by NNRTIs as is the generation of longer products. These results have suggested that the NNRTIs act in a stochastic manner with longer templates providing more opportunities for the inhibitors to act.

In this report we demonstrate that NNRTIs potently and specifically inhibit plus-strand initiation in vitro under conditions in which little or no inhibition of minus-strand DNA synthesis is observed, and we identify the structural features of the plus-strand initiation substrate that contribute to NNRTI sensitivity. Based on the observations reported here, we propose a model for the action of NNRTIs on the initiation of plus-strand viral DNA synthesis.

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**Inhibition of Plus-strand Initiation by NNRTIs**

**EXPERIMENTAL PROCEDURES**

Reagents—All oligonucleotides were synthesized by Integrated DNA Technologies unless otherwise noted. Wild-type HIV-1 NL4.3 RT and mutant enzymes were purified as described previously (11, 14).

RT Polymerase Assays—For all assays, HIV-1 RT (10 nM) was preincubated for 5 min at 37 °C with inhibitor in an assay buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 6 mM MgCl₂, 80 mM KCl, 0.2% polyethylene glycol 8000, 0.1 mM EGTA, 25 μM each dCTP, dGTP, and dTTP, and 1 μM [³²P]dATP. Reactions were initiated by the addition of the primer-template substrate to a final concentration of 5 nM and incubated at 37 °C for the indicated times. The coupled assay for minus- and plus-strand synthesis utilized an RNA template comprising the HIV-1 HXB2 sequence encompassing the 3’-PPT annealed to a DNA primer (5’-ATCTTGTCCTGGTGGAGTAGAATTAGC-3’) at its 3’-end. All other assays used as template a PAGE-purified 80-nt synthetic oligodeoxyribonucleotide (5’-ATCTTGTCCTGGTGGAGTAGAATTAGCCCTTCCAGTCCCCCCTTTTTTAAAAGTGGCTAA-GATCTACAGCTGCCC-3’) to which was annealed either the full-length RNA complement generated by in vitro transcription, an RNA PPT primer (5’-rUrUrArArArGrArArGrArGrG-rGrG-rGrG-3’), or a DNA PPT primer (5’-TAAAAGAAAGGGGGG-3’). For gel-based assays, reactions were quenched by the addition gel loading buffer (95% formamide, 10 mM Tris HCl, pH 8.0, 40 mM EDTA) and heating to 70 °C for 10 min. Products were resolved by electrophoresis using 16% denaturing polyacrylamide gels containing 7 M urea in Tris-borate-EDTA buffer. Results were quantified by phosphorimaging analysis (Amersham Biosciences).

In some experiments a homogeneous scintillation proximity assay (SPA, Amersham Biosciences) was used to characterize the effects of NNRTIs on plus-strand initiation. For this assay format, a 5’-biotinylated 80-nt DNA template of the same sequence of that described above annealed to the various PPT primers was utilized, and [³H]dATP was used as the radiolabel instead of [³²P]dATP. Assay buffer was otherwise identical to that used for the gel-based assay. For the homogeneous assay format, reactions were initiated by the addition of enzyme. The elimination of the preincubation step in the homogeneous assay format resulted in approximately a doubling of the apparent IC₅₀ compared with those observed with the gel-based assay. Reactions were quenched by addition of scintillant-impregnated streptavidin-coated polyvinyltoluene beads in a quench solution containing 100 mM EDTA. Plates were allowed to rest for 8 h prior to counting radioactivity using a Topcount plate reader (Packard).

Ligand Binding Assay—We used an adaptation of the scintillation proximity assay described above to measure the affinity of incoming dNTPs to RT assembled on PPT primer-template substrates. For these studies, we employed dideoxy-terminated RNA and DNA primers to prevent incorporation of the incoming dNTP, and we used RT containing the D443N mutation in the RNase H activity site to preempt any degradation of the nucleic acid. 20 nM RT (D443N) and 10 nM primer-template were mixed in assay buffer and incubated at room temperature for 30 min, and then streptavidin-coated SPA beads were added to a final concentration of 0.5 mg/ml in assay buffer. Plates were allowed to rest for 8 h prior to counting in a Topcount plate reader as described above.

**RESULTS**

Coupled Assay for Minus- and Plus-strand Synthesis—For our initial studies we employed a gel-based assay that allows tracking of several biochemical steps required for successful reverse transcription of the HIV-1 genome (Fig. 1A). The assay employed as substrate an RNA template that is derived from the HIV-1 HXB2 genome and that includes the 3’-polypurine tract and a complementary DNA primer that anneals to the 3’-end of the RNA. Upon initiation of the reaction, RT catalyzed RNA-dependent DNA synthesis (minus-strand synthesis), cleavage of the RNA template to generate the PPT primer, PPT-primer-dependent DNA polymerization (plus-strand synthesis), and removal of the RNA PPT primer from the nascent DNA product. A time course for the assay is shown in Fig. 1B. Within 15 s (the earliest time point measured) minus-strand replication intermediates are observed, and at 30 s after initiation, the
full-length minus-strand product is visible. Minus-strand DNA synthesis is essentially complete after ~5 min. Plus-strand DNA synthesis is delayed relative to minus-strand synthesis. The plus-strand DNA product is not visible until ~1 min after initiation of the reaction and continues to increase for 20 min. It had been shown previously that after incorporating 12 nt, RT dissociates from the nucleic acid, cleaves the nascent DNA from the PPT primer, and then initiates synthesis anew from the 3’-end of the 12-nt DNA (15). This 12-nt intermediate first appears ~1 min after initiation, peaks at ~10 min, and then apparently is chased into the 37-nt product. In similar experiments using RT with the D443N RNase H-inactivating mutation, only the minus-strand product was observed (data not shown). RT containing the D185N mutation in the polymerase active site did not yield any product (data not shown). Interestingly, when an equimolar mixture of RT D185N and RT D443N was used in the assay, plus-strand products were observed, demonstrating that the polymerase and RNase H activities could be provided in trans.

**Effects of NNRTI L-697661 on Reverse Transcription**—The assay described above provides a tool for interrogating the effects of RT inhibitors on each of the steps required for initiation of plus-strand synthesis including the RNA- and DNA-dependent polymerase activities and the RNase H-dependent generation and removal of the PPT primer. In this assay system the aminopyridinone NNRTI L-697661 (16) has strikingly different effects on plus-strand and minus-strand syntheses (Fig. 2). Although weakly inhibiting minus-strand synthesis with an IC₅₀ of 1400 nM, this NNRTI was significantly more potent on plus-strand synthesis (IC₅₀ = 35 nM). No products corresponding to aborted initiation products were observed, indicating that once polymerization was initiated, it proceeded to either the 12-nt product or the 37-nt nucleotide product. This observation suggests that L-697661 does not effectively inhibit processive polymerization but rather appears to have a significant impact on the initiation of polymerization. We also observed that in the presence of L-697661 both the full-length plus-strand product and the 12-nt intermediate were extended by one or two nucleotides. These extended products presumably result from altered RNase H cleavage specificity, resulting in PPT primers that are truncated by one or two nucleotides. This observation is consistent with previous reports that NNRTIs alter the cleavage specificity of RNase H (11).

**L-697661 Potently Inhibits Initiation of RNA PPT but Not DNA PPT-primed Plus-strand Synthesis**—In the experiments described above, L-697661 inhibited plus-strand synthesis more effectively than minus-strand synthesis. The RNA PPT-DNA hybrid duplex has some unusual structural features including misaligned base pairs that make it resistant to cleavage by RNase H and that allow it to act as primer for plus-strand synthesis (17, 18). The observation that L-697661 selectively inhibited plus-strand initiation prompted us to explore the structural features of the substrate that could contribute to increased sensitivity. Specifically we tested whether the nature of the primer used to initiate synthesis contributed to the different sensitivities. We thus compared the effects of L-697661 on the initiation of plus-strand synthesis using substrates comprising a DNA template with either an RNA or a DNA PPT primer (Fig. 3). The RNA-DNA duplex substrate requires RNase H activity to generate the PPT primer in addition to the polymerase activity, whereas the latter two substrates require only a functional polymerase. Initiation of plus-strand synthesis using the RNA-DNA hybrid substrate and initiation with the RNA PPT-primed substrate were equally sensitive to inhibition by L-697661 with IC₅₀ values of ~150 nM. In con-
Inhibition of Plus-strand Initiation by NNRTIs

**TABLE 1**

|                | RNA/DNAM duplex IC₅₀ (µM) | RNA PPT primer IC₅₀ (µM) | DNA PPT primer IC₅₀ (µM) |
|----------------|---------------------------|--------------------------|--------------------------|
| L-697661 NNRTI| 230 ± 50                  | 430 ± 120                | >5000                    |
| Nevirapine NNRTI| 450 ± 60                  | 900 ± 400                | >5000                    |
| Efavirenz NNRTI| ND                        | 17 ± 0                   | 350 ± 0                  |
| Foscarnet PRTI| >5000                     | >5000                    | >5000                    |
| AZTTP NRTI     | 360 ± 40                  | 320 ± 50                 | 240 ± 40                 |

ND, not determined.

**TABLE 2**

|                | IC₅₀ (µM) RT (wt) | IC₅₀ (µM) RT (D443N) |
|----------------|-------------------|-----------------------|
| PPT            | 0.29              | No product            |
| 1              | 0.28              | 0.44                  |
| 5              | >10               | >10                   |
| 2              | 0.22              | 0.24                  |
| 3              | 0.28              | 0.23                  |
| 6              | 0.27              | 0.24                  |
| 9              | ND                | 0.27                  |
| 12             | 0.32              | 0.13                  |
| 12             | 0.13              | 0.15                  |
| >10            | ND                | >10                   |

ND, not determined.

Contrast, L-697661 only weakly inhibited initiation of plus-strand synthesis when a DNA PPT-primed substrate was used (IC₅₀ > 10 µM).

**Selective Inhibition of Initiation of RNA PPT-primed Plus-strand Synthesis by NNRTIs**—To facilitate further characterization of the structural elements that confer increased sensitivity of RNA PPT-primed initiation to inhibition by NNRTIs, we adapted the assay described above to a scintillation proximity format. For this format we utilized an 80-nt 5'-biotinylated DNA template annealed to either an 80-nt complementary RNA, an RNA PPT primer, or a DNA PPT primer and [³H]dATP. DNA synthesis is quantified by scintillation counting following capture of the radiolabeled product with scintillant impregnated, streptavidin coated beads. Results obtained using this homogeneous assay format were qualitatively and quantitatively identical to those obtained in gel based assays.

Following the observation that L-697661 selectively inhibited plus-strand initiation using an RNA PPT primer, we next tested whether initiation of plus-strand synthesis was sensitive to other NNRTIs or to other mechanistically distinct classes of RT inhibitors using the scintillation assay format. L-697661 inhibited synthesis with RNA-DNA hybrid duplex and RNA PPT-primed substrates with IC₅₀ values of 0.23 ± 0.05 µM and 0.43 ± 0.12 µM, respectively (Table 1). Likewise, nevirapine inhibited synthesis on the same substrates with IC₅₀ values of 0.45 ± 0.06 µM and 0.9 ± 0.4 µM, respectively. L-697661 and nevirapine did not inhibit the activity of RT containing either the K103N or Y181C NNRTI resistance-conferring mutations (IC₅₀ > 5 µM with all substrates tested with both RT mutants). With the DNA PPT-primed substrate, neither L-697661 nor nevirapine appreciably inhibited DNA synthesis at concentrations up to 5 µM. Efavirenz inhibited synthesis using an RNA PPT-primed substrate with an IC₅₀ of 17 nm (Fig. 4). In contrast to L-697661 and nevirapine, efavirenz also inhibited synthesis with the DNA PPT-primed substrate. However, with an IC₅₀ of 345 nm, the activity of efavirenz was more than 20-fold less potent as an inhibitor of DNA PPT-primed synthesis compared with reactions in which an RNA PPT primer was used. Under the assay conditions used here, no significant inhibition was observed with foscarnet (IC₅₀ > 5 µM for all substrates). With limiting dTTP concentrations, AZTTP was equally effective with all three substrates (Table 1). The mutations K103N and Y181C, which conferred resistance to L-697661 and nevirapine in this assay, had no effects on the inhibitory activity of AZTTP with any of the substrates tested. These results demonstrate that the selective inhibition of plus-strand synthesis is a property common to the NNRTIs but not to other mechanistically distinct classes of RT inhibitors.

**Chimeric Replication Intermediates Are Sensitive to Inhibition by NNRTIs**—Incorporation of the first two deoxyribonucleotides in the initiation of plus-strand synthesis from the RNA PPT primer has previously been shown to be rate-limiting because of a higher Km for the incoming dNTP and a corresponding decrease in the catalytic efficiency (19). These rate-limiting steps could be bypassed using chimeric primers in which the first two incorporation events were bypassed. Using a series of chimeric substrates with RNA PPT primers extended at their 3'-end with up to 12 deoxyribonucleotides, we tested whether 3' DNA extensions could reduce sensitivity to L-697661. As shown in Table 2, all of the chimeric substrates tested were equally sensitive to inhibition by the NNRTI. As noted above, L-697661 causes aberrant cleavage of the RNA PPT, yielding a primer truncated by 1 or 2 nt. To rule out the
trivial possibility that the rate effect of the NNRTI on plus-strand initiation is a consequence of this miscleavage, we also conducted the assay using RT containing the RNase H active site mutation D443N, which is devoid of RNase H activity. The results obtained using the RT with the inactive RNase H were similar to those observed with the wild-type enzyme (data not shown) and indicate that the inhibition is a consequence of the effects on the polymerase activity.

NNRTIs Are Competitive with dNTPs on RNA PPT but Not DNA PPT Substrates—Previous studies on NNRTI mechanism of action have demonstrated that NNRTIs do not significantly affect binding of the incoming dNTP (6). The RNA PPT-DNA hybrid duplex substrate used in these studies is distinct from those used in previous studies in that RT can bind to the substrate in either an RNase H cleavage mode or in a DNA polymerase mode, whereas with the substrate used in previous studies, the enzyme could bind to substrate only in a polymerase mode (20). We designed an assay to explore the effects of NNRTIs on the binding of incoming dNTPs to RT associated under conditions in which little or no inhibition of minus-strand synthesis is observed. Related substrates in which the RNA PPT primer is replaced by a DNA primer of the same sequence are at least 20-fold less sensitive to inhibition by NNRTIs. (ii) Chimeric substrates in which the RNA PPT primer has been extended by up to 12 deoxyribonucleotides remain as sensitive to NNRTI inhibition as the unextended RNA PPT primer. (iii) NNRTIs compete for binding to RT when the enzyme is associated with RNA PPT-DNA hybrid duplex substrate but not with a corresponding DNA PPT primer-DNA template substrate. These observations show that NNRTIs manifest their effects differently on an RNA PPT-primed substrate than on a corresponding DNA PPT-primed substrate.

RT has previously been shown to bind RNA-DNA hybrid duplexes in different modes depending on the structure of the substrate (20). With substrates that contain a recessed DNA 3′-OH, RT engages the nucleic acid in a polymerase-dependent mode of binding where the 3′-end of the DNA is positioned in

**FIGURE 5.** Effect of L-697661 on the binding of [3H]dATP to RT associated with RNA or DNA PPT-primed substrates. A, binding of [3H]dATP to RT associated with a DNA PPT-primed substrate was measured in the absence of NNRTI (○), with 0.5 μM L-697661 (□), and with 1.0 μM L-697661 (△). Following measurement of [3H]dATP binding in the presence or absence of L-697661, EDTA was added to 50 mM in each sample, resulting in the dissociation of the nucleotide. Shown are the results of the addition of EDTA on ligand binding in the presence of 0.5 μM L-697661 (△). B, binding of [3H]dATP to RT associated with a RNA PPT-primed substrate in the absence of NNRTI (○) and in the presence of 0.5 μM L-697661 (□).

**DISCUSSION**

In this study, we have explored the effects of NNRTIs on plus-strand initiation and have demonstrated the following. (i) NNRTIs selectively inhibit plus-strand initiation from an RNA PPT primer containing DNA substrates. For this assay dideoxy-terminated PPT primers were annealed to biotinylated DNA templates. Dideoxy-terminated primers were employed to prevent incorporation of the incoming dNTP. Binding studies were performed using RT that contained the RNase H active site mutation D443N, to prevent aberrant cleavage of the RNA PPT substrate in the presence of NNRTIs as described above.

In binding assays, saturable binding of [3H]dATP to RT was observed was observed with both the RNA and DNA PPT substrates (Fig. 5). Modest decreases in the affinity of the enzyme for the nucleotide were observed upon addition of L-697661 in a dose-dependent manner. The increase in apparent Kd is consistent with previous observations. No change in the number of apparent dNTP binding sites was observed. In contrast, L-697661 abrogated binding of [3H]dATP to RT associated with RNA PPT primer-DNA template substrates.
the polymerase active site poised to be extended. For RNA-DNA hybrid duplex substrates in which the 3'-end of the DNA is not recessed, RT preferentially engages substrate in a polymerase-independent mode where the 5'-end of the RNA strand, and not the 3'-end, is situated in the polymerase active site (Fig. 6). Thus, most recessed RNA oligonucleotides annealed to DNA templates do not act as efficient primers for initiation of polymerization. RNA PPT hybrid duplexes are selectively utilized as primers by RT because binding of the 3'-end of the RNA in the polymerase active site is not as disfavored as with non-RNA PPT primers. Nevertheless, RT binds RNA PPT-primed substrates preferentially in an RNase H cleavage mode with the 5'-end of the RNA situated in the polymerase active site.

Previous studies have demonstrated that NNRTIs inhibit polymerization by inducing a conformational change in the enzyme that prevents the covalent coupling of the incoming dNTP to the end of the product strand but do not prevent binding of either the nucleic acid substrate or dNTP. In these studies, substrates invariably contained DNA primers annealed to either an RNA or DNA template. With the substrates used in those NNRTI mechanism-of-action studies, RT would almost exclusively bind in a polymerase-dependent mode.

The effects of NNRTIs that we observed using RNA PPT-primed substrates are distinct from those observed previously in two significant ways. First, the particular sensitivity to inhibition was not altered by extending the RNA primer with oligodeoxynucleotides of varying lengths up to 12 nt. This observation suggests either that the unique structural features that distinguish the RNA PPT-DNA hybrid duplexes extend well beyond the 3'-end of the hybrid region or that the RNA PPT sequence acts as a trap by some other mechanism. Second, although NNRTIs do not affect binding of incoming dNTPs to DNA-primed substrates, they clearly have profound effects on the binding of the incoming dNTP with RNA PPT-primed substrates. These observations suggest that on RNA PPT-primed substrates, NNRTIs may be working by another mechanism in addition to interfering with the chemical step of dNTP incorporation. One possibility, which is consistent with all the observations described here, is that NNRTI binding stabilizes binding of RT to this substrate in an RNase H cleavage mode rather than in a polymerase-dependent mode. Such a complex would not be capable of binding incoming dNTPs. In addition, if NNRTIs stabilize binding of RT to the RNA in this mode, it provides a plausible explanation for the observation that extensions of RNA PPT do not influence the potency of the compound. Instead, the RNA PPT appears to be a trap on extended substrates.

The observations described here and the model that we propose to explain them raise many questions that have not been addressed in the current study. For example, in in vitro assays to characterize the effects of NNRTIs on RT-catalyzed polymerization, it has previously been shown that generation of full-length reverse transcription products is template length-dependent (12, 13). NNRTI concentrations required for effective inhibition of full-length product synthesis by RT are decreased with increasing template length. NNRTIs do not alter the apparent pattern of pause sites normally observed with both RNA and DNA heteropolymeric substrates due to stalling or dissociation at discrete sites. We propose that some component of this inhibition is the result of trapping the enzyme in a polymerase-independent RNase H-dependent mode of binding. Also, in cell-based replication assay, NNRTIs have little or no effect on the production of minus-strand strong stop DNA when present at concentrations greatly exceeding those required to inhibit complete reverse transcription. These observations raise the possibility that NNRTIs may in part manifest their effect on HIV-1 replication by mechanisms other than inhibition of the chemical reaction of dNTP incorporation. In addition, the finding that different steps in the RT process can display differential sensitivity to inhibitors also suggests that there may be additional opportunities to identify other novel classes of RT inhibitors using assays that more fully replicate the complex biological process.

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