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Efavirenz Stimulates HIV-1 Reverse Transcriptase RNase H Activity by a Mechanism Involving Increased Substrate Binding and Secondary Cleavage Activity

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Abstract

Efavirenz is a non-nucleoside reverse transcriptase inhibitor used for treating HIV/AIDS. We found that polymerization activity of a reverse transcriptase (RT) with the E478Q mutation that inactivates the RNase H catalytic site is much more sensitive to efavirenz than the wild type RT, indicating that a functional RNase H attenuates the effectiveness of efavirenz. Moreover, efavirenz actually stimulated wild type RNase H binding and catalytic functions, indicating another link between efavirenz action and RNase H function. During reverse transcription *in vivo*, the RT that is extending the DNA primer also periodically cleaves the genomic RNA. The RNase H makes primary template cuts about 18 nucleotides from the growing DNA 3' end and, when the RT pauses synthesis, it shifts to make secondary cuts about 9 nucleotides from the DNA 3' end. After synthesis, RTs return to bind remaining template RNA segments at their 5' ends, and make primary and secondary cuts, 18 and 9 nucleotides in, respectively. We found that efavirenz stimulates both 3' and 5'-directed RNase H activity. Use of specific substrates revealed a particular acceleration of secondary cuts. Efavirenz specifically promoted binding of the RT to RNase H substrates, suggesting that it stabilizes the shifting of RTs to make the secondary cuts. We further showed that efavirenz similarly stimulates the RNase H of an RT from a patient-derived virus that is highly resistant and grows more rapidly in the presence of low concentrations of efavirenz. We suggest that for efavirenz resistant RTs, stimulated RNase H activity contributes to increased viral fitness.

Treatment and prevention of HIV/AIDS remains a challenging endeavor and a current focus of basic research. A major target of antiretroviral therapies against HIV-1 is the viral reverse transcriptase (RT) ¹. HIV-RT is a low fidelity DNA polymerase and highly error prone^{2,3}. Because of this characteristic, mutations are common during reverse transcription. Furthermore, HIV packages two similar RNA genomes, allowing for facile production of recombinant progeny ^{4,5}.

Currently two main classes of approved drugs antagonize the RT, nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors

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(NNRTIs) ⁶. NRTIs inhibit RT through competitive binding to the active site, while NNRTIs bind to a separate pocket away from the active site ⁷⁻⁹. Both classes of drugs target RT polymerase activity. Development of resistance to existing NRTIs and NNRTIs is common during treatment as a result of the high rates of both mutagenesis and recombination during reverse transcription. Therefore, there is a continuing search for new drugs. RT also possesses RNase H, strand transfer and strand displacement activities ^{10,11}. There are currently no approved drugs directed toward these additional activities, so they remain available targets for drug development.

HIV-1 converts its single stranded RNA genome into double stranded DNA that is incorporated into the host cell genome, serving to transcribe more viral RNA ¹². The viral RNA genome is first converted into an RNA/DNA hybrid, and then the RNA is degraded to make way for synthesis of the second strand of DNA ¹³. The RT, having both polymerization and RNase H functions, is able to make cuts in the RNA of the RNA/DNA hybrid as it is being made. This is called DNA 3'-end-directed, polymerization-dependent cleavage ¹⁴. The cleavage mechanism involves making primary cuts about 18 nt from the DNA 3' end. At frequent synthesis pause sites, the RT shifts to make secondary cuts about 9 nt from the end. To complete RNA degradation, additional RTs position at the 5' ends of the RNA fragments to carry out RNA 5'-end-directed cleavage ¹⁵. Again, primary cuts are made about 18 nt from the RNA 5' and then secondary cuts are made about 9 nt from the end. All of these classes of cuts are thought to be necessary for efficient genomic RNA removal. RNase H activity is a particularly attractive drug target because of this central role in reverse transcription ^{13,16}.

Efavirenz (Figure 1a) is one of the most frequently prescribed NNRTIs, used as a first-line treatment for HIV in highly active antiretroviral therapy, HAART ^{17,18}. As with other NNRTIs, rapid development of resistance is a major problem ^{5,19}. A more recent disturbing observation was the ability of efavirenz to stimulate virus growth after the virus acquired certain NNRTI resistant mutations ^{20,21}. Although the mechanism of stimulation is largely unknown, it was shown that the effect was caused by changes in the early stages of the viral life cycle ²². Efavirenz has also been shown to stimulate viral RT RNase H activity ²³⁻²⁵, a characteristic that may serve as a basis of virus growth stimulation. Another NNRTI nevirapine was also reported to stimulate HIV-1 RNase H activity, and the mechanism was probed in detail ²⁶, with results suggesting that nevirapine specifically alters DNA 3'-directed RNase H cleavage among other functions.

Since, RNase H activity is an important component of the reverse transcription pathway, a potential drug target, and may connect NNRTI activity to more efficient viral replication; we examined the relationships between efavirenz and HIV-1 RNase H in more detail. We report that a RNase H negative mutant of RT is much more sensitive to efavirenz than the wild type RT, and that efavirenz specifically stimulates certain RNase H functions in both the wild type and a patient-derived drug resistant mutant RT. We discuss how the drug and the RNase H activity influence each other and how these influences might explain promotion of mutant virus growth by efavirenz.

Materials and Methods

Materials

DNA and RNA templates were purchased from Integrated DNA Technologies (Coralville, IA) and reconstituted in nuclease free water and 1x TBE, respectively. T4 polynucleotide kinase (Life Technologies), *E. coli* DNA polymerase I *Klenow* fragment, (Roche Molecular Biochemicals), and shrimp alkaline phosphatase (SAP) and SAP buffer, (Thermoscientific) were used in the preparation and labeling of primers and templates. HIV-1 RT wild type (WT) protein (p66/p51 dimer, NL4-3), (specific activity = 5400 U/mg), patient RT isolate,

K101E+G190S+M41L+T215Y, (D10) (specific activity = 7500 U/mg) were expressed and purified in our laboratory as previously described²⁷. HIV-1 E478Q RT (specific activity = 40,000 U/mg) was provided by Dr. Stuart F.J. Le Grice. The non-nucleoside reverse transcriptase inhibitor, efavirenz (EFV) was obtained through the NIH AIDS Research and Reference program, Division of AIDS, NIAID. It was reconstituted in dimethyl sulfoxide (DMSO) to achieve a final concentration of 5 mM and stored at -20 °C. All other buffers and diluents were prepared using molecular grade reagents using manufacturer's protocols.

Template/Primer substrates

A number of templates and primers were used in our experiments to allow measurement of either RNA 5'-directed RNase H activity or DNA 3'-directed RNase H activity (Table 1). For RNA 5'-directed activity, a 40 nt-RNA (RNAJ1) was annealed to a 35 nt-DNA primer (DNAJ2) forming a recessed RNA 5'-end; and for DNA 3'-directed RNase H activity, RNAJ1 was annealed to a 35 nt-DNA primer (DNAJ3) with a 25 nt region of complementarity. To measure the effect of efavirenz on primary and secondary cleavages we used a 45 nt-RNA template (RNAJ4) and the same 45 nt-RNA (RNAJ4[#]) containing 2'-O-methyl bases within and around the primary cleavage site (Table 1). These were either annealed to a 45 nt-DNA primer (DNAJ5) to produce a blunt substrate or a 61 nt-DNA primer (DNAJ6) to produce a RNA 5'-directed substrate.

Preparation of 5'-end labeled RNA and DNA

DNA primers were 5'-end labeled using [γ -³²P] ATP, (6,000 Ci/mmol) and T4 polynucleotide kinase. Excess radionucleotides were removed using a Tris RNase-free P30 Micro Bio-Spin column (Bio-Rad). Gel purified RNA was first treated with shrimp alkaline phosphatase for 60 min, at 37 °C, then the enzyme was inactivated at 65 °C for 25 min. 5'-end labeling was then performed as described above. Further purification and concentration of the RNA was performed using the GeneJET RNA clean up and concentration Microkit (Thermoscientific).

Preparation of 3'-end labeled RNA

RNA template was pre-annealed to the DNA primer. A single nucleotide extension was done using [α -³²P] pCp, (6,000Ci/mmol) and Klenow fragment. The reaction was incubated at 37 °C for 2 hrs then deactivated by incubating at 65°C for 30 min. The DNA was then digested using turbo DNase (Thermoscientific) followed by RNA purification and clean up as described above.

Hybridization

Annealing of the RNA to DNA (1RNA: 4 DNAs) was performed in 50 mM Tris-HCl (pH 8.0), 80 mM KCl, and 1 mM DTT. Components were constituted, heated at 95 °C for 7 min, and slow-cooled to room temperature.

RNase H activity assay

The assays were performed as described previously with modifications²⁸. Briefly, for the DNA 3'-directed RNase H assay, a 15 μ l reaction assay was performed containing 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1.0 mM EDTA, 34 mM KCl, 6 mM MgCl₂, 8 mM substrate/primer, 17 nM RT and varying amounts of efavirenz. In all cases, RT was pre-incubated with substrate for 3 min at 37 °C and then the reaction was initiated by adding MgCl₂. Reactions were terminated with 15 μ l of 2x termination mixture (90% formamide (v/v), 10 mM EDTA (pH 8.0) and 0.1% each of xylene cyanole and bromophenol blue). Reactions for RNA 5'-directed RNase H assays were carried out in the same manner except that 10 nM RT was needed. For time course experiments, a master mix of all reagents was

prepared without MgCl_2 . Following initiation with MgCl_2 , aliquots were taken at various time intervals and mixed with stop buffer as described above. Samples were resolved by 15% denaturing PAGE, analyzed by PhosphorImager (GE Healthcare) and quantitated using ImageQuant software version 5.2.

DNA polymerase activity assay

Substrate RNAJ1 was annealed as described above to 5'-end ^{32}P labeled DNAJ3 (Table 1) in the ratio 4:1. This substrate was then used for primer extension assays using 17 nM of RT and different concentrations of efavirenz (Figure 1c) in an assay reaction containing 8 nM template/primer, 100 μM dNTPs and 6 mM MgCl_2 .

Binding assay

To test the effect of efavirenz on the binding of RT to template/primer, a gel mobility shift assay (EMSA) was performed. In this assay, RNase H substrates were incubated in the presence or absence of efavirenz and RT. The binding buffer contained 20 mM Tris-HCl (pH 8.0), 6 mM NaCl, 1 mM EDTA, and 10% v/v glycerol. The reaction was performed at 4 or 37 °C for 10 min then the mobility shift was assessed by 6% native PAGE at 150V at 4 °C for 2 hours.

Results

RNase H negative mutant E478Q RT is more sensitive to efavirenz than wild type

In an effort to define the relationship between efavirenz and the catalytic activities of HIV-1 RT, we questioned whether functional RNase H activity affects the inhibition of polymerization. E478Q is an RNase H negative mutant with functional polymerase activity²⁹. This mutation is in the active site of the RNase H, and produces an RT with no RNase H functions, but with normal polymerization functions^{30–32}. For this reason, it has been frequently used in biochemical characterizations of HIV-1 RT, to analyze the properties of an RNase H deficient enzyme²⁹. HIV-1 with this mutation cannot be grown, since RNase H activity is essential. We titrated efavirenz into polymerization assays with either wild type or E478Q RT. Both wild type and E478Q RTs were sensitive to efavirenz in a concentration dependent manner (Figure 1b and 1d). However, we observed that the E478Q showed a much greater sensitivity to efavirenz inhibition and was completely inhibited at 2.5 μM (Figure 1c and 1d). The greater drug sensitivity of E478Q RT implies that a functional RNase H active site helps to counteract the inhibitory properties of efavirenz. Although the mechanism by which this occurs is not evident, the result suggests that the functional RNase H active site protects substrate binding, translocation, or conformational properties of the RT that support polymerization. This observation indicated that further analysis of the relationship between efavirenz and RT RNase H was warranted.

Efavirenz stimulates DNA 3'-end-directed RNase H activity, particularly secondary cuts

NNRTIs were reported to alter RNase H activity in a substrate-dependent manner, stimulating DNA 3'-directed RNase H activity^{26,33}. It was further shown that the presence of specific point mutations in the NNRTI binding site could change this specificity²⁶.

We designed substrates that would allow measurement of the generation of primary and secondary products, and determine the effect of efavirenz (Figure 2a). We measured the generation of products over time at various concentrations of efavirenz. There was a general increase in total RNase H products (Figure 2b) but, in particular, secondary product generation was greatly enhanced. The effect on the rate of primary product generation was difficult to assess in these assays, because the RNA strand was labeled at its 5' end and increased secondary product generation depleted the pool of primary products.

Efavirenz alters RNA 5'-end-directed RNase H activity in a drug concentration-dependent manner

In order to measure how efavirenz affects 5' primary and secondary cleavages, we designed substrates that allowed the RT to position specifically on the 5' end of an RNA strand recessed on a DNA strand (Figure 2c). We then measured the generation of products over time at various concentrations of efavirenz (Figure 2d). Efavirenz enabled RT to move from primary to secondary cleavage sites so that the proportion of secondary products was increased. Above the 5 μ M level of efavirenz both products of RNase H cleavage were increased, with secondary products increasing to a greater extent than primary products, suggesting that efavirenz is able either to increase binding of the RT to the substrate so that the slower secondary cleavages can be completed, or to facilitate movement of the RT along the template to acquire the secondary cleavage site.

Blunt substrate reveals that efavirenz stimulates primary cleavages

Both 3' and 5' secondary cuts were reported to occur independent of primary cuts^{34, 35}. However, since secondary cuts occur more slowly than primary cuts, their occurrence depletes the pool of primary cut products. In a reaction with a standard 3' or 5' end directed cleavage substrate, primary cut products are simultaneously being created and destroyed; making it impossible to determine whether the primary cut is being stimulated. To examine whether efavirenz stimulates primary cuts, we used a 5' end ³²P labeled RNAJ4 annealed to DNAJ5 to form a blunt end as depicted in Figure 3a. This substrate allows RT to bind and make cuts at the primary site but does not allow for RT to slide and make the secondary cut³⁶. Since neither the RNA nor the DNA strand is recessed, it is not possible to know whether 5' RNA or 3' DNA positioning elements in the substrate are the main determinant of RT binding location. Possibly, the RT is immobilized because both sets of substrate positioning contacts collaborate. In the absence of efavirenz, RT could make the primary cut but not secondary cuts, while in the presence of efavirenz, the primary cut was made more efficiently (Figure 3b). More so in the presence of efavirenz, an additional cut was made, suggesting a change in binding characteristics of the RT.

We further measured the effects of efavirenz on cleavage of this substrate when the primary site was blocked. We used substrate RNAJ4[#] that contained 2'-O-methyl bases between positions 16–22 that block primary cuts (Figure 3c). While no cuts were made in the absence of drug, the presence of efavirenz altered the cleavage profile resulting in products larger (28 nt product) and smaller (14 nt product) than the expected primary products (Figure 3d). This result shows that efavirenz partly overcame the immobilizing effects of the blunt ended structure, promoting some translocation of the RT in both directions from the normal primary cut site. While this is further evidence of the translocation-promoting properties of efavirenz, the new cleavages seen here depend on both the drug and the 2'-O-methyl modification, and so are probably not relevant to viral growth or the clinical use of efavirenz.

Efavirenz stimulation of 5'-end-directed secondary cuts happens independently of primary cuts

Although primary and secondary cuts were shown to be independent in the absence of efavirenz, the efavirenz-induced increases in secondary products accompanied the disappearance of primary products in the above assays. In view of this observation, we wanted to determine whether the stimulation of secondary cuts required making primary cuts. To test this, 5' labeled RNAJ4 was annealed to DNAJ6 to form a 5'-directed cleavage substrate (Figure 4a) that could allow primary cuts and subsequent secondary cuts when the RT slides back. Subsequently, we applied 2-O-methyl modified substrate RNAJ4[#], which has a blocked primary cleavage site (Figure 4b), with which we observed that secondary cuts

were stimulated by efavirenz even though the primary cuts were absent (Figure 4c; compare standard *versus* blocked panels). This shows that efavirenz exerts its influence on secondary RNase H cleavage independent of the primary cut. The RT may bind initially to the primary cut site, but does not have to cleave before efavirenz can facilitate sliding to the secondary cut site.

Second primary cuts, and their stimulation by efavirenz, do not depend on first primary cuts or even first secondary cuts

Efficient degradation of the viral RNA genome is a rate-limiting step in reverse transcription and involves several successive cuts made in long RNA segments to convert them to oligomeric RNA products that can efficiently dissociate³⁷. Therefore, we asked whether efavirenz affected only the initial primary and secondary cuts or could also influence subsequent primary cuts. To examine this issue, we used 3' ³²P C radiolabeled substrates RNAJ4 and RNAJ4[#] annealed to DNAJ6 (Figure 5a and b). Because the label is on the 3' position it was possible to see the first primary product (28–30 nt product) but not the first secondary product, which would be made without the radiolabel as the RT slides towards the 5' RNA end after making a primary cut. We then observed the second primary product (8–10 nt). In Figure 5c (standard substrate), the first primary products and second primary products had lengths of 28 nt and 12 nt respectively. When substrate RNAJ4[#], which blocks the first primary cut was used (Figure 5c, (blocked template), the second primary cut was made, and its formation was stimulated by efavirenz, meaning that this cut, and its stimulation, did not require cleavage at the first primary site.

We then applied a 3' ³²P C radiolabeled RNAJ4 and RNAJ4[#] annealed to DNAJ5 to produce unblocked and blocked blunt-ended substrates (Figure 6a and b). We observed that it was possible to make first primary cuts with no additional cuts in the absence of efavirenz but efavirenz enabled both primary and secondary primary cuts (Figure 6c (standard substrate). When the primary cut was blocked and sliding to the secondary site was inhibited (Figure 6b), no second primary product was made, presumably because there was no 5' end that the RT could use to orient for the second primary cut. Surprisingly, some second primary cut product was made in the presence of efavirenz (Figure 6c (blocked). The ability of RT to make a second primary cut in this case suggests that the drug allows unique binding orientations, possibly based on positioning from the DNA 5' end, the RNA 3' end or both, that can promote cleavage of the HIV-1 genomic RNA.

Efavirenz increases the binding of RT to template/primer

Our findings indicate that efavirenz alters the binding or translocation of RT such that increased secondary or increased alternative cleavages can be made, resulting in accelerated RNA degradation. In order to determine whether the motions of RT in the presence of efavirenz are the result of altered binding affinity of the RT for the template, we performed an EMSA binding assay as described in materials and methods. We used the 5' ³²P radiolabeled substrate RNAJ4 annealed to the DNAJ6; to produce a 5'-end-directed RNA substrate. Figure 7a shows gel products in the presence or absent of efavirenz at increasing levels of RT protein that indicate a change in interaction caused by the drug. There was a dose dependent increase in the amount of RT bound to the template/primer in the presence of efavirenz at low concentrations of RT (Figure 7b). This difference was reduced as the RT concentration was increased suggesting that the drug effect may be more relevant in drug-resistant mutant viruses that have low RT content, as are commonly found in patients³⁸. Very likely the higher affinity allows the RT to move to secondary sites and cut before it dissociates.

A drug resistant RT shows similar stimulation of RNase H activity

Stimulation of RNase H suggests that efavirenz can promote more efficient removal of genomic RNA during the steps of HIV-reverse transcription, which would result in increased rates of plus strand DNA synthesis. This property may not alter the rate of wild type virus growth given that the drug inhibits DNA polymerization steps, but, when the RT is drug resistant, the removal of genomic RNA may limit the rate of reverse transcription, and its stimulation would be relevant to the rate of virus growth. Therefore, we tested whether efavirenz also stimulates the RNase H activity of an NNRTI-resistant RT. The mutant D10 (K101E+G190S+M41L+T215Y) was isolated from a patient who had failed efavirenz treatment³⁹. We performed polymerase extension assays with D10 and, as expected, this RT was more resistant to efavirenz relative to wild type. Resistance of D10 to efavirenz was evident at 2.5 mM drug concentration (compare Figure 1a (WT) to 8a (D10)). At 25 μ M efavirenz, the mutant was still 50% active, while the WT was <10% active (Figure 8b). Both 3' DNA-directed and 5' RNA-directed RNase H activities, in particular secondary product formation, were also stimulated (Figure 8c and 8d). We note, however, that stimulation of 5' cleavage is not uniformly dose dependent, indicating that the RT responds in a complex manner to increasing drug interactions. The observed stimulations may be the basis of previous findings that virus containing the D10 RT sequence replicates better in low concentrations of NNRTI than in the absence of drug⁴⁰. Indeed the authors mapped the stimulation to early stages of virus replication making the role of RNase H a possible explanation⁴¹.

Discussion

The search for alternative drug targets for the disruption of HIV-1 will remain an active area of research until a cure can be found. The development of drugs that target the RNase H activity of HIV-1 RT is one part of this search because to date there are no approved antiretrovirals that inhibit RNase H activity despite its essential function in the HIV-1 life cycle. Our results showed that E478Q RT, with a non-functional RNase H, is more sensitive to the commonly used NNRTI antiviral efavirenz than the wild type RT, implying that an active RNase H attenuates drug inhibition. We suggest that targeting viral replication through the inhibition of RNase H will serve the dual purpose of enhancing the effect of existing RT inhibitors and inhibiting the essential viral replication functions of RNase H, and so should be explored. There is considerable data to show that RNase H plays a significant role in influencing the potency of NRTIs and NNRTIs^{26,42,43}. Our study advances these findings and offers additional biochemical insights relevant to utilizing drugs that bind the existing NNRTI binding pocket to influence RNase H function therapeutically.

Efavirenz was reported to stimulate DNA 3'-end-directed RNase H activity and partially inhibit 5'-end-directed cleavages³³. In addition, nevirapine was reported to stimulate 3'-end-directed secondary cleavages²⁶ suggesting similar effects to those of efavirenz. The inhibition or stimulation of RNase H activity by NNRTIs is also substrate dependent^{33,36}. Moreover, the role of RNase H in reverse transcription is complex. In order to accomplish its ultimate purpose of removing genomic RNA to make way for plus strand synthesis, the RT must carry out a series of nucleolytic functions. The genomic RNA is initially cleaved into long oligomers during minus strand DNA synthesis by the polymerization-dependent DNA 3'-end-directed RNase H activity. Additional RTs then return to degrade these oligomer segments by progressive RNA 5'-end-directed cleavages^{13,44}. All these cleavages involve primary and secondary site positioning. The need for this series of functions prompted us to examine the full range of mechanisms by which efavirenz influences RT RNase H.

Our initial results showed that inactivation of the RNase H active site greatly enhanced the ability of efavirenz to inhibit RT-directed DNA polymerization. This implies a strong functional interaction between the drug and both the polymerization and RNase H functions, worthy of additional examination. Recent structural and biochemical data does indeed confirm the existence of such a relationship^{43,45} and our study therefore fits with these findings.

We have found that efavirenz stimulation of overall RNase H activity, assessed by depletion of starting substrate, is moderate. However, a large stimulatory effect occurs through an efficient conversion of primary products to secondary products. It was particularly interesting to see that, in the presence of efavirenz, RT could make secondary cuts even when we blocked the primary cuts. Moreover, efficient secondary cuts were evident even when we used a blunt substrate that would not support secondary cleavages in the absence of drug³⁶. This result strongly suggests that binding of efavirenz causes a structural distortion of RT in addition to increased binding that allows easy sliding on the template to make the secondary cuts. It is possible that this distortion effect, when fully understood structurally, could be advanced to target inhibition of RT RNase H for treatment.

We previously reported that the patient-derived NNRTI resistant mutant virus expressing the D10 RT grows more rapidly in the presence of low concentrations of efavirenz^{20,22}. One potential explanation for this effect is that the removal of the genomic RNA is limiting the rate of the reverse transcription pathway, and that, at specific drug concentrations; efavirenz stimulates critical steps of RNA removal. Indeed we have shown this drug concentration-dependent pattern for the D10 mutant RT, whereby as the drug concentration was increased, the 5' RNA end-directed RNase H activity increased or remained unchanged at low levels of efavirenz, dipped at intermediate drug levels, and finally was stimulated as the concentration of drug was increased further. We propose that at the efavirenz concentrations at which stimulation is observed, the stimulation of multiple RNase H functions is sufficient to cause an overall acceleration of reverse transcription.

One possible explanation for an inhibition followed by stimulation is the existence of a secondary drug-binding site. This is yet to be demonstrated and was beyond the scope of the current studies though the existence of such a site could offer an additional target for viral inhibition.

Degradation of the viral RNA genome during reverse transcription serves an essential role in reverse transcription, allowing the complete formation of double stranded DNA for integration into the host chromosome^{14,46}. RNase H activity also contributes to viral recombination and evolution⁴⁷. It is clear that mutations affecting RNase H or compounds that target RNase H will impact the integrity of the transcribed viral genome and will cause changes in virus proliferation. Efavirenz is a first line therapy for HIV/AIDS and any changes in response to its use could directly affect the outcome of the treatment process⁴⁸. In patients who develop high levels of NNRTI resistance, efavirenz might have the undesirable side effect of stimulating virus growth through efficient removal of the parental RNA genome. The observations made here and in other studies have implications in drug administration and adherence especially in resource limited environments where monitoring viral loads is not routinely performed. Our studies have provided a possible explanation to the stimulation of virus growth in the presence of NNRTIs. However, it is also true that accelerated template removal, if not timed properly, may have the opposite effect of making a virus less fit in the presence of drug because the process prematurely degrades the viral genome during reverse transcription.

In summary, our results highlight how small molecule interactions at the NNRTI binding site can influence both the polymerase and RNase H active sites, and we propose that proper targeting of all three sites could provide a more effective inhibition of viral replication. Perhaps a new class of NNRTIs targeting RNase H is feasible.

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Abbreviations

RT	reverse transcriptase
RNase	ribonuclease
EFV	efavirenz
NNRTI	non nucleoside reverse transcriptase inhibitors
EMSA	electrophoretic mobility shift assay
PP	primary products
SP	secondary products
S	substrate
nt	nucleotide

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Figure 1. The E478Q RNase H negative mutant RT is more sensitive to efavirenz
 Primer extension was performed using a 35 nt 5'-³²P labeled primer (DNAJ3; Table 1) annealed to a 40 nt RNA template (RNAJ1) in the presence of Mg²⁺ and ATP. (a) Structure of non nucleoside inhibitor, efavirenz (b) A representative gel of wild type polymerization activity performed in the presence of 2.5 μM efavirenz (right) or 0 μM efavirenz (left). TP and EP indicate unextended template-primer and extended product, respectively. The molecular marker (M; lane 1) sizes are indicated to the left. Time is shown above in seconds. (c) A representative gel of the E478Q mutant RT polymerization and RNase H activities. TP1 indicates the template/primer substrate in the primer extension assay and EP indicates the extension products. TP2 represents the template/primer used for the RNase H assay showing no cuts made in the presence of E478Q. (d) Primer extension reactions were performed at least in triplicate at different concentrations of efavirenz and quantitated using ImageQuant 5.2. The y-axis represents the percentage of TP converted to EP calculated as %EP = EP/(TP+EP)*100, where EP = extended products, TP = non-extended template/primer. The x-axis shows the different concentrations of efavirenz tested in μM. Error bars represent standard deviations from the mean.

Figure 2. Efavirenz stimulates DNA 3'- and RNA 5'-directed RNase H activity of wild type RT
 (a) Representation of the RNA and DNA template/primer. RT is shown aligned on the DNA 3' end, to make the primary cut typically 15–20 nt from the 3' end and secondary cuts 7–9 nt away. The square indentation represents the polymerization active site. The grooved indentation represents the RNase H active site. (b) Analysis in percent of primary products, secondary products and combined total products on the Y-axis at different concentrations of efavirenz in μM shown on the X-axis. Error bars represent standard deviations from the mean. (c) Representation of the RNA and DNA template/primer aligned such that RT positions on the 5' end of the RNA. RT makes primary cuts 13–19 nt from the 5' end and then slides to make secondary cuts 7–9 nt from the 5' end. (d) Analysis in percent of cleavage products on the Y-axis and concentrations of efavirenz in μM shown on the X-axis. Percentage products was calculated using the formula $\% \text{ PP} = \text{PP}/(\text{PP} + \text{SP} + \text{S}) \times 100$, where PP = primary cleavage products, SP = secondary cleavage products and S = undigested substrate. Error bars represent standard deviations from the mean based on at least three assays.

Figure 3. Efavirenz stimulates 5' end-directed RNase H primary cuts

(a) Representation of RNAJ4-DNAJ5 template/primer forming a blunt end substrate. RT is shown aligned to the 5'-³²P labeled RNA. Products sizes obtained with this substrate are indicated. (b) A 15% polyacrylamide gel showing the cleavage of the blunt substrate over time (seconds) in the presence or absence of 10 μM efavirenz. The expected 18-nt primary product and an alternative 23-nt product are marked on the right. Lane 1, molecular markers (M) sizes shown to the left. (c) RNAJ4[#]-DNAJ5 template primer, forming a blunt substrate with first primary cut site blocked (X) by 2'-O-methyl bases. (d) A representative gel of cleavage of the 2'-O-methyl substrate over time (seconds), with 10 μM efavirenz (+EFV) or without efavirenz (-EFV). New product sizes 14 and 28 nt generated in the assay with efavirenz are marked on the right.

Figure 4. Efavirenz stimulates secondary cuts independently of primary cuts

(a) 3'-³²P labeled 46mer-RNAJ4 annealed to 61mer DNAJ6 showing expected primary (18 nt) and secondary (13 nt) cleavage positions, respectively. (b) RNAJ4[#]- DNAJ6 template is shown with first primary cleavage site blocked (X) by 2'-O-methyl nts and secondary product (13 nt). (c) Gel products with templates described in (a) and (b) in the presence of 10 μ M efavirenz showing substrates with blocked primary sites alongside unblocked substrates (standard). Substrate, primary and secondary products sizes are shown to the right.

Figure 5. Efavirenz accelerates second primary cuts without the requirement for the first primary cut

(a) Depicts the 3'-³²P labeled 46mer-RNAJ4 annealed to the 61mer-DNAJ6 showing expected cuts for first primary (28 nt), first secondary, (35 nt), second primary (12 nt) and second secondary (8 nt). (b) Depicts the template/primer set described in (a) except template is RNAJ4[#], where the first primary cut PP1 is blocked. (c) A 15% polyacrylamide gel showing a time course (seconds) of RNase H activity in the presence of 10 μ M efavirenz (+EFV) or its absence (-EFV). The panel to the left shows results obtained with substrate depicted in Figure 5(a), while to the right are results obtained with the substrate depicted in Figure 5(b).

Figure 6. Efavirenz accelerates second primary cuts without first primary or secondary cuts
 (a) Depicts the 3'-³²P labeled 46mer-RNAJ4 annealed to the 46mer-DNAJ5 to form a blunt ended substrate, where 28 nt = first primary cut, 8 nt = second primary and 12 nt = second secondary cut. (b) Depicts the template set described in (a) except use of RNAJ4[#] in which the first primary cut is blocked. (c) A 15% polyacrylamide gel showing cleavage products over time (seconds) in the presence of 10 μ M efavirenz (+EFV) or its absence (-EFV). The panel to the left shows results obtained with the substrate depicted in Figure 6(a), while to the right are results obtained with the substrate depicted in Figure 6(b).

7(a)

7(b)

Figure 7. Efavirenz alters binding of RT to substrate

The effect of efavirenz on RT binding to template/primer was tested by an electrophoretic mobility shift assay (EMSA) using the 5'-³²P labeled RNAJ4-DNAJ6 at 37 °C. Products were resolved on a 6% native polyacrylamide gel. (a) Representative gel showing products with 10 μM efavirenz (+EFV) or its absence (-EFV) and increasing levels of RT, [RT] nM. RT/S marks the position of RT bound to substrate, while S is unbound substrate. (b) Analysis of product shift at constant levels of 10 nM RT and increasing levels of efavirenz in μM. Error bars represent standard deviations from the mean based on at least three replicates.

Figure 8. Efavirenz resistant and WT RTs have similar RNase H stimulation characteristics
K101E+G190S+M41L+T215Y (D10) was isolated and cloned from a patient failing efavirenz treatment. (a) A 12% polyacrylamide gel of a three minute primer extension assay without (0 μ M) and with 2.5 μ M efavirenz. TP and EP indicate unextended template/primer and extended product, respectively. The molecular marker (M; lane 1) sizes are indicated to the left. Time is shown above in seconds. (b) The percentage of primer extended as a function of increasing efavirenz concentrations comparing WT and D10 RT. (c) Analysis of products from DNA 3'-end-directed RNase H cleavage products of D10 in increasing levels of efavirenz and (d) analysis of RNA 5' end directed RNase H cleavage products.

Table 1

Strands of RNA (5'-3') templates and DNA (3'-5') primers used in the study

Name	Sequence	Length
RNAJ1	CUACGUAUCGAACUCCUAAUCCGGCCUGGGUAGCCUCU	40
DNAJ2	GATGCATAGCGATGCAGAGCTTGAGGATTAAGGCC	35
DNAJ3	GATTAAGGCCGGGACCCATCGGAGAGTCAACCATG	35
RNAJ4	GUGAAUUCGACCUUCGAUACCCUAGGAUCCACUAUAGCUAGCCUG	45
RNAJ4[#]O	GUGAAUUCGACCUUC <u>GUAACCC</u> UAGGAUCCACUAUAGCUAGCCUG	45
DNAJ5	CACTTAAGCTGGAAGCTATGGGATCCTAGGTGATATCGATCGGAC	45
DNAJ6	TAAGATTACTATTCGCACTTAAGCTGGAAGCTATGGGATCCTAGGTGATATCGATCGGACG	61

Strands are named J1 through J6 indicating order of use.

[#] Shows modified substrate, 2-O-methylbases added shown in underlined bold. Sequences are aligned to show regions of complementarity.