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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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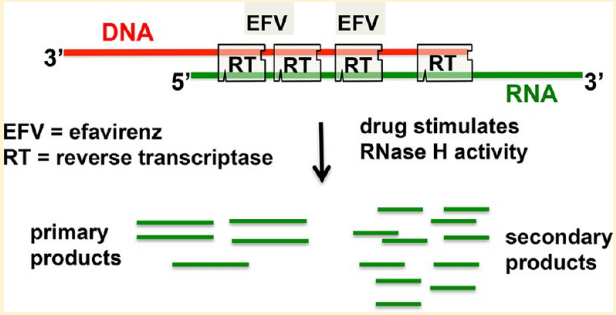
ABSTRACT: Efavirenz is a non-nucleoside reverse transcriptase inhibitor used for treating HIV/AIDS. We found that polymerization activity of a reverse transcriptase (RT) with the E478Q mutation that inactivates the RNase H catalytic site is much more sensitive to efavirenz than wild-type RT, indicating 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 ~18 nucleotides from the growing DNA 3′-end, and when the RT pauses synthesis, it shifts to make secondary cuts ~9 nucleotides from the DNA 3′-end. After synthesis, RTs return to bind the 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 (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 the 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 ~18 nucleotides from the DNA 3′-end. At frequent synthesis pause sites, the RT shifts to make secondary cuts ~9 nucleotides from the end. To complete RNA degradation, additional RTs are positioned at the 5′-ends of the RNA fragments to conduct RNA 5′-end-directed cleavage.[15] Again, primary cuts are made ~18

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Table 1. Strands of RNA (5′−3′) Templates and DNA (3′−5′) Primers Used in This Study[a]

Name	Sequence	Length
RNAJ1	CUACGUAUCGAACUCCUAAUUCCGGCCCUGGGUAGCCUCU	40
DNAJ2	GATGCATAGCGATGCAGAGCTTGAGGATTAAGGCC	35
DNAJ3	GATTAAGGCCGGGACCCATCGGAGAGTCAACCATG	35
RNAJ4	GUGAAUUCGACCUUCGAUACCCUAGGAUCCACUAUAGCUAGCCUG	45
RNAJ4[b]O	GUGAAUUCGACCUUC**GAUACCC**UAGGAUCCACUAUAGCUAGCCUG	45
DNAJ5	CACTTAAGCTGGAAGCTATGGGATCCTAGGTGATATCGATCGGAC	45
DNAJ6	TAAGATTACTATTCGCACTTAAGCTGGAAGCTATGGGATCCTAGGTGATATCGATCGGACG	61

[a]Strands are named J1−J6 indicating the order of use. [b]Shows the modified substrate in which added 2-*O*-methyl bases are shown in bold. Sequences are aligned to show regions of complementarity.

nucleotides from the RNA 5′-end, and then secondary cuts are made about 9 nucleotides 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, the 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.

Because RNase H activity is an important component of the reverse transcription pathway and 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 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 RNase H activity influence each other and how these influences might explain the 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 1× TBE, respectively. T4 polynucleotide kinase (Life Technologies), *Escherichia coli* DNA polymerase I Klenow fragment (Roche Molecular Biochemicals), and shrimp alkaline phosphatase (SAP) and SAP buffer (Thermo scientific) 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 of 5400 units/mg), patient RT isolate, and K101E/G190S/M41L/T215Y (D10) (specific activity of 7500 units/mg) were expressed and purified in our laboratory as previously described.[27] HIV-1 E478Q RT (specific activity of 40000 units/mg) was provided by S. F. J. Le Grice. The non-nucleoside reverse transcriptase inhibitor, efavirenz (EFV), was obtained from the National Institutes of Health AIDS Research and Reference program, Division of AIDS, National Institute of Allergy and Infectious Diseases. 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 the protocols of the manufacturers.

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

Preparation of 5′-End-Labeled RNA and DNA. DNA primers were 5′-end-labeled using [γ-^{32}P]ATP (6000 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, and then the enzyme was inactivated at 65 °C for 25 min. The 5′-end labeling was then performed as described above. Further purification and concentration of the RNA were performed using the GeneJET RNA cleanup and concentration Microkit (Thermoscientific).

Preparation of 3′-End-Labeled RNA. The RNA template was preannealed to the DNA primer. A single-nucleotide extension was made using [α-^{32}P]pCp (6000 Ci/mmol) and Klenow fragment. The reaction mixture was incubated at 37 °C for 2 h and then deactivated by being incubated at 65 °C for 30 min. The DNA was then digested using turbo DNase (Thermoscientific) followed by RNA purification and cleanup as described above.

Hybridization. Annealing of the RNA to DNA (one RNA per four 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 slowly cooled to room temperature.

RNase H Activity Assay. The assays were performed as described previously with modifications.[28] Briefly, for the DNA 3′-directed RNase H assay, a 15 μL reaction assay was performed containing 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1.0 mM EDTA, 34 mM KCl, 6 mM $MgCl_2$, 8 nM

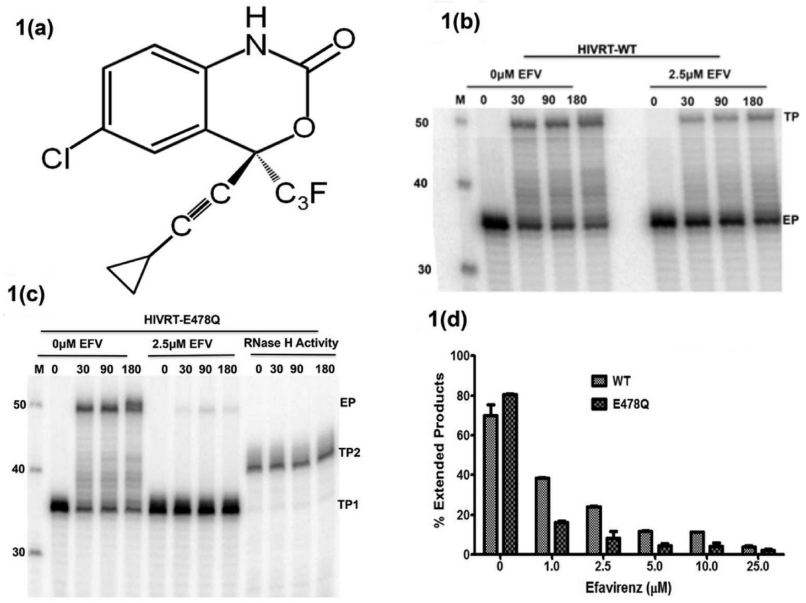

Figure 1. E478Q RNase H negative mutant RT that is more sensitive to efavirenz. Primer extension was performed using a 35-nucleotide 5′-^{32}P-labeled primer [DNAJ3 (Table 1)] annealed to a 40-nucleotide RNA template (RNAJ1) in the presence of Mg^{2+} and ATP. (a) Structure of non-nucleoside inhibitor efavirenz. (b) 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 at the left. The time is shown above in seconds. (c) 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 the E478Q mutant. (d) Primer extension reactions were performed in at least triplicate at different concentrations of efavirenz and quantitated using ImageQuant version 5.2. The *y*-axis represents the percentage of TP converted to EP calculated as %EP = EP/(TP + EP) × 100, where EP denotes extended products and TP the nonextended template/primer. The *x*-axis shows the different concentrations of efavirenz tested. Error bars represent standard deviations from the mean.

substrate/primer, 17 nM RT, and varying amounts of efavirenz. In all cases, RT was preincubated with substrate for 3 min at 37 °C and then the reaction was initiated by adding $MgCl_2$. Reactions were terminated with 15 μL of 2× termination mixture [90% formamide (v/v), 10 mM EDTA (pH 8.0), 0.1% xylene cyanole, and 0.1% bromophenol blue]. Reactions for RNA 5′-directed RNase H assays were conducted 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 polyacrylamide gel electrophoresis (PAGE), analyzed with a PhosphorImager (GE Healthcare), and quantitated using ImageQuant version 5.2.

DNA Polymerase Activity Assay. Substrate RNAJ1 was annealed as described above to DNAJ3 labeled at the 5′-end with ^{32}P (Table 1) in a 4:1 ratio. This substrate was then used for primer extension assays using 17 nM RT and different concentrations of efavirenz (Figure 1c) in an assay reaction mixture 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 the 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, and then the mobility shift was assessed by 6% native PAGE at 150 V and 4 °C for 2 h.

RESULTS

RNase H Negative Mutant E478Q RT Is More Sensitive to Efavirenz Than the 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 activity.[29] 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 enzyme.[29] HIV-1 with this mutation cannot be grown, because 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,d). However, we observed that E478Q exhibited a much greater sensitivity to efavirenz inhibition and was completely inhibited at 2.5 μM (Figure 1c,d). 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

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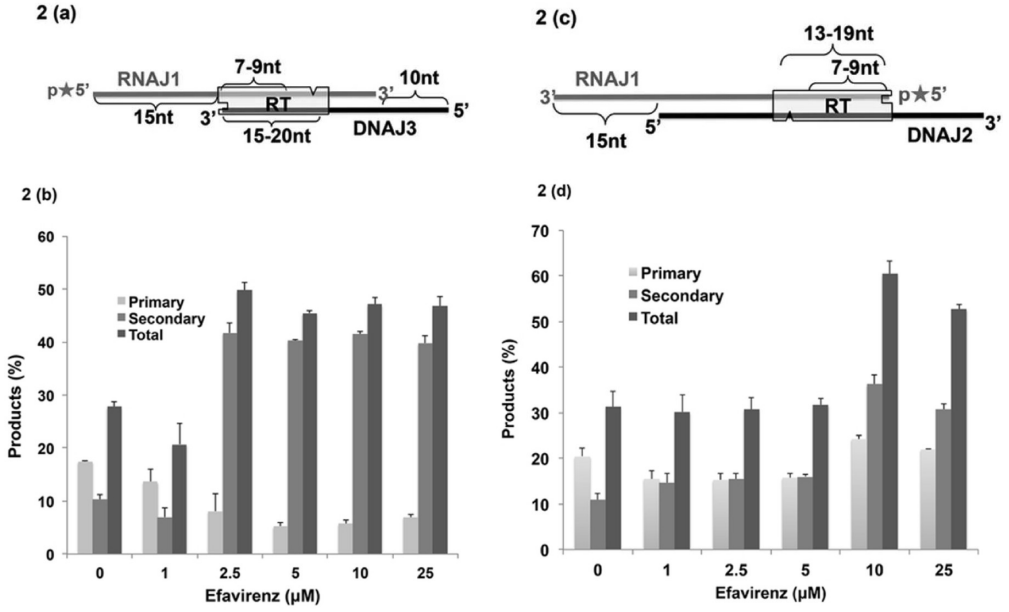

Figure 2. Efavirenz stimulates DNA 3′-directed and RNA 5′-directed RNase H activity of wild-type RT. (a) Representation of the RNA and DNA template/primer. RT is shown aligned on the DNA 3′-end, to make the primary cut typically 15−20 nucleotides from the 3′-end and secondary cuts 7−9 nucleotides from the 3′-end. 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 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 is positioned on the 5′-end of the RNA. RT makes primary cuts 13−19 nucleotides from the 5′-end and then slides to make secondary cuts 7−9 nucleotides from the 5′-end. (d) Analysis in percent of cleavage products on the *y*-axis and concentrations of efavirenz shown on the *x*-axis. The percentage of products was calculated using the formula %PP = PP/(PP + SP + S) × 100, where PP denotes 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.

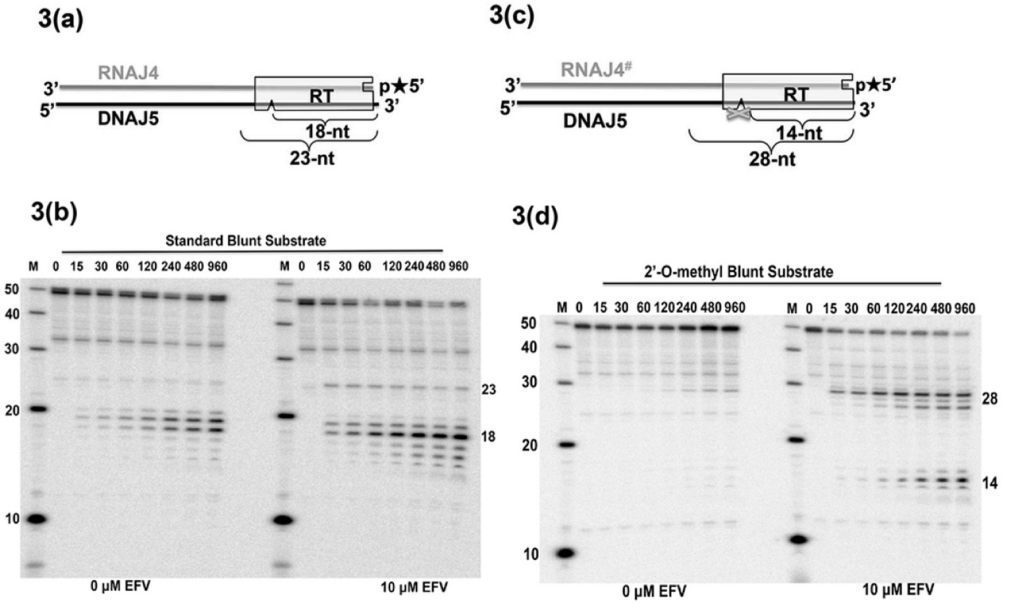

Figure 3. Efavirenz stimulates 5′-end-directed RNase H primary cuts. (a) Representation of the RNAJ4−DNAJ5 template/primer forming a blunt end substrate. RT is shown aligned to the 5′-^{32}P-labeled RNA. Product sizes obtained with this substrate are indicated. (b) A 15% polyacrylamide gel showing the cleavage of the blunt substrate over time (seconds) in the presence or absence of 10 μM efavirenz. The expected 18-nucleotide primary product and an alternative 23-nucleotide product are marked on the right. Lane 1 contained molecular markers (M) (sizes at the left). (c) RNAJ4$^{\#}$−DNAJ5 template primer, forming a blunt substrate with the first primary cut site blocked (X) by 2′-*O*-methyl bases. (d) 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 of 14 and 28 nucleotides generated in the assay with efavirenz are marked at the right.

was further shown that the presence of specific point mutations in the NNRTI binding site could change this specificity.[26]

We designed substrates that would allow measurement of the generation of primary and secondary products and determine

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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 the total level of RNase H products (Figure 2b); 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 an increased level of secondary product generation depleted the pool of primary products.

Efavirenz Alters RNA 5′-End-Directed RNase H Activity in a Drug Concentration-Dependent Manner. To measure how efavirenz affects 5′-primary and -secondary cleavages, we designed substrates that allowed the RT to be positioned 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 allowed RT to move from primary to secondary cleavage sites, so that the proportion of secondary products was increased. At >5 μM efavirenz, the levels of both products of RNase H cleavage were increased, with the level of secondary products increasing to a greater extent than the level of primary products, suggesting that efavirenz is able either to increase the level of 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.

A Blunt Substrate Reveals That Efavirenz Stimulates Primary Cleavages. Both 3′- and 5′-secondary cuts were reported to occur in a manner independent of primary cuts.[34,35] However, because 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 RNAJ4 labeled at the 5′-end with ^{32}P 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 RT to slide and make the secondary cut.[36] Because 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 determinants of the 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 the 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 and 22 that block primary cuts (Figure 3c). While no cuts were made in the absence of the drug, the presence of efavirenz altered the cleavage profile, resulting in products larger (28-nucleotide product) and smaller (14-nucleotide 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 Occurs in a Manner Independent of Primary Cuts. Although primary and secondary cuts were shown to be independent in the absence of efavirenz, the efavirenz-induced increases in the level of secondary products accompanied the disappearance of primary products in the assays described above. In light of this observation, we wanted to determine whether the stimulation of secondary cuts required that primary cuts be made. To test this, 5′-labeled RNAJ4 was annealed to DNAJ6 to form a 5′-directed cleavage substrate (Figure 4a)

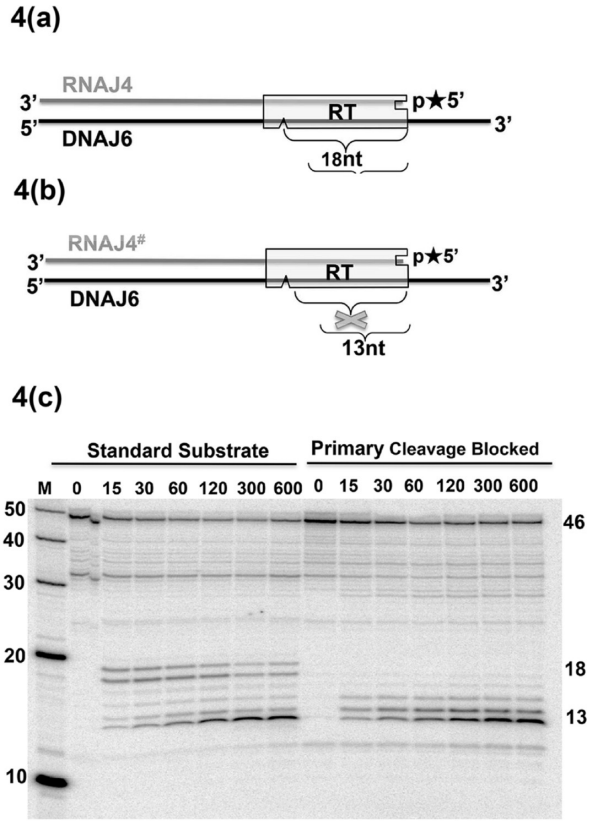

Figure 4. Efavirenz stimulates secondary cuts in a manner independent of primary cuts. (a) The 3′-^{32}P-labeled 46mer RNAJ4 annealed to 61mer DNAJ6 showing expected primary (18 nucleotides) and secondary (13 nucleotides) cleavage positions, respectively. (b) RNAJ4$^{\#}$—DNAJ6 template shown with the first primary cleavage site blocked (X) by 2′-*O*-methyl nucleotides and the secondary product (13 nucleotides). (c) Gel products with templates described in panels a and b in the presence of 10 μM efavirenz showing substrates with blocked primary sites alongside unblocked substrates (standard). Substrate, primary, and secondary products sizes are shown at the right.

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 (in Figure 4c, compare standard and blocked panels). This shows that efavirenz exerts its influence on secondary RNase H cleavage in a manner independent of the primary cut. The RT may bind initially to the primary cut

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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 dissociate.[37] 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′-^{32}P C-radiolabeled substrates RNAJ4 and RNAJ4$^{\#}$ annealed to DNAJ6 (Figure 5a,b). Because the label is at the

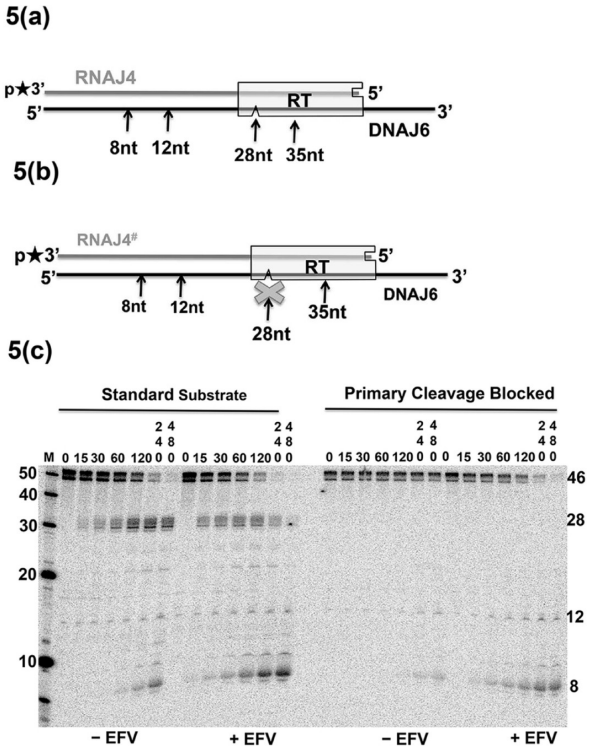

Figure 5. Efavirenz accelerates second primary cuts without the requirement for the first primary cut. (a) The 3′-^{32}P-labeled 46mer RNAJ4 annealed to the 61mer DNAJ6 showing expected cuts for first primary (28 nucleotides), first secondary (35 nucleotides), second primary (12 nucleotides), and second secondary (8 nucleotides) positions. (b) Template/primer set described in panel a except the template is RNAJ4$^{\#}$, in which 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 μM efavirenz (+EFV) or in its absence (−EFV). The left panel shows results obtained with the substrate depicted in panel a, while the right panel shows the results obtained with the substrate depicted in panel b.

3′-position, it was possible to see the first primary product (28−30-nucleotide product) but not the first secondary product, which would be made without the radiolabel as the RT slides toward the 5′-RNA end after making a primary cut. We then observed the second primary product (8−10 nucleotides). In Figure 5c (standard substrate), the first primary products and second primary products had lengths of 28 and 12 nucleotides, 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′-^{32}P C-radiolabeled RNAJ4 and RNAJ4$^{\#}$ annealed to DNAJ5 to produce unblocked and blocked blunt-ended substrates (Figure 6a,b). We observed that it was

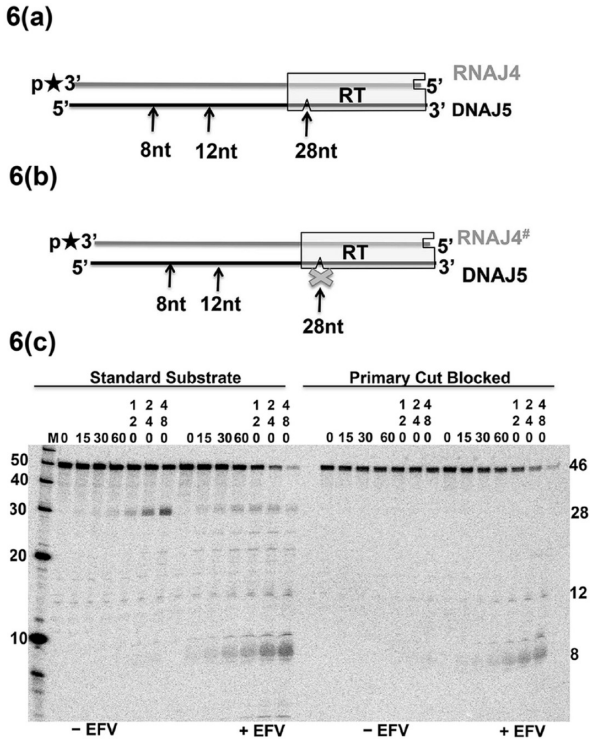

Figure 6. Efavirenz accelerates second primary cuts without first primary or secondary cuts. (a) The 3′-^{32}P-labeled 46mer RNAJ4 annealed to the 46mer DNAJ5 to form a blunt-ended substrate, where 28 nucleotides constitute the first primary cut, 8 nucleotides the second primary cut, and 12 nucleotides the second secondary cut. (b) Template set described in panel a except for the 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 μM efavirenz (+EFV) or in its absence (−EFV). The left panel shows results obtained with the substrate depicted in panel a, while the right panel shows results obtained with the substrate depicted in panel b.

possible to make first primary cuts with no additional cuts in the absence of efavirenz but efavirenz allowed 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 Level of Binding of RT to the Template/Primer. Our findings indicate that efavirenz alters the binding or translocation of RT such that increased levels of secondary or increased alternative cleavages can be reached, resulting in accelerated RNA degradation. To determine whether the motions of RT in the presence of efavirenz are the result of the altered binding affinity of the RT for the

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template, we performed an EMSA binding assay as described in Materials and Methods. We used the 5′-^{32}P-radiolabeled substrate RNAJ4 annealed to DNAJ6, to produce a 5′-end-directed RNA substrate. Figure 7a shows gel products in the presence or absence of efavirenz at increasing levels of RT protein that indicate a change in the 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, which are commonly found in patients.[38] Very likely, the higher affinity allows the RT to move to secondary sites and cut before it dissociates.

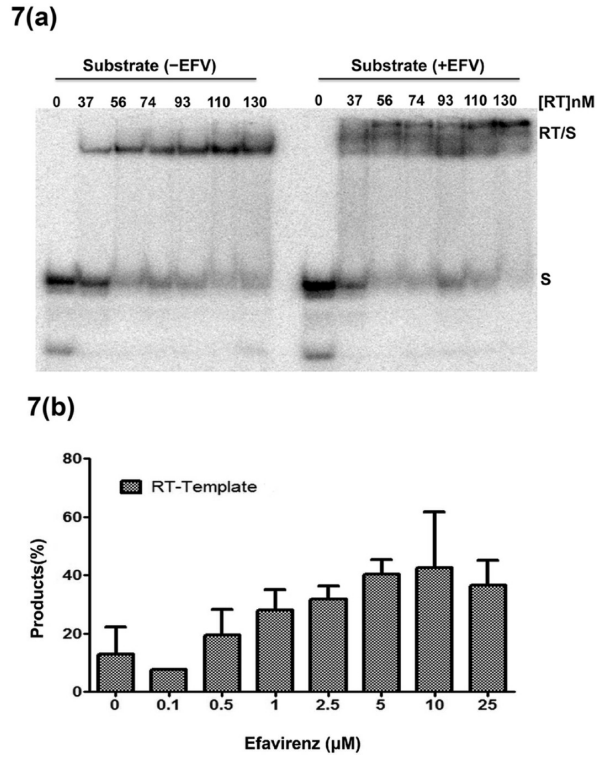

Figure 7. Efavirenz alters the binding of RT to substrate. The effect of efavirenz on binding of RT to template/primer was tested by an electrophoretic mobility shift assay (EMSA) using the 5′-^{32}P-labeled RNAJ4−DNAJ6 complex at 37 °C. Products were resolved on a 6% native polyacrylamide gel. (a) Representative gel showing products with 10 μM efavirenz (+EFV) or without it (−EFV) and increasing levels of RT. RT/S marks the position of RT bound to substrate, while S is unbound substrate. (b) Analysis of the product shift at constant levels of 10 nM RT and increasing levels of efavirenz. Error bars represent standard deviations from the mean based on at least three replicates.

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. Mutant D10 (K101E/G190S/M41L/T215Y) was isolated from a patient who had failed efavirenz treatment.[39] We performed polymerase extension assays with D10, and as expected, this RT was more resistant to efavirenz than the wild type was. Resistance of D10 to efavirenz was evident at a drug concentration of 2.5 mM [compare Figures 1a (WT) and 8a (D10)]. At 25 μM 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,d). We note, however, that stimulation of 5′-cleavage is not uniformly dose dependent, indicating that the RT responds in a complex manner to an increasing level of drug interaction. The observed stimulations may be the basis of previous findings that virus containing the D10 RT sequence replicates better at low concentrations of NNRTI than in the absence of drug.[40] Indeed, the authors mapped the stimulation to early stages of virus replication, making the role of RNase H a possible explanation.[41]

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 nonfunctional RNase H, is more sensitive to the commonly used NNRTI antiviral efavirenz than 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 are 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 cleavages.[33] In addition, nevirapine was reported to stimulate 3′-end-directed secondary cleavages,[26] suggesting effects similar 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. To accomplish its ultimate purpose of removing genomic RNA to make way for plus strand synthesis, the RT must conduct 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

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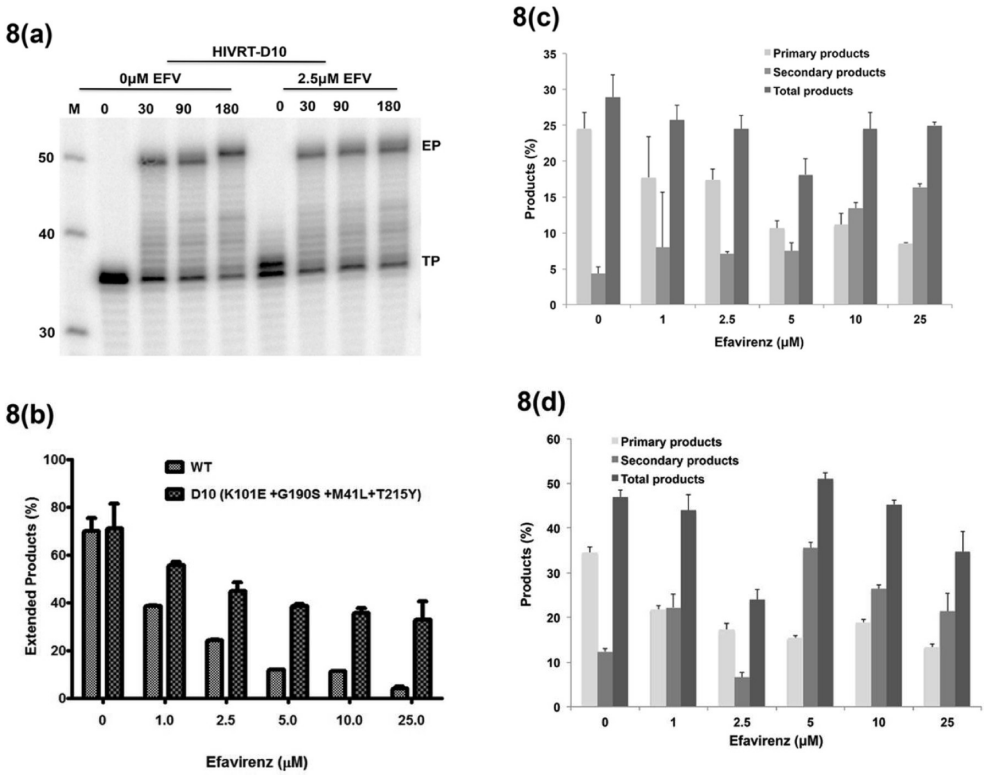

Figure 8. Efavirenz-resistant and WT RTs have similar RNase H stimulation characteristics. The K101E/G190S/M41L/T215Y mutant (D10) was isolated and cloned from a patient who had failed efavirenz treatment. (a) A 12% polyacrylamide gel of a 3 min primer extension assay without (0 μM) and with 2.5 μM efavirenz. TP and EP indicate unextended template/primer and extended product, respectively. The molecular marker (M, lane 1) sizes are shown at the left. The time is shown above in seconds. (b) Percentage of primer extended as a function of increasing efavirenz concentration comparing WT and D10 RT. (c) Analysis of products from DNA 3′-end-directed RNase H cleavage products of D10 with increasing levels of efavirenz. (d) Analysis of RNA 5′-end-directed RNase H cleavage products.

polymerization and RNase H functions, which are worthy of additional examination. Recent structural and biochemical data do 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 drug.[36] This result strongly suggests that binding of efavirenz causes a structural distortion of RT in addition to an increased level of 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, decreased at intermediate drug levels, and finally was stimulated as the concentration of the 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 this study, 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 evolution.[47] 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 process.[48] 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

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have provided a possible explanation for 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 the 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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Notes

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ABBREVIATIONS

RT, reverse transcriptase; RNase, ribonuclease; EFV, efavirenz; NNRTI, non-nucleoside reverse transcriptase inhibitor; EMSA, electrophoretic mobility shift assay; PP, primary products; SP, secondary products; S, substrate.

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