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HIV-1 Reverse Transcriptase Can Simultaneously Engage its DNA/RNA Substrate at both the DNA Polymerase and RNase H Active Sites: Implications for RNase H Inhibition

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Abstract

Reverse transcriptase (RT) of the human immunodeficiency virus (HIV) possesses DNA polymerase and ribonuclease (RNase) H activities. Although the nucleic acid binding cleft separating these domains can accommodate structurally-diverse duplexes, it is currently unknown whether regular DNA/RNA hybrids can simultaneously contact both active sites. In this study we demonstrate that ligands capable of trapping the 3'-end of the primer at the polymerase active site affect specificity of RNase H cleavage without altering the efficiency of the reaction. Experiments under single turnover conditions reveal that complexes with a bound nucleotide substrate show specific RNase H cleavage at template position -18, while complexes with the pyrophosphate analogue foscarnet show a specific cut at position -19. This pattern is indicative for post- and pre-translocated conformations. The data are inconsistent with models postulating that the substrate toggles between both active sites, such that the primer 3'-terminus is disengaged from the polymerase active site when the template is in contact with the RNase H active site. In contrast, our findings provide strong evidence to suggest that the nucleic acid substrate can engage both active sites at the same time. As a consequence, the bound and intact DNA/RNA hybrid can restrict access of RNase H active site inhibitors. We have mapped the binding site of the recently discovered inhibitor β -thujaplicinol between the RNase H active site and Y501 of the RNase H primer grip and show that the inhibitor is unable to bind to a pre-formed RT-DNA/RNA complex. In conclusion, the bound nucleic acid substrate, and in turn, active DNA synthesis can represent an obstacle to RNase H inhibition with compounds that bind to the RNase H active site.

Keywords

HIV; Reverse Transcriptase; RNase H; drug resistance; inhibitors

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Introduction

The human immunodeficiency virus type-1 (HIV-1) reverse transcriptase (RT) is a heterodimeric (p66/p51), multifunctional DNA polymerase that converts the single-stranded (ss) viral RNA genome into integration-competent double-stranded DNA. This process requires coordination of the RT-associated DNA polymerase and ribonuclease (RNase) H activities 1. Both active sites reside in the large subunit p66, and accommodate divalent metal ions required for catalyzing the nucleotide incorporation and strand scission events 2; 3; 4; 5. The RNase H activity of HIV-1 RT degrades the RNA moiety of RNA/DNA replication intermediates during synthesis of the first, or minus DNA strand. The rate of nucleotide incorporation is approximately an order of magnitude faster than that of RNase H cleavage 6; however, specific RNase H cuts can be seen at a fixed distance of 18 base pairs (bp) upstream of the primer 3'-terminus in the absence of nucleoside triphosphates (dNTPs) or when DNA synthesis is arrested by chain-terminating nucleotides 7; 8; 9; 10; 11; 12. This specific cleavage between template positions -18 and -19 is referred to as polymerase-dependent RNase H activity and reflects the distance between the polymerase and RNase H active sites. HIV-1 RNase H also functions in a polymerase-independent mode, which collectively refers to cleavage events that occur when the 3'-end of the primer is not located in the vicinity of the polymerase active site, although variations of this mechanism have been reported 13. This activity is required to remove viral RNA during and following minus-strand DNA synthesis, as well as to specifically remove tRNA^{Lys3} and the polypurine tracts (PPTs), the primers for minus- and plus-strand DNA synthesis, respectively 14; 15; 16; 17; 18; 19.

Despite its crucial role in viral replication, the RT-associated RNase H activity has yet to be explored as a pharmaceutical target for drug development efforts. All currently approved RT inhibitors interfere with the polymerase activity of the enzyme 20. Some of these inhibitors also affect RT-associated RNase H activity. Non-nucleoside analogue RT inhibitors (NNRTIs), for example, have been shown to alter the specificity and efficiency of RNase H cleavage 21, while nucleoside analogue RT inhibitors (NRTIs) can alter the RNase H cleavage pattern, without affecting cleavage efficiency 22. Phosphonoformic acid (PFA, foscarnet), a pyrophosphate analogue, also inhibits both the polymerase and RNase H activities of RT in cell-free assays 23, as do N-acyl hydrazones, some of which have been shown to inhibit RT-associated RNase H activity exclusively 24; 25; 26. Some diketo acids 27, N-hydroxyimides 28; 29, and dihydroxytropolones 30; 31 show inhibition of RNase H cleavage in the submicromolar range and share common structural features capable of divalent metal ion chelation. These compounds may therefore interfere with positioning of Mg²⁺ ions at the RNase H active site 32. In contrast, vinylogous ureas show inhibition of RNase H cleavage through a different mechanism of action 33.

Crystallographic data of an RNase H-competent complex of HIV-1 RT are not available, which complicates mechanistic studies on RNase H inhibition. HIV-1 RT has been crystallized with duplex DNA 3, and a PPT-derived DNA/RNA hybrid 4. In each of these structures, the 3'-end of the primer is engaged at the polymerase active site, with most protein-nucleic acid contacts observed between the first 6 base pairs and the fingers, palm and thumb subdomains of the N-terminal polymerase domain of p66. Residues that

constitute the RNase H primer grip contact primarily the primer strand in a region of duplex 11 to 15 nucleotides upstream from the primer 3' terminus 4. The RNase H active site does not contact the scissile bond in any of the crystallized complexes, which is consistent with the expectation that the nucleic acids in these complexes would not be cleaved *in vivo*. However, recent modeling studies in which a polymerase-competent RT-DNA/RNA complex and a truncated RNase H-competent complex of human RNase H1 (containing the active site mutant D210N) are overlaid suggest that the RNA/DNA hybrid cannot simultaneously engage the DNA polymerase and RNase H active sites, regardless of the sequence context 34. The model predicts that positioning an RNA/DNA hybrid for cleavage at the RNase H domain of HIV RT requires that the substrate can shift positions such that the primer 3'-terminus is disengaged from the polymerase active site. As a consequence, ligands that are trapped at the polymerase active site and engage the 3'-end of the primer could indirectly inhibit RNase H cleavage by preventing the conformational change necessary for the DNA/RNA hybrid to contact the RNase H active site.

In the present study, we concurrently examined how engagement of substrate at the polymerase active site affects RNase H activity, and explored the mechanism of action of β -thujaplicinol, a dihydroxytropolone (Figure 1A) recently shown to directly inhibit RT-associated RNase H activity 30. We demonstrate that stabilizing the DNA polymerase active site at the 3'-end of the primer can affect the position of RNase H cleavage in a highly specific manner without inhibiting the activity. These findings are inconsistent with models postulating that the substrate can shift positions or toggles between polymerase and RNase H active sites. Rather, these data show that the bound DNA/RNA hybrid can simultaneously engage both active sites. Primer/template binding restricts access of β -thujaplicinol to its target site that we map between the metal binding sites and Y501 of the RNase H primer grip, suggesting that the nucleic acid substrate can be an obstacle for RNase H active site inhibitors.

Results

Experimental design

In order to study whether the nucleic acid substrate can simultaneously engage both active sites of HIV-1 RT, we utilized RT-primer/template complexes that are stabilized through ligand binding at the DNA polymerase active site. We recently showed that the pyrophosphate-analogue PFA affects the translocation status of HIV-1 RT 35. Translocation of RT relative to its nucleic acid substrate follows a complete cycle of nucleotide incorporation to allow binding of the next complementary dNTP 36; 37. This process frees the nucleotide binding site and moves the 3'-end of the primer into the adjacent priming site. The position of the RNase H active site is shifted in concert by a single nucleotide (Figure 1B). High resolution footprinting studies with HIV-1 RT bound to duplex DNA show that PFA binds to the DNA polymerase active site and traps the pre-translocated complex, while the nucleotide substrate traps the post-translocated complex 38. Both ternary complexes are stabilized, and resist challenge with heparin, relative to the binary RT-primer/template complex. Here we asked (i) whether ligand binding at the DNA polymerase active site can

affect specificity and efficiency of RNase H cleavage, and (ii) what role the bound nucleic acid substrate plays in RNase H inhibition by β -thujaplicinol.

Effects of ligands on polymerase-dependent RNase H cleavage

We devised a DNA/RNA substrate with a DNA primer (22-mer) recessed at both 5'- and 3'-ends on a 52-mer RNA template and monitored primary, polymerase-dependent cleavage events under steady-state conditions. Two major RNase H cuts are evident in the absence of small molecule ligands (Figure 2A, left). The first is located between residues -18 and -19, and the second is between residues -19 and -20 (defined here as positions -18 and -19, respectively). Saturating concentrations of β -thujaplicinol inhibited both cuts (Figure 2A, middle and C). The addition of PFA also showed considerable inhibition of RNase H activity (Figure 2A, right and C). However, in contrast to β -thujaplicinol, the pyrophosphate analogue shifted the cleavage pattern. The cut at position -18 is weaker than the cut at -19, which is reminiscent of our previous footprinting data 35.

We conducted the same experiment under single turnover conditions to exclude potential effects of repeated complex dissociation and association on the cleavage patterns. The RT-DNA/RNA complex was pre-formed and the reaction was initiated with divalent metal ions in the absence and presence of inhibitor. Under these conditions, β -thujaplicinol failed to inhibit RNase H activity (Figure 2B, middle and D). RNase H cleavage in the presence of PFA is likewise not inhibited (Figure 2B, right and D). The intensity of the -19 cleavage product is almost identical to the sum of the two cuts seen in the absence of PFA. However, the cleavage pattern shows again a strong bias towards -19 cleavage, demonstrating that the DNA/RNA hybrid simultaneously contacts the DNA polymerase and RNase H active sites. The data suggest at the same time that the bound nucleic acid can represent an obstacle that limits access of β -thujaplicinol to its binding site.

To fully define the influence of the translocation status of HIV-1 RT on RNase H cleavage, we next monitored activity in the presence of increasing concentrations of PFA and in the presence of the next complementary dNTP, respectively (Figure 3A). The 3'-end of the primer contained a 2'-deoxy-thymidine-monophosphate (dTMP) in the former case, while a 2',3'-dideoxy-thymidine-MP (ddTMP) was used in the latter case to prevent nucleotide incorporation. Increasing concentrations of PFA biased cleavage to position -19, while increasing concentrations of the dNTP biased cleavage towards position -18. However, in spite of these alterations in cleavage patterns, binding of PFA or the nucleotide substrate, and in turn, formation of stable ternary complexes, does not diminish the efficiency of the RNase H activity under single turnover conditions (Figure 3B). We next studied whether β -thujaplicinol retains its ability to inhibit RNase H cleavage under steady-state conditions in the presence of PFA and the incoming dNTP, (Figure 3C). The two ligands diminish RNase H cleavage under these conditions; however, the addition of β -thujaplicinol does not cause further reductions in RNase H activity, which is consistent with the notion that stable RT-DNA/RNA complexes restrict access of the dihydroxytropone to its binding site.

Binding of β -thujaplicinol requires divalent metal ions

We performed order-of-addition experiments to analyze whether β -thujaplicinol can bind to the free enzyme. The following conditions were used: (i) pre-incubation of RT with inhibitor and Mg^{2+} , and initiating with primer/template, (ii) pre-incubation of RT with inhibitor only, and initiating the reaction with primer/template and Mg^{2+} , and (iii) pre-incubation with RT, primer/template, and β -thujaplicinol, and initiating the reaction with Mg^{2+} alone (Figure 4). Reactions were monitored under pre-steady-state conditions, prior to establishing equilibrium with all components. Differences in the order of addition do not significantly influence the rate of the reaction in the absence of inhibitor (Figure 4A). In keeping with the aforementioned data, β -thujaplicinol does not inhibit the reaction when the RT-DNA/RNA complex is pre-formed and the compound is added at the start of the reaction with Mg^{2+} (Figure 4B). Inhibition is also negligible when RT is pre-incubated with the compound in the absence of divalent metal ions. However, inhibition is seen when the RT-inhibitor complex is formed in the presence of Mg^{2+} . Thus, β -thujaplicinol appears to bind solely to the free enzyme, and binding is metal ion dependent.

The rate constants for the three reactions ($k_{RNase\ H}$) are $0.11\ s^{-1}$ with a pre-formed RT-inhibitor- Mg^{2+} complex, compared to $0.35\ s^{-1}$ when the enzyme-inhibitor complex was pre-incubated in the absence of metal ions, and $0.38\ s^{-1}$ with a pre-formed enzyme-primer/template complex. The maximum product generated after 20 seconds remains unchanged under these conditions, suggesting that the components of the reaction equilibrate at longer reaction times. Thus, there is a modest ~3-fold inhibitory effect before the primer/template substrate can bind to the enzyme. The template is eventually cleaved, once the RT-DNA/RNA complex is formed, which in turn, suggests that the inhibitor is released from the complex within a single turnover. Measurements of the equilibrium dissociation constant (K_d) for the nucleic acid substrate revealed values of 3 nM and 15 nM, respectively, depending on whether nucleotide incorporation or RNase H cleavage was used as readout. Thus, although binding of RT to its DNA/RNA substrate is locally weakened at the RNase H domain, the inhibitor appears unable to compete with the intact nucleic acid substrate.

β -thujaplicinol inhibits predominantly secondary RNase H cleavage

The dependence on divalent metal ions for RNase H inhibition points to two possible binding sites for β -thujaplicinol: binding to the RNase H active site, which provides a mechanism for direct inhibition of RNase H cleavage, or alternatively, binding to the polymerase active site. To distinguish between the two possible scenarios, we used a chimeric DNA/DNA-RNA substrate that is cleaved in polymerase-independent fashion in the vicinity of the DNA-RNA junction (Figure 5A). This substrate mimics the tRNA-primer removal reaction. In agreement with previous data, the primary cut is seen a single residue upstream of the RNA-DNA junction, and ensuing secondary cuts follow with time (Figure 5B, left) 19; 39; 40. β -thujaplicinol inhibits ensuing secondary cleavages to a much greater degree than the primary cut (Figure 5B, middle). The ensuing secondary cleavages showed 50% inhibitory concentrations (IC_{50}) of approximately 150 nM, while the primary cleavage displayed an IC_{50} of $> 25\ \mu M$. The low value of 150 nM for the secondary cuts is in keeping with the value of 210 nM previously reported, based on a FRET-assay that does not distinguish between primary and ensuing secondary cuts 30; 41. Due to its short length, the

substrate is never in contact with the polymerase active site when RNase H cleavage takes place. The presence of PFA does not inhibit RNase H cleavage (Figure 5B, right), supporting previous findings that β -thujaplicinol appears to target divalent metal ions at the RNase H active site.

Benzoyl-L-Phe at position 501 confers resistance to β -thujaplicinol inhibition

Previous modelling studies have suggested that binding of N-acylhydrazone inhibitors of HIV RNase H requires the presence of divalent metal ions, as well as stacking interactions between the aromatic ring of a prototype inhibitor and the highly conserved Y501 of the RNase H primer grip 24. To study whether this residue likewise facilitates binding of β -thujaplicinol via its ring system, we constructed mutant enzymes with natural and unnatural amino acid substitutions at this position (Figure 6). All IC_{50} values reported in Figure 6 were obtained using a FRET-based RNase H assay, as described under Materials and Methods. Retention of RNase H activity despite replacement of a conserved RNase H primer grip residue allowed us to determine IC_{50} values in the context of the various mutant enzymes. Replacing Y501 with either W or F only marginally raised the IC_{50} , (470 and 356 nM, respectively), when compared with wild type RT (308 nM). This data demonstrates that the hydroxyl function of Y501 is not critical for inhibitor binding. However, the Y501W mutant showed diminished RNase H activity per se when compared with wild type RT and Y501F, respectively, which is consistent with the aforementioned study. Of the two non-natural amino acid substitutions, introducing an azido function (p66^{AzF}/p51 RT) also had little to no effect on β -thujaplicinol sensitivity. In contrast, inserting a benzophenone into the RNase H primer grip (p66^{501BpF}/p51 RT) created an enzyme that was resistant to β -thujaplicinol at inhibitor concentrations as high as 300 μ M, i.e. three orders of magnitude greater than the IC_{50} of the wild type enzyme. This substantial alteration in sensitivity to β -thujaplicinol suggests that the benzophenone moiety of p66^{BpF}/p51 RT restricts access of the inhibitor to its binding site, while the activity of this enzyme was comparable with the Y501W mutant that was fully sensitive to the inhibitor.

Modeling the binding site for β -thujaplicinol

The experimental observations could be reconciled in a model for binding of β -thujaplicinol, and mechanisms of inhibition and resistance (Figure 7). Inhibitor binding to wild type RT may be mediated by coordination of a catalytic Mg^{2+} ion by carbonyl and hydroxyl oxygens of β -thujaplicinol (Figure 7A). In addition, π -stacking between the central ring of the inhibitor and the side chain of Y501 may provide a second critical element in inhibitor binding (Figure 7B). The latter interaction requires rotation of the Y501 side chain around the C α -C β and C β -C χ bonds from the position observed in RT apoenzyme and co-crystal structures, as suggested for the N-acylhydrazone 24. Positioning of the inhibitor in this manner would be expected to be sterically incompatible with substrate binding, with the principal clash occurring between an exocyclic hydroxyl group of β -thujaplicinol and C4' of the nucleotide immediately 5' to the scissile phosphate.

The structural basis for Y501BpF resistance to inhibition by β -thujaplicinol may be that the inhibitor is unable to recruit benzophenone to a position amenable for stacking (Figure 7B and 7C). The increased length of the unnatural side chain may allow for accommodation of

the distal ring of benzophenone into a hydrophobic pocket flanked by L479 and the peptide backbone of K476. In addition, the ϵ -amino group of the latter may participate in hydrogen bonding with the benzophenone carbonyl moiety. Taken together, these interactions may prevent rotation of the side chain altogether, rendering it unavailable for stacking with β -thujaplicinol, and as a consequence, rendering the inhibitor incapable of stably binding RT. It is unlikely that benzophenone substitution at position 501 would itself preclude substrate binding, given the predicted trajectory of the unnatural side chain.

Discussion

Binding of DNA/RNA substrate by HIV-1 RT

Here we studied the coordination of interaction at the polymerase and RNase H active sites of HIV-1 RT with its nucleic acid substrate. Two conflicting models have been proposed for the HIV-1 RT-DNA/RNA complex. The first model suggests that both the polymerase active site and the RNase H active site can simultaneously engage the substrate. This model is supported by several biochemical studies that show RNase H cleavage at a fixed distance of 18 base pairs upstream of the 3'-end of the primer 7; 8; 9; 10; 11; 12. These findings imply that the polymerase active site can interact with the primer terminus at the same time when the template is cleaved. The second proposal suggests that the substrate cannot simultaneously interact with both active sites, based on modeling studies designed to reconcile the structure of the RT complex with a PPT-derived DNA/RNA hybrid and the structure of human RNase H1-substrate complex 34. The former provides the specific interaction at the polymerase active site, while the latter provides the specific interaction at the RNase H active site. With these constraints, it appears impossible to connect the RNA strands, which led the authors to propose that the nucleic acid substrate toggles between the two active sites. Such differences in substrate binding described by the two models, will impact on the binding properties and mechanisms of action of small molecule RNase H inhibitors. One would predict that substrate toggling increases access of small molecules to the RNase H active site, while substrate binding at both active sites restricts inhibitor accessibility. In this study, we demonstrate that a regular DNA/RNA hybrid occupies both active sites at the same time, which can be an obstacle in the development of RNase H active site inhibitors.

Previous studies under steady-state conditions have shown that binding of the PPI-analogue PFA or the dNTP substrate can diminish RNase H cleavage 8; 23. At first glance, these findings are consistent with the model suggesting that the DNA/RNA substrate that is specifically poised at the polymerase active site cannot simultaneously interact with the RNase H active site. However, we show here that RNase H activity is not inhibited when the experiments were conducted under single turnover conditions. Ligand binding is unambiguously shown through differences in the translocation status of RT. The presence of PFA yields a single RNase H cut at position -19 of the template, while the presence of the incoming dNTP yields a single cut further downstream at -18. The absence of ligands shows an even distribution of the two cleavage events, which points to the existence of two isoenergetic conformations in this particular sequence context. Consistent with a Brownian ratchet model for polymerase translocation 35; 42, we suggest that the two complexes exist

in a dynamic equilibrium, and represent pre- and post-translocational states. Nucleotide hydrolysis is not required for translocation and as such the enzyme can freely oscillate between the two positions. Interconversion of the two conformations in the absence of ligands is kinetically invisible 6. Ligands such as PFA or the nucleotide decrease the forward motion or the reverse, respectively. Thus, PFA traps the pre-translocated complex, which provides a mechanism of inhibition of DNA synthesis, while the nucleotide traps the post-translocated complex, which provides a mechanism for RT translocation associated with productive DNA synthesis 35; 36; 38; 43; 44. Thus, a single RNase H cut is indicative for specific interactions with the ligand, the 3'-end of the primer, and the DNA polymerase active site. Our observation that the efficiency of RNase H cleavage is not reduced when compared to the unliganded RT-DNA/RNA complex provides strong evidence for a model that allows simultaneous substrate binding to the DNA polymerase and RNase H active sites.

Coordination of polymerase and RNase H activities

Reduction in RNase H activity under steady-state conditions in the presence of dNTP substrate or PFA can be explained by the increased stability of ternary complexes 35; 44. The increased stability of the complex with one or the other ligand diminishes the turnover of the reaction, and, consequently, overall efficiency of RNase H cleavage. However, our experiments under single turnover conditions show that the RNase H activity *per se* is not affected in the presence of PFA or dNTP.

The ability of the DNA polymerase and RNase H active sites to interact simultaneously with the DNA/RNA substrate does not imply that both activities are temporally coordinated. RNase H cleavage was shown to be slower than DNA synthesis 6. Here, we show that the affinity around the two active sites is likewise not identical. Equilibrium dissociation constants (K_d) were measured at both active sites, and values obtained at the RNase H active site are approximately 5-fold higher compared to measurements at the polymerase active site. This is consistent with crystallographic data showing that most interactions with the nucleic acid substrate are mediated through the polymerase domain 3; 4; 5. In light of the collective data discussed above, we propose a model that allows partial dissociation and re-association or “breathing” of enzyme-nucleic acid interaction around the RNase H active site, while contacts around the polymerase active site are retained. This model remains consistent with the observation that RNase H cleaves its substrate predominantly during RT pausing events 45. However, our data are inconsistent with a model that postulates mutually exclusive binding at each active site.

Consequences for RNase H inhibition

While different classes of DNA polymerase active site inhibitors, including nucleotide analogues, pyrophosphate-analogues, and nucleotide competing RT inhibitors require the primer/template for binding 46, our data show that the nucleic acid substrate can be an obstacle for RNase H active site inhibitors. Order-of-addition experiments suggest that β -thujaplicinol binds in close proximity to the RNase H active site of the free enzyme, while the nucleic acid substrate restricts access to the inhibitor binding site. Conversely, inhibition of ensuing secondary RNase H cuts is much more efficient than inhibition of primary RNase

H cuts, which points to increased access following the first cut. The increased flexibility of the cleaved substrate may facilitate binding of the inhibitor. The various product complexes are structurally distinct from the original enzyme-substrate complex, which translates into non-competitive inhibition under steady-state conditions, as previously published 30. However, our data show that under pre-steady state conditions, binding of β -thujaplicinol and the intact DNA/RNA substrate is mutually exclusive and therefore competitive. We have observed a similar kinetic behavior with PFA, where binding of the inhibitor to the pre-translocated RT-substrate complex prevents the enzyme from shifting to the post-translocational state 35. Thus, PFA and the nucleotide substrate interact with the active site of conformationally distinct complexes. As with β -thujaplicinol, competitive inhibition is solely observed under single turnover conditions. Steady-state kinetics point to a non-competitive mode of inhibition, because PFA stabilizes the product complex in the pre-translocational state that prevents nucleotide binding. The nucleotide can only bind to the distinct post-translocated complex following enzyme dissociation 35.

Most importantly, our data show that the nucleic acid substrate engages the RNase H active site at the same time when the nucleotide substrate is bound at the polymerase active site and stabilizes the complex. These findings suggest that active DNA synthesis can represent an obstacle to RNase H inhibition. The model shown in Figure 7 is consistent with our data and provides a possible structural explanation for the competition with the nucleic acid substrate. The proposed conformational change of the side chain of Y501 appears to lock the inhibitor in a position that provokes a steric conflict with the template. In contrast, locking the side-chain in its natural orientation provides a plausible mechanism for resistance to β -thujaplicinol.

Implications for drug discovery and development efforts

Although high throughput screening of chemical libraries has led to the discovery of several compounds that inhibit the RT-associated RNase H activity, our data make clear that evaluation of RNase H inhibition under steady-state conditions has shortcomings. The design of more stringent secondary screening assays under single-turnover conditions may help to focus on the discovery of compounds that interfere directly with the RNase H activity rather than indirectly through turnover reduction. The use of stable ternary complexes with the bound nucleotide substrate or PFA provides simple versions of such an assay, and may facilitate screening for compounds capable of blocking RNase H activity during DNA synthesis. It remains to be seen whether the proposed steric clash with the intact nucleic acid substrate can be prevented with different classes of RNase H inhibitors, e.g. compounds that may not interact with Y501 of the RNase H primer grip as inferred for β -thujaplicinol and N-acylhydrazones 24. At the same time, this work raises the question of whether potent inhibition of HIV RNase H in cell culture or *in vivo* requires blockage of primary/internal RNase H cuts.

Materials and Methods

Expression and Purification of HIV-1 RT Variants

Heterodimeric p66p51 HIV-1 RT was expressed and purified as described 47. The following protocol was used to introduce natural and unnatural amino acids in p66. The coding region of RT p66 was cloned into the vector pRSET to introduce a C-terminal H₆-affinity tag. The coding region of p51 RT was incorporated into the bacterial expression vector pPR-IBA2, introducing a Strep-tag at the N-terminus to facilitate purification. For incorporation of non-natural amino acids, the codon for Y501 was mutated to TAG, generating plasmid pRSET-p66His-501Stop. pRSET-p66His-501Stop was co-transformed with either pSup-BpaRS-6TRN or pSup-pAzPheRS-6TRN into bacterial strain BL21(DE3) 48. A single colony was used to inoculate in the presence of 50 µg ml⁻¹ ampicillin, 50 µg ml⁻¹ chloramphenicol and 1 mM of the unnatural amino acid. Mutations Y501W and Y501F were introduced using the QuikChange site-directed mutagenesis kit. Protein expression and purification was conducted essentially as described 49.

Nucleic Acids

Oligodeoxynucleotides used in this study were chemically synthesized and purchased from Invitrogen. The following sequences were used: PBS-22Dpol, 5' AGGTCCTGTTCGGGCGCCACT 3'; PBS-21Dpol 5' AGGTCCTGTTCGGGCGCCAC 3'. PBS-22Dchi, 5' CTAGCAGTGGCGCCCGAACAGG 3'. Both PBS-22Dpol and PBS-21Dpol are referred to as polymerase-dependent substrates, unless otherwise indicated. PBS-52R oligoribonucleotide was synthesized by *in vitro* transcription using T7 polymerase, 5' GGAAAUUCUCUAGCAGUGGCGCCCGAA CAGGGACCUGAAAGCGAAAGGGAAAC 3'. The chimeric RNA-DNA oligonucleotide, PBS-14R8D, 5' cuguucggcgccaCTGCTAGA 3' was purchased from Trilink. Nucleotides, deoxynucleotides and dideoxynucleotides were purchased at Fermentas Life Sciences, phosphonoformic acid was purchased from Sigma.

Time-Course of RNase H Activity

3'-radiolabeled RNA template (or RNA/DNA chimera) was heat annealed to a 2-fold excess of DNA primer. The RNA/DNA hybrid was added to the reaction at a final concentration of 150 nM containing 25 nM RT in a buffer of 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 100 µM EDTA and 6 mM MgCl₂, in the absence or presence of β-thujaplicinol (50 µM) or PFA (200 µM) unless otherwise indicated. Reactions in the absence of β-thujaplicinol contain 0.1% dimethyl sulfoxide (DMSO). The reaction is allowed to proceed at 37°C and stopped by the addition of 100% formamide containing 0.01% (w/v) xylene cyanol and bromophenol blue. RNA fragments were resolved on a 12% denaturing polyacrylamide gel and visualized by phosphorimaging.

Pre-Steady-State Single Turnover Reactions

Experiments conducted in pre-steady-state used a Kin-Tek RQF-3 rapid quench-flow apparatus (www.kintek-corp.com). RNA/DNA hybrids were prepared as described above to a final concentration of 50 nM with 500 nM RT in a buffer containing 50 mM Tris-HCl, pH

7.8, 50 mM NaCl, 200 μ M EDTA and 6 mM $MgCl_2$, in the presence or absence of β -thujaplicinol or PFA. Reactions in the absence of β -thujaplicinol contain 0.1% DMSO. Single-turnover conditions were provided by a 10-fold excess of RT over primer/template, and confirmed by non-linear regression analysis and the addition of heparin at 4 mg/mL when possible. All reactions are terminated by addition of 0.5M EDTA, and 100 μ L of 100% formamide. Curves were fitted to a single-phase exponential association equation [$Y=Y_{max}*(1-\exp(-K*X))$] using the program GraphPad Prism 4.0. The rate constant $k_{(RNase\ H)}$ was obtained from this function.

Kinetic Analysis

Kinetic parameters are obtained using the program GraphPad Prism 4. $K_{d(RNase\ H)}$ values were obtained by adding variable amounts of heat annealed RNA/DNA hybrid (1.2 μ M to 0.01 μ M) to 1 μ M RT in a buffer containing 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, and 500 μ M EDTA. $MgCl_2$ and heparin were added simultaneously at final concentrations of 6 mM and 4 mg/mL, respectively. $K_{d(Pol)}$ values were obtained similarly, except the next templated nucleotide (dGTP) was added together with $MgCl_2$ and heparin at a final concentration of 25 μ M. The resulting points were plotted and fit to a quadratic equation [$Y=0.5(K+E+X)-(0.25(K+E+X)^2-(E)X)^{0.5}$] using the program GraphPad Prism 4.0 to obtain the equilibrium binding constant K_d .

Single-Turnover Dose-Responses

Reactions were prepared as described above except 300 μ M EDTA was used, and heparin was added at 4 mg/mL. Dideoxythymidine triphosphate (ddTTP) was added at a concentration of 10 μ M, while increasing concentrations of dGTP were added at 0, 0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5 and 10 μ M when ddTTP was added. PFA was added at concentrations of 0, 0.049, 0.098, 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5 μ M.

Ternary Complex Formation

DNA/RNA primer/template hybrids were prepared as described above. β -thujaplicinol was added at 100 μ M and PFA at 50 μ M. dGTP was added at a final concentration of (100 μ M). The DNA primer was PBS-22Dpol except for reactions containing dGTP, in which case the primer was PBS-21Dpol, and the reaction includes ddTTP at a final concentration of 20 μ M. RNA/DNA hybrid was added at a final concentration of 300 nM to 50 nM RT in a buffer containing 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 100 μ M EDTA and 6 mM $MgCl_2$. Reactions were allowed to proceed at 37°C and stopped with excess of 100% formamide containing 0.01% (w/v) xylene cyanol and bromophenol blue. Samples collected after 0.5, 1, 2, 3, 4, 5 and 6 minutes, were fractionated on a 12% polyacrylamide gel and visualized by phosphorimaging.

Order-of-Addition Experiments

Reactions were prepared essentially as described above. Reactions were started with either RNA/DNA hybrid, $MgCl_2$, or RNA/DNA hybrid as well as $MgCl_2$. Heparin trap was not added due to a conflict with the experimental design. Single turnover conditions were ensured by nonlinear regression analysis using the software GraphPad Prism 4.0.

RNase H IC₅₀ determination for primary and secondary cleavages

Reactions were prepared essentially as described above. To determine IC₅₀ values for secondary cleavages, 50 nM RNA-DNA/DNA chimeric hybrid (PBS-14r8d/PBS-22d) labelled at the 5' RNA end was added to 50 nM RT in increasing concentrations of β -thujaplicinol (0, 0.098, and doubling until 100 μ M). Reactions were allowed to proceed at 37° for 8 minutes then stopped with 100% formamide containing 0.01% (w/v) xylene cyanol and bromophenol blue. For primary cleavages, 600 nM of chimeric hybrid (PBS-14r8d/PBS-22d) labelled at the 3' DNA end was added to 50 nM RT in increasing concentrations of β -thujaplicinol (same as above). Reactions were allowed to proceed and were stopped as described above.

FRET-Based RNase H Assay

RNaseH assays based on fluorescence-resonance energy transfer were performed as described 41.

Molecular Modeling

Molecular models were generated using Discovery Studio 7.0 (DS 7.0; Accelrys) using structural coordinates of HIV RT-RNA/DNA (1HYS; 4) and human RNase H1-RNA/DNA (2KQ9; 34) complexes downloaded from the protein data bank. The two complexes were overlaid by superposition of four carboxylate-containing catalytic residues common to the RNase H domains of the two enzymes. The panels of Figure 7 depict some or all of the following components of the overlaid complexes: The HIV-1 RNase H domain (from 1HYS); RNA/DNA (from 2KQ9); and site A and B Mg ions (2KQ9). β -thujaplicinol was constructed using the 'build' features of DS 7.0. Because it possesses structural features suggesting metal ion chelation, the inhibitor was manually docked into the RNase H active site at a position which permits contact with the B-site Mg²⁺ ion and stacking with Y501 (panel [A]) 24. The benzophenone side chain (panel [B]) was constructed by deleting the hydroxyl group of and extending the Y501 side chain. The trajectory of the unnatural side chain reflects original positioning of Y501 (as given in 1HYS), which otherwise has not been altered.

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Figure 1. Structures of Small Molecules used in this Study and Schematic of the Polymerase-Dependent Binding Mode of HIV RT

(A) Structures of inhibitors used in this study: β -thujaplicinol and PFA.

(B) Schematic representation of pre- and post-translocated complexes of HIV-1 RT. The polymerase active center is represented by the green cylinder, which is either occupied by the 3' end of the primer (pre-translocation) or available for nucleotide binding (post-translocation). RT is shown here shifted relative to its nucleic acid substrate by a single nucleotide. This difference ($\Delta 1nt$) is also reflected at the RNase H active site (green arrow).

Figure 2. Inhibition of RNase H Activity by β -thujaplicinol and PFA

A) Inhibition of RNase H activity under steady-state conditions. Time-course reaction (0-12 mins) in the absence and presence of β -thujaplicinol (50 μ M) and PFA (200 μ M) under steady-state conditions. RNase H cleavages at positions -18 and -19 are marked post- and pre-translocation, respectively. The figure focuses on this part of the gel.

(B) Time-course experiment (0.05 – 20 sec) in the presence and absence of β -thujaplicinol (50 μ M) and PFA (200 μ M). The protein trap heparin was added to all reactions at 4 mg/mL.

(C) and (D) Results from (A) and (B) represent graphically, respectively. Error bars represent the standard deviation between three independent experimental replicates.

Figure 3. Effects of Ternary Complex Formation on RNase H Inhibition

(A) Left: Increasing dose-response of PFA (0 – 10 μ M) on the polymerase-dependent substrate with a dTMP-terminated primer. Right: Increasing concentrations of the next template nucleotide (dGTP) were added from 0 to 10 μ M to reactions containing a polymerase-dependent substrate terminated with ddTMP.

(B) The data from (A) presented graphically showing both translocational trends and RNase H activity.

(C) Steady-state RNase H activity measured in the linear phase of the reaction in the absence of ligands, and with PFA or dGTP, in the absence and presence of β -thujaplicinol.

Figure 4. Effects of Order-of-Addition on RNase H Inhibition

Time-course assay under pre-steady-state conditions. % Activity refers to the percentage of the total substrate converted to the -18 and -19 major reaction products. (Top) Reactions were pre-incubated with RT, Mg²⁺ and the RNA/DNA substrate in the orders shown in the absence of inhibitor. (Bottom) Same as top but in the presence of 50 μM β-thujaplicinol.

Figure 5. Effects of β -thujaplicinol on Polymerase-Independent RNase H activity

(A) Sequence of the polymerase-independent substrate. A primary RNase H cut is expected at the RNA/DNA junction +1, while ensuing secondary cuts are expected to occur upstream of the primary cut.

(B) Time-course of RNase H activity (0 – 40') on the chimeric substrate PBS-14r8d. β -thujaplicinol was added at a concentration of 50 μ M, while PFA was added at a concentration of 200 μ M. Primary and secondary cuts are indicated.

Amino acid at position 501		IC ₅₀ (nM) ± SD
Y		308 ± 18 nM
F		356 ± 49 nM
W		470 ± 135 nM
Az-F		386 ± 123 nM
Bp-F		> 300 μM

Figure 6. Altering Y501 of the RNase H primer grip affects β-thujaplicinol sensitivity
Structures of natural (F and W) and unnatural amino acid insertions (AzF and BpF) for Y501 are illustrated in addition to the IC₅₀ for the mutant enzyme. IC₅₀ values are the average of triplicate analyses.

Figure 7. Model of β -thujaplicinol binding site

An RT-substrate complex generated by superposition of HIV RT-RNA/DNA and human RNase H-RNA/DNA co-crystal structures 4; 34 is depicted in all panels. (A) Surface representation of β -thujaplicinol (pink, with red oxygen atoms) and the RNA nucleotide 17 bp from the primer 3' terminus (blue). Steric interference between the inhibitor and the RNA is evident at the junction between the two surfaces, as well as in the stick representations near the ribose C4' atom. RNA (dark blue ball and stick), active site Mg ions (red spheres), and active site residues D443, E478, D498, D549 (light blue ball-and-stick) are also highlighted. (B) Y501 rotation from the position observed in published crystal structures (grey) to stack with β -thujaplicinol (rotated Y501, white; β -thujaplicinol, pink). For illustrative purposes, RNA and DNA strands are shown as blue and red tubes, respectively; however, binding of substrate and β -thujaplicinol at the RNase H active site is thought to be mutually exclusive. (C) Same as B, except the complex is rotated to highlight the positioning of Y501 relative to L479, K476, and Q475 (yellow ball-and-stick), and β -thujaplicinol is not shown. (D) Same as C, with benzophenone substituted for the Y501 side chain. Note that while the extended side chain permits potential hydrophobic interactions with L479 and K476 that cannot occur with Y501, it is not likely to directly affect substrate binding.