Molecular Pharmacology Fast Forward. Published on November 16, 2007 as doi:10.1124/mol.107.038596

MOL #38596

Efavirenz Accelerates HIV-1 Reverse Transcriptase Ribonuclease H Cleavage Leading to Diminished Zidovudine Excision

Jessica Radzio and Nicolas Sluis-Cremer

Department of Infectious Disease and Microbiology, Graduate School of Public Health (J.R.) and Division of Infectious Diseases, Department of Medicine (J.R., N.S.-C.), University of Pittsburgh, Pittsburgh, PA, USA, 15261

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

MOL #38596

Running Title: **Inhibition of AZT Excision by Efavirenz**

Corresponding Author: Nicolas Sluis-Cremer

University of Pittsburgh School of Medicine, Division of Infectious Diseases, 817 Scaife Hall, 3550 Terrace Street, Pittsburgh, PA 15261

Phone: 412-648-8457 ; Fax: 412-648-8521; Email: CremerN@dom.pitt.edu

Number of text pages: **20**

Number of tables: **0**

Number of figures: **4**

Number of references: **22**

Number of words in "Abstract": **250**

Number of words in "Introduction": **464**

Number of words in "Results and Discussion": **1186**

Abbreviations: RT, reverse transcriptase; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; RNase H, ribonuclease H; T/P, template/primer; AZT, zidovudine or 3'-azido-3'deoxythymidine; AZT-MP, AZT-5'-monophosphate; AZT-TP, AZT-5'-triphosphate

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Previous biochemical studies have demonstrated that synergy between nonnucleoside reverse transcriptase (RT) inhibitors (NNRTI) and nucleoside RT inhibitors (NRTIs) is due to the NNRTI inhibiting the rate at which HIV-1 RT facilitates ATP-mediated excision of NRTIs from chain-terminated template/primers (T/P). However, these studies did not take into account the possible effects of NNRTI on the ribonuclease H (RNase H) activity of RT, despite recent evidence which suggests an important role for this activity in the NRTI excision phenotype. Accordingly, in this study we compared the ability of efavirenz to inhibit the incorporation and excision of zidovudine (AZT) by HIV-1 RT using DNA/DNA and RNA/DNA T/Ps that were identical in sequence. Whereas IC_{50} values for the inhibition of AZT-triphosphate incorporation by efavirenz were essentially similar for both DNA/DNA and RNA/DNA T/P, a 19-fold difference in IC_{50} was observed between the AZT-monophosphate excision reactions, with the RNA/DNA T/P substrate being significantly more sensitive to inhibition. Analysis of the RNase H cleavage events generated during ATP-mediated excision reactions demonstrated that efavirenz dramatically increased the rate of appearance of a secondary cleavage product that decreased the T/P duplex length to only 10 nucleotides. Studies designed to delineate the relationship between T/P duplex length and efficiency of AZT excision demonstrated that RT could not efficiently unblock chain-terminated T/P if the RNA/DNA duplex length was less than 12 nucleotides. Taken together, these results highlight an important role for RNase H activity in the NRTI excision phenotype and in the mechanism of synergy between NNRTI and NRTI.

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

Reverse transcriptase (RT) facilitates the conversion of the HIV-1 single stranded RNA genome into double stranded DNA. HIV-1 RT is a multifunctional enzyme that exhibits RNA-dependent and DNA-dependent DNA polymerase activity and ribonuclease (RNase) H activity. Because of its crucial role in viral replication, RT is a major target for chemotherapeutic intervention. To date, two distinct groups of RT inhibitors have been approved by the US FDA for the treatment of HIV-1 infection. These include (i) the nucleoside/nucleotide inhibitors (NRTI) that bind to the active site of RT and act as competitive inhibitors of DNA polymerization, and (ii) the nonnucleoside inhibitors (NNRTI) that bind to a non-active site pocket in HIV-1 RT (termed the NNRTI-binding pocket) and act as allosteric inhibitors of DNA polymerization.

The USA Panel of the International AIDS Society recommends combination therapies that comprise an NNRTI or protease inhibitor boosted with low-dose ritonavir, each combined with two NRTIs for the treatment of adult HIV infection (Hammer et al., 2006). In this regard, the efficacy of regimens that include both an NNRTI and NRTIs may be explained, in part, by the observed synergistic interactions between these two classes of drugs. (Richman et al., 1991; Chong et al., 1994; Pauwels et al., 1994; Merrill et al., 1996; Borkow et al., 1999; Maga et al., 2000; King et al., 2002). Biochemical studies designed to address the mechanistic basis of synergy between NRTIs and NNRTIs have proposed a general mechanism in which the NNRTI inhibits the ATP-dependent removal of NRTIs from primer termini, thus prolonging the effect of chain-termination (Borkow et al., 1999; Basavapathruni et al., 2004; Cruchaga et al., 2005). Pre-steady-state kinetic studies further demonstrated that the NNRTI inhibit the ability of RT to unblock chain-terminated template/primers (T/P) by negatively affecting both affinity of ATP for RT and the rate of the chemical step in the excision reaction (Basavapathruni et al., 2004).

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

However, these studies were carried out using DNA/DNA T/P only and, as such, they ignored the potential contribution of the enzyme's RNase H activity in the NRTI excision phenotype.

Recently, Nikolenko *et al* (2005) reported that a balance between nucleotide excision and template RNA degradation plays an important role in NRTI resistance. This group elegantly demonstrated that any mutation (and by inference drug) that affects the enzyme's RNase H activity also affects its ability to excise chain-terminating nucleotides. In this regard, several studies have reported that NNRTIs can modulate the RNase H activity of RT (Gopalakrishnan and Benkovic, 1994; Palaniappan et al., 1995; Shaw-Reid et al., 2005; Hang et al., 2007). In light of these findings, we were interested to compare and contrast the ability of NNRTIs to inhibit the ability of RT to unblock chain-terminated primers using both RNA/DNA and DNA/DNA T/P substrates. The results of these studies are described below.

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Materials. HIV-1 RT was over-expressed as an N-terminal hexa-histidine fusion protein and purified to homogeneity as described previously (Le Grice et al., 1995). Enzyme concentration was determined spectrophotometrically at 280 nm using an extinction co-efficient (ε280) of 260,450 M^{-1} cm^{-1}. Efavirenz ((4S)-6-chloro-4-cyclopropylethynyl-4-trifluoromethyl-1,4-dihydro-benzo[d][1,3]oxazin-2-one) was obtained from the NIH AIDS Research and Reference Reagent Program. Both RNA and DNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). AZT-triphosphate (AZT-TP) was purchased from Sierra Bioresearch (Tuscon, AZ). All other reagents were of the highest quality available and were used without further purification.

Template/primer (T/P) substrates. All assays were carried out using a 26-nucleotide DNA primer (**pr26**: 5'-CCTGTTCGGGCGCCACTGCTAGAGAT-3') annealed to either a 35-nucleotide RNA template (**RNA-T**: 5'-AGAAUGGAAAAUCUCUAGCAGUGGCGCCCGAACAG-3') or to a DNA template that was identical in sequence to the RNA template (**DNA-T**: 5'-AGAATGGAAAATCTCTAGCAGTGGCGCCCGAACAG-3'). The pr26 primer was chain-terminated with AZT-monophosphate (AZT-MP) to generate pr26-AZT, as described previously (Sluis-Cremer et al., 2005; Sluis-Cremer et al., 2007). Depending on the nature of the assay (described below), the 5'-end of the DNA primer or RNA template was radioactively labeled with γ-^{32}P-ATP (GE Healthcare, Piscataway, NJ).

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

Assays for inhibition of AZT-TP incorporation and AZT-MP excision by HIV-1 RT. Inhibition of AZT-TP incorporation by efavirenz was determined using a fixed-time assay. Briefly, 200 nM of HIV-1 RT was pre-incubated with 20 nM T/P (RNA-T/pr26 or DNA-T/pr26) and varying concentrations of efavirenz (0 to 100 nM) in 50 mM Tris-HCl, pH 7.5 and 50 mM KCl at 37ºC for 5 min. Reactions were initiated by the addition of 0.1, 1 or 10 μM AZT-TP and 10 mM $MgCl_2$. Reactions were quenched after a defined time (15 s, 1 min or 15 min) by the addition of an equal volume of sample loading buffer (98% deionized formamide, 10 mM EDTA, and 1 mg/ml each of bromphenol blue and xylene cyanol). Inhibition of AZT-MP excision by efavirenz was determined using both fixed time and time-course assays. Briefly, 200 nM of HIV-1 RT was pre-incubated 20 nM T/P (DNA-T/pr26-AZT or RNA-T/pr26-AZT) in 50 mM Tris-HCl, pH 7.5 and 50 mM KCl and varying concentrations of efavirenz (0 to 500nM) at 37ºC for 5 min. Reactions were initiated by the addition of 3 mM ATP and 10 mM $MgCl_2$. Excision reactions carried out on the DNA-T/pr26-AZT and RNA-T/pr26-AZT were quenched with an equal volume of sample loading buffer after 30, 60 or 90 minutes, respectively. Reaction products were separated using denaturing polyacrylamide gel electrophoresis and analyzed by densitometry using a Bio-Rad GS525 Molecular Imager ® FX (Bio-Rad Laboratories, Hercules, CA). Data analyses were carried-out using SigmaPlot 8.02 and/or SigmaStat 3.00 (SPSS Inc., Chicago, IL). Statistical significance was determined using the two-sample Student's *t* test. The results demonstrated that the IC_{50} values for efavirenz calculated at different AZT-TP concentrations (i.e. 0.1, 1.0 or 10 μM), or time points (15 s, 1 min or 15 min), were similar. Similarly, IC_{50} values calculated for AZT-MP excision from the fixed time assay (from different time points) or time-course assays were also similar. Accordingly, for inhibition of AZT-TP incorporation data is reported from 1 min assays that contained 1.0 μM AZT-TP, and for

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

inhibition of AZT-MP excision data is reported from a fixed time assay that was carried out for 30 min (Figure 1).

RNase H assays. The effect of efavirenz on RNase H activity was evaluated using the RNA-T/pr26-AZT T/P that was used in the ATP-mediated excision assays described above. 200 nM of HIV-1 RT was pre-incubated with 20 nM T/P in 50 mM Tris-HCl, pH 7.5 and 50 mM KCl and varying concentrations of efavirenz (0 and 150 nM) at 37ºC for 5 min. Reactions were initiated by the addition of 3 mM ATP and 10 mM $MgCl_2$. Aliquots were removed and quenched at varying times and analyzed as described above.

Gel mobility shift assays. Gel mobility shift assays were used to evaluate the thermodynamics of RT-T/P interactions. In these assays, the amount of T/P-bound RT present in an equilibrium solution was assayed by native gel electrophoresis. RT (0 to 10μM total) was equilibrated with 100 nM T/P in 50 mM Tris-Cl pH 7.5, 50 mM KCl for 15 min as 37ºC. Gels were run at room temperature for 30 min (100 V constant voltage) and quantified as described above. Discontinuity of sample and gel buffers can cause severe streaking of the bands. To correct for this, the area of the unshifted band was estimated from the lane containing DNA alone, and the area between shifted and unshifted bands was counted as the shifted band. The percent DNA-bound RT was calculated assuming that the amount of DNA in the shifted band represented a 1:1 complex of RT-T/P.

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

Results and Discussion

In this study we compare the ability of the NNRTI efavirenz to inhibit HIV-1 RT catalyzed incorporation and excision of the NRTI zidovudine (AZT) in reactions carried out on DNA/DNA and RNA/DNA T/P that are identical in length and sequence. In this regard, previous detailed biochemical studies designed to delineate the molecular mechanism of synergy between NRTIs and NNRTIs measured inhibition of NRTI-MP excision on DNA/DNA T/P only (Basavapathruni et al., 2004). Other studies (Borkow et al., 1999; Odriozola et al., 2003), evaluated NNRTI-mediated inhibition of NRTI-MP excision on RNA/DNA T/P, but did not provide a direct comparison with results obtained from complementary DNA/DNA T/P. Furthermore, none of these studies considered the possible effects of RNase H activity on the NRTI excision phenotype, despite ample evidence in the literature that this activity was modulated by NNRTI binding to RT (Gopalakrishnan and Benkovic, 1994; Palaniappan et al., 1995; Temiz and Bahar, 2002; Shaw-Reid et al., 2005; Hang et al., 2007).

To determine whether efavirenz differentially inhibited the incorporation and/or excision of AZT by HIV-1 RT on RNA/DNA and DNA/DNA T/Ps, IC_{50} values for these reactions were determined (Figure 1). In all experiments described, the same DNA primer (pr26 or pr26-AZT) was annealed to DNA or RNA templates (RNA-T or DNA-T) that were identical in length and sequence (Figure 1A). The IC_{50} values for efavirenz inhibition of AZT-TP incorporation by HIV-1 RT were calculated to be 19.6 ± 8.5 nM and 10.2 ± 4.0 nM for the DNA/DNA and RNA/DNA T/P, respectively (Figure 1C). The small difference (~1.9-fold) between these values was found to be statistically not significant. By contrast, the IC_{50} values for efavirenz inhibition of AZT-

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

MP excision by HIV-1 RT were calculated to be 108.1 ± 32.3 nM and 5.8 ± 1.1 nM for the DNA/DNA and RNA/DNA T/P, respectively (Figure 1B, C). The large (~19-fold) difference between these values is statistically significant ($p < 0.005$). This large difference in IC_{50} value can not be explained by pre-existing large differences in the rates of AZT-MP excision from RNA/DNA or DNA/DNA T/Ps in the absence of drug (the apparent rates of AZT-MP excision were calculated to be 0.067 ± 0.005 min^{-1} and 0.045 ± 0.002 min^{-1} for DNA/DNA and RNA/DNA T/P, respectively) or by differences in apparent affinity of RT for the RNA/DNA and DNA/DNA T/P (Figure 1D, 4A). Studies from the Anderson lab (Spence et al., 1995; Basavapathruni et al., 2004) and ours (Xia et al., 2007) have demonstrated communication between the NNRTI-binding pocket and the DNA polymerase active site in HIV-1 RT, which accounts for the inhibition of RT catalyzed nucleotide incorporation and excision reactions on a DNA/DNA T/P via a remote effect on the chemical step of these reactions. However, the data in Figure 1 suggest that additional parameters may also contribute toward the ability of efavirenz to inhibit NRTI-MP excision on RNA/DNA T/P.

Recent reports have suggested that the NRTI excision phenotype might also be influenced by the RNase H activity of RT (Nikolenko et al., 2005). In this regard, several studies have demonstrated that NNRTIs modulate the enzyme's RNase H activity. For example, both Shaw-Reid *et al* (2005) and Hang *et al* (2007) showed that this class of drugs can accelerate the enzyme's 3'-end directed (or DNA polymerase directed) RNase H activity. Accordingly, we were interested to assess the RNase H cleavage events that occurred during the AZT-MP excision reaction, and to delineate whether these affected the efficiency of the excision reaction. Figure 2 shows that efavirenz accelerates HIV-1 RT RNase H cleavage, data which is consistent

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

with the Shaw-Reid *et al* (2005) and Hang *et al* (2007) studies. Specifically, efavirenz increased the rate of appearance of a secondary RNase H cleavage event that reduced the RNA/DNA duplex length to 10 nucleotides (Figure 2A). Interestingly, we also find an inverse relationship between the efficiency of AZT-MP excision and the appearance of this secondary RNase H cleavage event in time-course assays carried out in the absence and presence of efavirenz (Figure 2B, C). Shaw-Reid *et al* (2005) further suggested that the NNRTI (±)4-(1-chloro-1,1-difluoromethyl)-4-(2-phenylethynyl)-6-chloro-2*H*-3,1-benzoxazin-2-one, an analogue of efavirenz, in addition to accelerating the rate of RT RNase H activity also altered the specific RNase H cleavage pattern. However, this analysis was based on data from a single time point and our data clearly show that the overall RNase H cleavage pattern of RT, including the primary and secondary cuts, was not affected by efavirenz (Figure 2A).

To define the relationship between the efficiency of NRTI-MP excision and RNase H activity, we next evaluated the ability of HIV-1 RT to excise AZT-MP from a chain-terminated DNA primer that was annealed to different RNA templates that were recessed from the 3'-end, therefore incrementally decreasing the RNA/DNA duplex length (Figure 3). These data show that the efficiency of AZT-MP excision (and AZT-TP incorporation) was severely compromised when the RNA/DNA duplex length was decreased to 12 nucleotides or less (Figure 3). If the RNA/DNA duplex was reduced to 10 nucleotides – a duplex length consistent with the secondary RNase H cleavage event described in Figure 2 – RT was essentially unable to carry-out ATP-mediated AZT-MP excision. Gel mobility shift assays demonstrate that RT exhibits a decrease in affinity for the RNA/DNA T/P each time the duplex length is decreased (Figure 4). This decrease in RT-T/P affinity provides a plausible explanation for the observed decrease in

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

the efficiency of AZT-MP excision (Figure 3). Taken together, these results provide convincing evidence that the sensitivity of the AZT-MP excision reaction on RNA/DNA T/P to efavirenz may be explained by the drug-induced accelerated RNase H activity of the enzyme in addition to effects on the chemistry step of AZT-MP excision reaction. It should be noted that our data also show that AZT-TP incorporation is affected by decreasing the RNA/DNA duplex length (Figure 3). However, the rate of AZT-TP incorporation is significantly faster than the rate of AZT-MP excision, (8.78 s^{-1} versus 0.54 x 10^{-3} s^{-1}; Sluis-Cremer, et al., 2005) and therefore we would not expect the observed increase in the secondary cleavage event that accumulates in a minute time-scale (see Figure 2) to adversely affect the IC_{50} for incorporation of AZT-TP.

Nikolenko *et al* (2005) recently proposed that an equilibrium exists between: (i) NRTI incorporation, NRTI excision and resumption of DNA synthesis; and (ii) degradation of the RNA template by RNase H activity that leads to dissociation of the template-primer and abrogation of HIV-1 replication. In this regard, the authors elegantly showed that mutations in the RNase H domain of RT that reduce RT's RNase H activity confer AZT resistance. Our study lends biochemical support to this model and clearly demonstrates that the efficiency of ATP-mediated excision reactions on RNA/DNA templates can be influenced by the enzyme's RNase H activity. However, the data in Figure 3 show that it is not a decrease in the absolute rate of RNase H activity that will contribute to increased NRTI-MP excision, but a decrease in the rate or appearance of secondary cleavage events that generate RNA/DNA T/P with duplexes less than 13 nucleotides.

References

Basavapathruni A, Bailey CM and Anderson KS (2004) Defining a molecular mechanism of synergy between nucleoside and nonnucleoside AIDS drugs. *J Biol Chem* **279**:6221-6224.

Borkow G, Arion D, Wainberg MA and Parniak MA (1999) The thiocarboxanilide nonnucleoside inhibitor UC781 restores antiviral activity of 3'-azido-3'-deoxythymidine (AZT) against AZT-resistant human immunodeficiency virus type 1. *Antimicrob Agents Chemother* **43**:259-263.

Chong KT, Pagano PJ and Hinshaw RR (1994) Bisheteroarylpiperazine reverse transcriptase inhibitor in combination with 3'-azido-3'-deoxythymidine or 2',3'-dideoxycytidine synergistically inhibits human immunodeficiency virus type 1 replication in vitro. *Antimicrob Agents Chemother* **38**:288-293.

Cruchaga C, Odriozola L, Andreola M, Tarrago-Litvak L and Martinez-Irujo JJ (2005) Inhibition of phosphorolysis catalyzed by HIV-1 reverse transcriptase is responsible for the synergy found in combinations of 3'-azido-3'-deoxythymidine with nonnucleoside inhibitors. *Biochemistry* **44**:3535-3546.

Gopalakrishnan V and Benkovic S (1994) Effect of a thiobenzimidazolone derivative on DNA strand transfer catalyzed by HIV-1 reverse transcriptase. *J Biol Chem* **269**:4110-4115.

Hammer SM, Saag MS, Schechter M, Montaner JS, Schooley RT, Jacobsen DM, Thompson MA, Carpenter CC, Fischl MA, Gazzard BG, Gatell JM, Hirsch MS, Katzenstein DA, Richman DD, Vella S, Yeni PG and Volberding PA (2006) Treatment for adult HIV infection: 2006 recommendations of the International AIDS Society-USA panel. *Jama* **296**:827-843.

Hang JQ, Li Y, Yang Y, Cammack N, Mirzadegan T and Klumpp K (2007) Substrate-dependent inhibition or stimulation of HIV RNase H activity by non-nucleoside reverse transcriptase inhibitors (NNRTIs). *Biochem Biophys Res Commun* **352**:341-350.

King RW, Klabe RM, Reid CD and Erickson-Viitanen SK (2002) Potency of nonnucleoside reverse transcriptase inhibitors (NNRTIs) used in combination with other human immunodeficiency virus NNRTIs, NRTIs, or protease inhibitors. *Antimicrob Agents Chemother* **46**:1640-1646.

Le Grice SF, Cameron CE and Benkovic SJ (1995) Purification and characterization of human immunodeficiency virus type 1 reverse transcriptase. *Methods Enzymol* **262**:130-144.

Maga G, Ubiali D, Salvetti R, Pregnolato M and Spadari S (2000) Selective interaction of the human immunodeficiency virus type 1 reverse transcriptase nonnucleoside inhibitor efavirenz and its thio-substituted analog with different enzyme-substrate complexes. *Antimicrob Agents Chemother* **44**:1186-1194.

Merrill DP, Moonis M, Chou TC and Hirsch MS (1996) Lamivudine or stavudine in two- and three-drug combinations against human immunodeficiency virus type 1 replication in vitro. *J Infect Dis* **173**:355-364.

Nikolenko GN, Palmer S, Maldarelli F, Mellors JW, Coffin JM and Pathak VK (2005) Mechanism for nucleoside analog-mediated abrogation of HIV-1 replication: balance between RNase H activity and nucleotide excision. *Proc Natl Acad Sci U S A* **102**:2093-2098.

Odriozola L, Cruchaga C, Andreola M, Dolle V, Nguyen CH, Tarrago-Litvak L, Perez-Mediavilla A and Martinez-Irujo JJ (2003) Non-nucleoside inhibitors of HIV-1 reverse

transcriptase inhibit phosphorolysis and resensitize the 3'-azido-3'-deoxythymidine (AZT)-resistant polymerase to AZT-5'-triphosphate. *J Biol Chem* **278**:42710-42716.

Palaniappan C, Fay PJ and Bambara RA (1995) Nevirapine alters the cleavage specificity of ribonuclease H of human immunodeficiency virus 1 reverse transcriptase. *J Biol Chem* **270**:4861-4869.

Pauwels R, Andries K, Debyser Z, Kukla MJ, Schols D, Breslin HJ, Woestenborghs R, Desmyter J, Janssen MA, De Clercq E and et al. (1994) New tetrahydroimidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-one and -thione derivatives are potent inhibitors of human immunodeficiency virus type 1 replication and are synergistic with 2',3'-dideoxynucleoside analogs. *Antimicrob Agents Chemother* **38**:2863-2870.

Richman D, Rosenthal AS, Skoog M, Eckner RJ, Chou TC, Sabo JP and Merluzzi VJ (1991) BI-RG-587 is active against zidovudine-resistant human immunodeficiency virus type 1 and synergistic with zidovudine. *Antimicrob Agents Chemother* **35**:305-308.

Shaw-Reid CA, Feuston B, Munshi V, Getty K, Krueger J, Hazuda DJ, Parniak MA, Miller MD and Lewis D (2005) Dissecting the effects of DNA polymerase and ribonuclease H inhibitor combinations on HIV-1 reverse-transcriptase activities. *Biochemistry* **44**:1595-1606.

Sluis-Cremer N, Arion D, Parikh U, Koontz D, Schinazi RF, Mellors JW and Parniak MA (2005) The 3'-azido group is not the primary determinant of 3'-azido-3'-deoxythymidine (AZT) responsible for the excision phenotype of AZT-resistant HIV-1. *J Biol Chem* **280**:29047-52.

Sluis-Cremer N, Sheen CW, Zelina S, Torres PS, Parikh UM and Mellors JW (2007) Molecular mechanism by which the K70E mutation in human immunodeficiency virus type 1

reverse transcriptase confers resistance to nucleoside reverse transcriptase inhibitors. *Antimicrob Agents Chemother* **51**:48-53.

Spence RA, Kati WM, Anderson KS and Johnson KA (1995) Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. *Science* **267**:988-993.

Temiz NA and Bahar I (2002) Inhibitor binding alters the directions of domain motions in HIV-1 reverse transcriptase. *Proteins* **49**:61-70.

Xia Q, Radzio J, Anderson KS and Sluis-Cremer N (2007) Probing nonnucleoside inhibitor induced active site distortion in HIV-1 reverse transcriptase by transient kinetic analyses. *Protein Sci*. **16**:1728-1737.

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes:

This study was supported by the NIH (National Institutes of Health) grant R01 GM068406-01.

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends:

Figure 1. Inhibition of RT mediated AZT-TP incorporation and AZT-MP excision on RNA/DNA and DNA/DNA T/P by efavirenz.

A) Sequences of RNA and DNA templates and DNA primer used in all experiments. In the ATP-mediated excision assays, the pr26 primer was chain terminated with AZT-MP (designated as **Z**).

B) Representative gel analysis of AZT-MP excision reaction carried out on RNA/DNA or DNA/DNA T/P in the absence and presence of varying concentrations of efavirenz (0 to 300 nM).

C) Graph of inhibition of AZT-MP excision by efavirenz on RNA/DNA T/P (o) and DNA/DNA T/P (•) from data in B. IC_{50} values determined from this isotherm were 11.9 nM and 100.7 nM for the RNA/DNA and DNA/DNA T/P, respectively.

D) HIV-1 RT AZT-MP excision isotherms from RNA/DNA T/P (o) and DNA/DNA T/P (•) in the absence of inhibitor. The apparent rates of AZT-MP excision were calculated to be 0.067 ± 0.005 min^{-1} and 0.045 ± 0.002 min^{-1} for DNA/DNA and RNA/DNA T/P, respectively. The burst amplitudes (total amount of product excised at an infinite time point) were 84 % and 45 % for the DNA/DNA and RNA/DNA T/P, respectively.

E) IC_{50} values for the inhibition of AZT-TP incorporation and AZT-MP excision by efavirenz under single turnover conditions. The concentrations of efavirenz used in the incorporation assays were 5, 10, 20, 30, 50, 100, 150, 250, 500 nM. The concentrations of efavirenz used in the excision assays were 2, 5, 10, 30, 50 and 100 nM and 25, 50, 100, 150, 200 and 500 nM for the RNA/DNA and DNA/DNA T/P, respectively. The calculated IC_{50} values for incorporation of AZT-TP were 19.6 ± 8.5 nM and 10.2 ± 4.0nM for the DNA/DNA and RNA/DNA T/P,

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

respectively. The calculated IC_{50}s for ATP-mediated excision of AZT-MP were 108.1 ± 32.3 nM and 5.8 ± 1.1 nM for the same two substrates respectively. The difference between these two values was found to be statistically significant (p-value < 0.005).

Figure 2. Efavirenz accelerates HIV-1 RT RNase H activity.

(A) Autoradiogram of RNase H products generated during ATP-mediated excision assays in the absence and presence of efavirenz. Experiments were carried out as described in Materials and Methods; the 5'-end of the RNA template is radioactively labeled in these experiments. The primary RNase H cleavage events occur at 17 or 18 nucleotides downstream from the polymerase, depending on the location of the AZT-MP moiety in the polymerase active site (17 nucleotides if in nucleotide (N)-site; 18 nucleotides if in priming (P)-site). These cleavages generate RNA/DNA duplex lengths of 19 and 18 nucleotides, respectively.

(B) Isotherm for the rate of appearance of the 19 nucleotide secondary RNase H cleavage product generated in the absence (●) or presence (○) of 150nM efavirenz. The intensity of the 19 nucleotide product was determined by densiometric analyses using Bio-Rad GS525 Molecular Imager ® FX software, and is reported as arbitrary units.

(C) Isotherm for the rate of ATP-mediated AZT-MP excision carried-out by HIV-1 RT in the absence (●) or presence (○) of 150nM efavirenz.

Figure 3. Ability of HIV-1 RT to incorporate or excise AZT on RNA/DNA T/P with decreasing duplex lengths.

Sequences of RNA templates that were annealed to pr26 (for incorporation of AZT-TP) or pr26-AZT (for excision of AZT-MP) are shown. The RNA/DNA duplex length is highlighted in bold.

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

Assays were carried out as described in Materials and Methods. Incorporation and excision activity on the RNA-T template was assumed as the reference (100%). Statistical difference is denoted as ** ($p < 0.01$) and *** ($p < 0.001$).

Figure 4. Mobility gel shift assays to assess RT-T/P interactions.

A) Representative gel for the binding of RT to RNA-T/pr26-AZT and DNA-T/pr26-AZT. Assays were carried out as described in Materials and Methods. The bound and free T/P substrates are labeled as RT-T/P-AZT and T/P-AZT, respectively. The concentrations of RT used in the assay are also indicated.

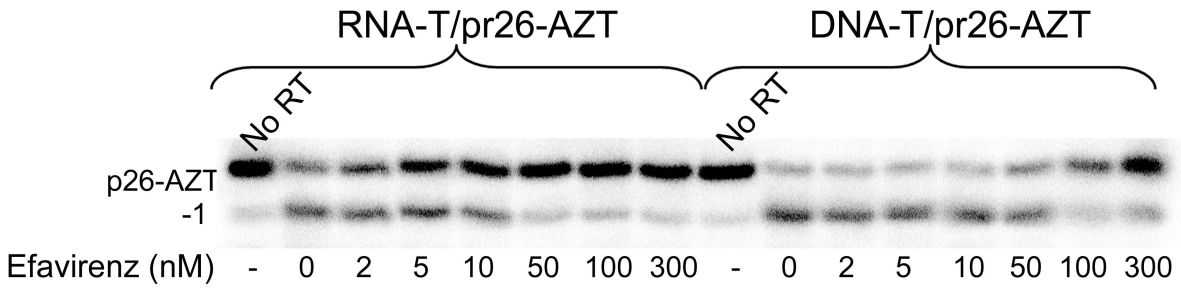
B) Plots of RT binding to the RNA/DNA T/P substrates with decreasing duplex lengths, as indicated in Figure 3A. RNA/DNA T/P with a duplex length of 26 nucleotides (•), 18 nucleotides (°), 16 nucleotides (▲), 14 nucleotides (Δ), 12 nucleotides (■), 10 nucleotides (□), and 8 nucleotides (solid ◊) and 6 nucleotides (◊) are shown.

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.

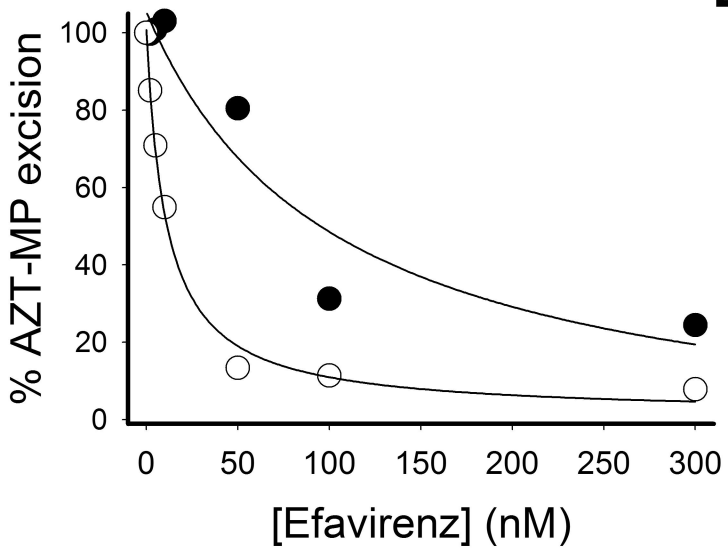
A

ZTAGAGATCGTCACCGCGGGCTTGTCC-5’ pr26-AZT
5’-AGAATGGAAAATCTCTAGCAGTGGCGCCCGAACAG-3’ DNA-T
ZTAGAGATCGTCACCGCGGGCTTGTCC-5’ pr26-AZT
5’-AGAAUGGAAAAUCUCUAGCAGUGGCGCCCGAACAG-3’ RNA-T

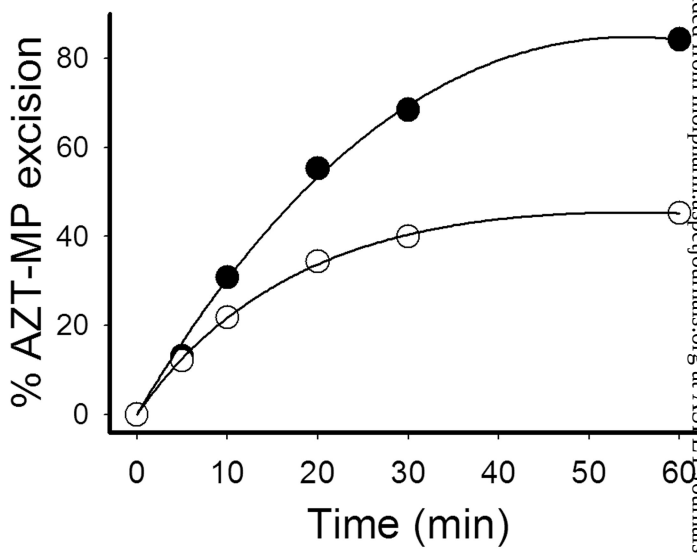
B



C



D



E

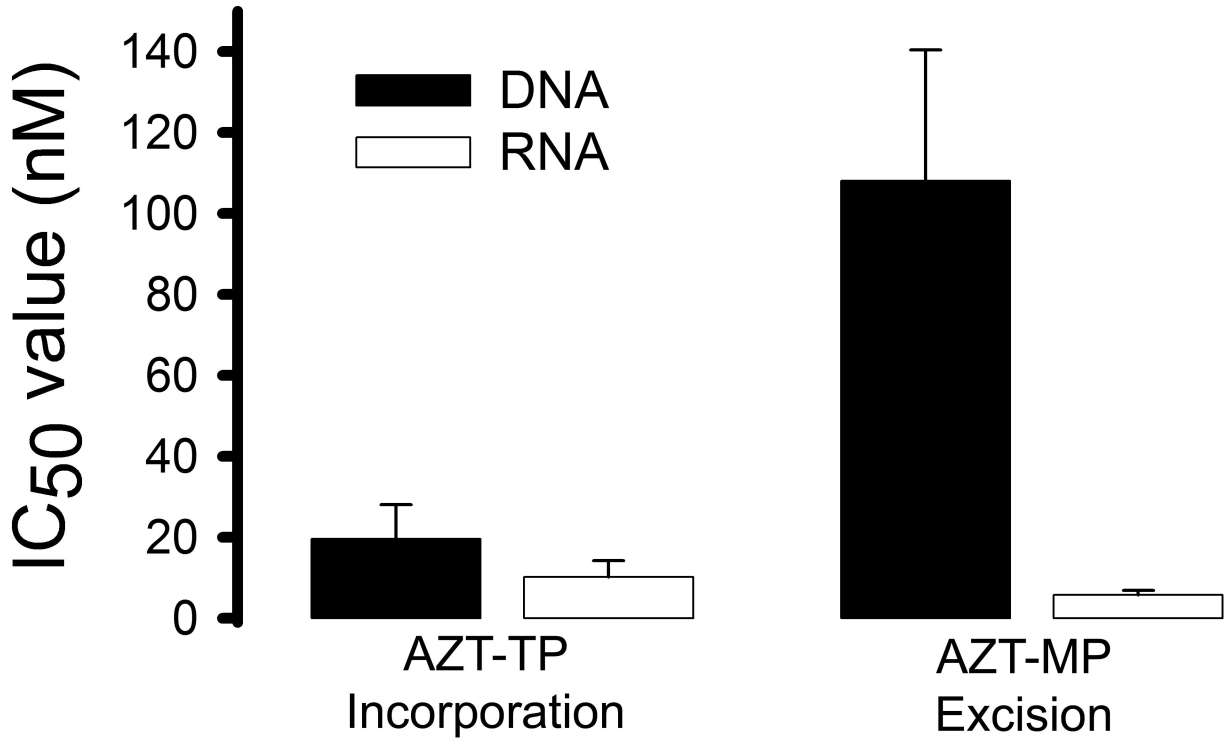
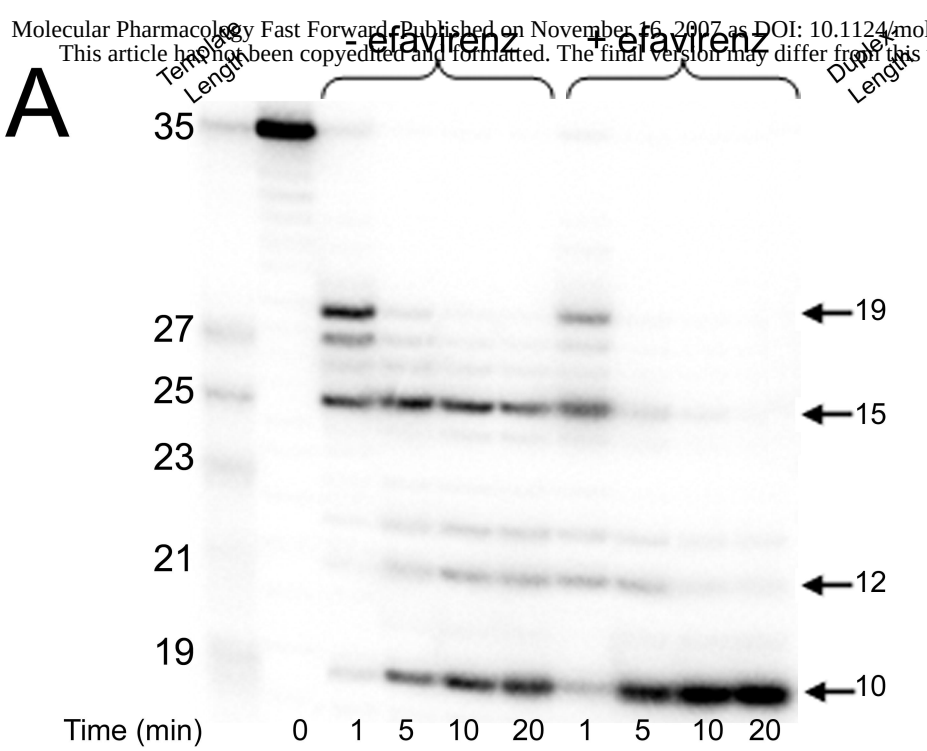
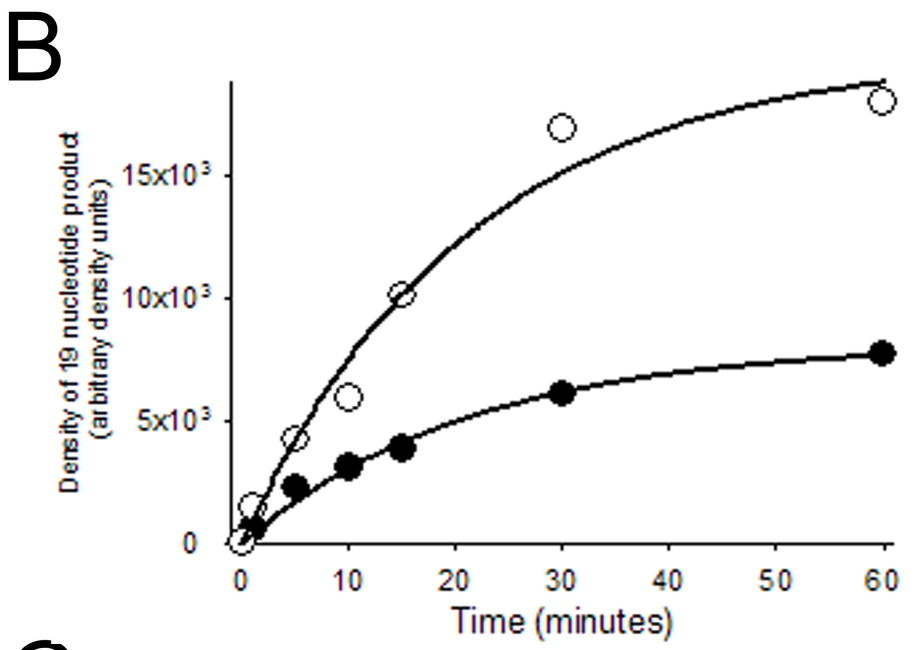


Figure 1

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.





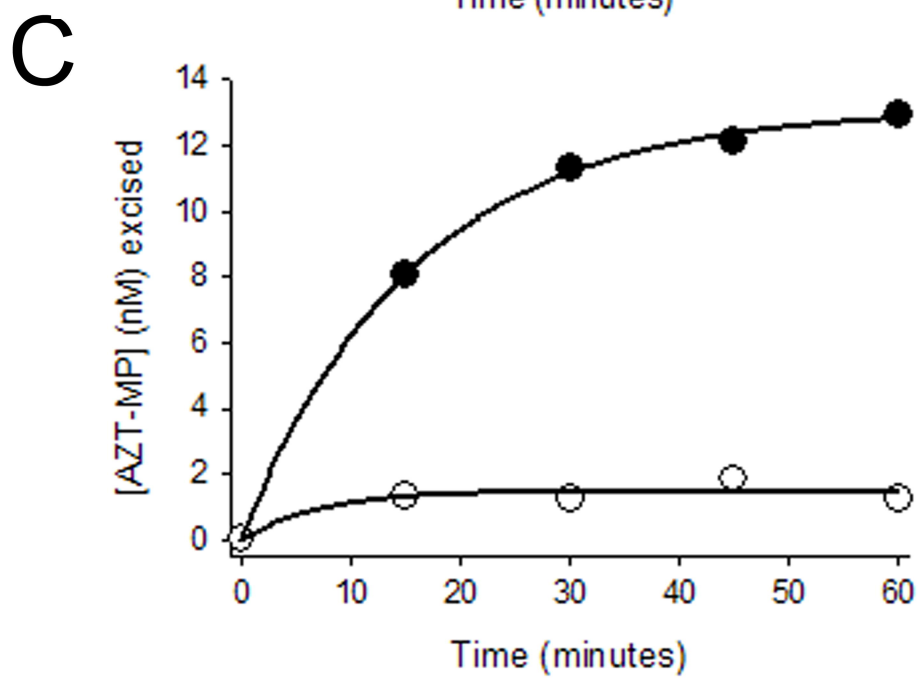


Figure 2

Duplex Length	
26	***ZTAGAGATCGTCACCGCGGGCTTGTC***C-5’ 5’-AGAAUGGAA***AAUCUCUAGCAGUGGCGCCCGAACAG***-3’
18	***ZTAGAGATCGTCACCGCG***GGCTTGTCC-5’ 5’-AGAAUGGAA***AAUCUCUAGCAGUGGCGC***-3’
16	***ZTAGAGATCGTCACCG***CGGGCTTGTCC-5’ 5’-AGAAUGGAA***AAUCUCUAGCAGUGGC***-3’
14	***ZTAGAGATCGTCAC***CGCGGGCTTGTCC-5’ 5’-AGAAUGGAA***AAUCUCUAGCAGUG***-3’
12	***ZTAGAGATCGTC***ACCGCGGGCTTGTCC-5’ 5’-AGAAUGGAA***AAUCUCUAGCAG***-3’
10	***ZTAGAGATCG***TCACCGCGGGCTTGTCC-5’ 5’-AGAAUGGAA***AAUCUCUAGC***-3’
8	***ZTAGAGAT***CGTCACCGCGGGCTTGTCC-5’ 5’-AGAAUGGAA***AAUCUCUA***-3’
6	***ZTAGAG***ATCGTCACCGCGGGCTTGTCC-5’ 5’-AGAAUGGAA***AAUCUC***-3’

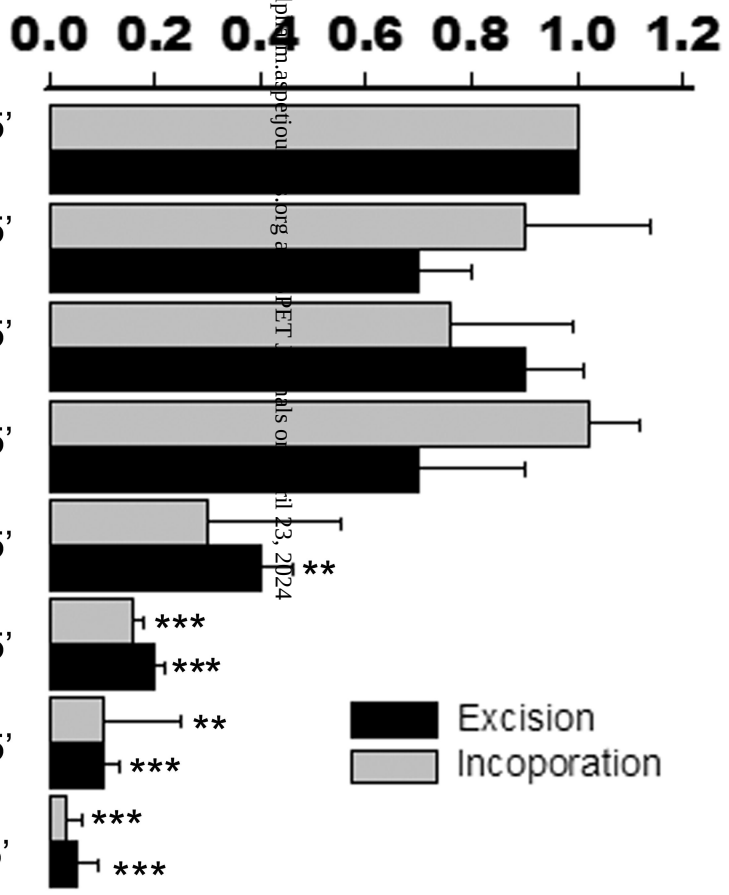
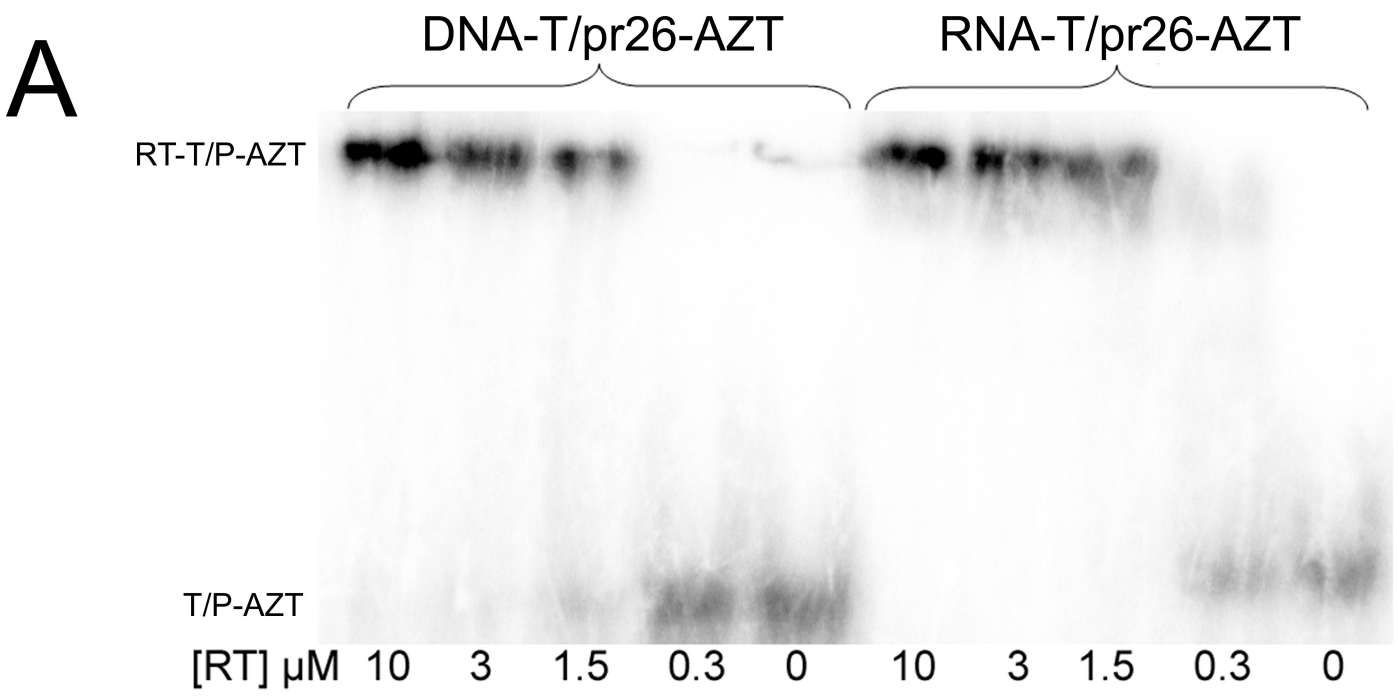


Figure 3

Molecular Pharmacology Fast Forward. Published on November 16, 2007 as DOI: 10.1124/mol.107.038596
This article has not been copyedited and formatted. The final version may differ from this version.



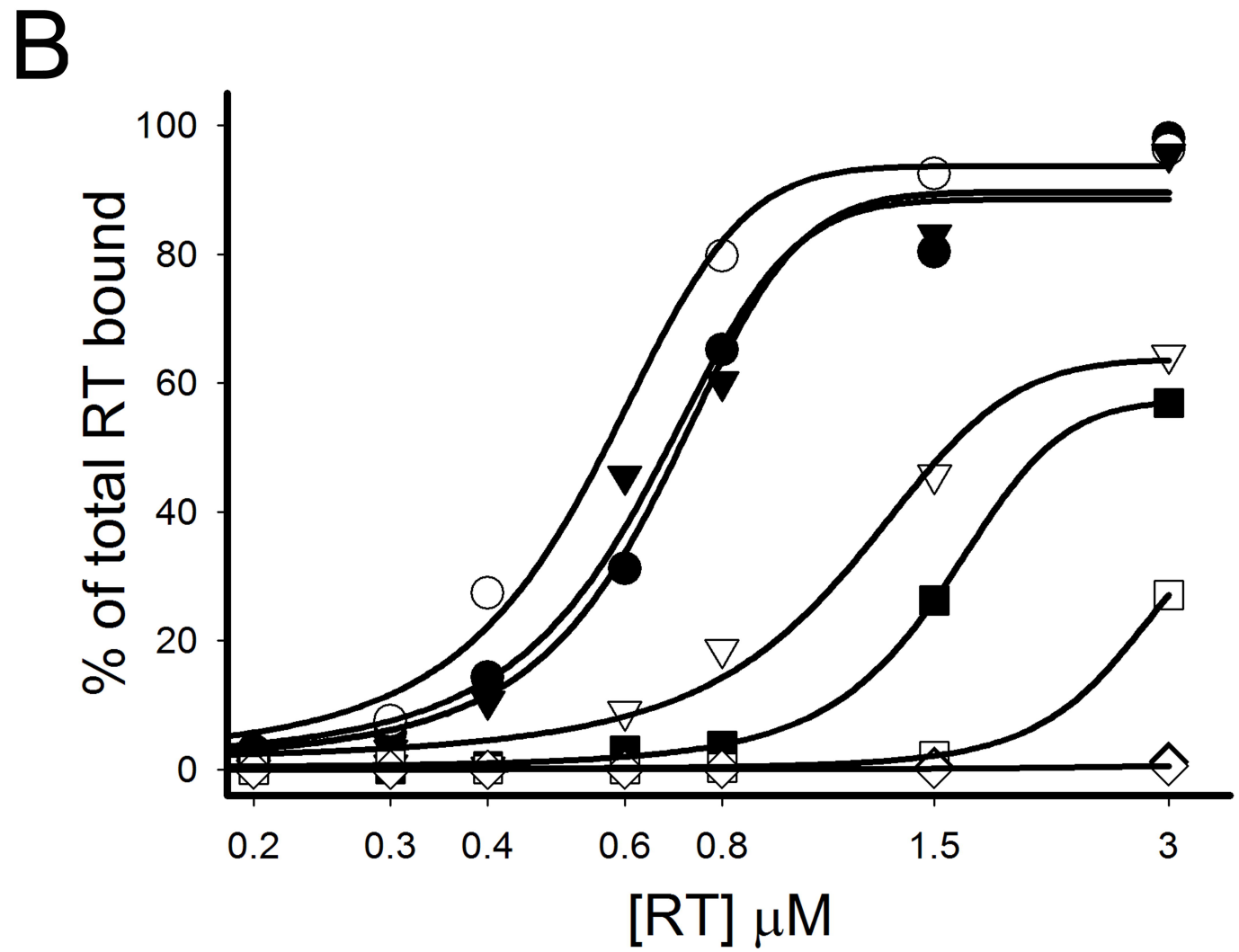


Figure 4