Modulation of Human Immunodeficiency Virus Type 1 Synergistic Inhibition by Reverse Transcriptase Mutations†

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ABSTRACT: Synergy between the anti-human immunodeficiency virus type 1 (HIV) nucleoside reverse transcriptase (RT) inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs) results from a general mechanism in which NNRTIs inhibit ATP-mediated removal of NRTIs from chain-terminated primers by decreasing the maximum rate of removal, thus sustaining NRTI chain termination. With this molecular mechanism of synergy, β-D-(+)-3′-azido-3′-deoxythymidine monophosphate (AZTMP) removal was examined in the context of clinically relevant RT mutants. The IC_{50} value for inhibition by nevirapine against wild-type (WT) RT in our removal assay was 3 μM, but this concentration had no effect on removal by the nevirapine-resistant Y181C mutant. Rather, a ∼83-fold increase in nevirapine was required to decrease the rate of removal by 50% for this mutant. Efavirenz displayed a 100 nM IC_{50} value against WT and the efavirenz-sensitive Y181C mutant, but the efavirenz-resistant mutants K103N and K103N/Y181C required a 6-fold increase in efavirenz concentration to achieve the same effect. A newer generation NNRTI, TMC125, showed potency (55 nM) against WT and all mutants, paralleling the activity of this inhibitor relative to nevirapine and efavirenz in cell culture. When tested against the AZT-resistant mutant, all NNRTIs inhibited removal by greater than 50%, showing that this mutant is hypersensitive to NNRTIs. Altogether these results illustrate that both the NNRTI and NRTI mutations can modulate chain termination. This demonstrates that sustaining synergistic HIV inhibition in combination NRTI/NNRTI therapy requires NNRTIs that are potent against WT virus and possess favorable activity profiles against clinically relevant mutations.

Highly active antiretroviral therapy (HAART) against HIV-1[1] infection focuses on delaying the development of drug-selected mutants and viral resistance (*1*, *2*). HAART entails administering patients with cocktails of drugs typically aimed at the viral reverse transcriptase (RT) and viral protease (PR), though inhibition of viral fusion grows to be another major therapeutic option. For integration of the virus's (+)-sense single-stranded RNA into the host cell genome to occur, the virus must first utilize its reverse transcriptase to convert its genome to a double-stranded DNA proviral copy. Though inhibitors have been developed against HIV-1 RT, PR, and viral fusion, the utility of these agents is limited by the emergence of viral resistance.

With respect to reverse transcription, FDA approved agents are categorized as nucleoside RT inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs). These inhibitors bind to distinct sites within the enzyme, yielding different inhibitory effects. NRTIs are analogues of endogenous cellular nucleosides and as prodrugs require phosphorylation by cellular kinases to generate the active triphosphate. Lacking a 3′-hydroxyl group, the triphosphates chain terminate DNA synthesis, and they achieve their therapeutic effect on viral replication by competing with natural dNTPs (deoxynucleoside triphosphates) at the polymerase active site (*3*). NNRTIs bind to a pocket approximately 10 Å from the active site (*4*, *5*), where they allosterically change the overall rate-limiting step by altering the rate of chemical catalysis (*6*, *7*). These inhibitors are selective against HIV-1 and are effective at nanomolar concentrations with large therapeutic windows. To date, eight NRTIs and three NNRTIs have been approved for anti-HIV-1 treatment. Combinations of these agents are given to patients, but the potency is ultimately limited by drug-resistant mutations.

Mutations within RT arise easily because HIV-1 RT lacks proofreading activity, yielding approximately 1 error in every 2000−5000 nucleotides incorporated (*8*). In the absence of inhibitors, this error frequency generates quasi-species of

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[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; PR, protease; NRTIs, nucleoside RT inhibitors; NNRTIs, nonnucleoside RT inhibitors; AZT, β-D-(+)-3′-azido-3′-deoxythymidine; AZTMP, β-D-(+)-3′-azido-3′-deoxythymidine monophosphate; d4T, β-D-(+)-2′,3′-didehydro-3′-deoxythymidine; IC_{50}, inhibitory concentration 50%; K_d, dissociation constant; AZTR, AZT-resistant; WT, wild-type; Nev, nevirapine; Efv, efavirenz; dNTP, deoxynucleoside triphosphate; (−)3TC, β-L-(−)-2′,3′-dideoxy-3′-thiacytidine; (−)FTC, β-L-(−)-2′,3′-dideoxy-5-fluoro-3′-thiacytidine.

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virus in infected individuals; in the presence of either NRTIs or NNRTIs, mutants are selected by these drugs that decrease the potency of inhibition, as observed both in patients and in cell culture. Against NRTIs, the virus generates mutants that affect incorporation, and our lab has studied the effect of mutations on the incorporation of inhibitors, for example, d4T, AZT, abacavir, ddC, (−)3TC and (−)FTC (*9*−*14*). More recently, it has been shown that RT can also catalyze the removal of chain terminators from the 3′ primer terminus in the presence of ATP, thus rescuing viral replication from chain termination (*15*−*20*). The addition of chain-terminating removal to the mechanistic understanding of resistance is a new paradigm in the field of viral resistance, because it was once thought that chain termination was the endpoint of NRTI activity. Similar to NRTI resistance, the path to mutant generation against NNRTIs can develop quickly. Resistance to the NNRTIs results in a decreased binding affinity for the inhibitor and appropriately a decrease in potency (*21*−*25*).

Combination therapy with NRTIs and NNRTIs (and protease inhibitors) has been shown to delay the onset of resistance (*1*). Synergy has been observed between NRTIs, NNRTIs, and protease inhibitors (*26*), but the observations that NRTIs and NNRTIs, which target the same viral enzyme, are synergistic is quite remarkable. For example, the NRTI AZT (zidovudine, Retrovir) and the NNRTI efavirenz (Sustiva) are synergistic in cell culture (*26*, *27*); in addition, synergy is observed between d4T (stavudine, Zerit) and nevirapine (Viramune) (*27*). It is likely that the synergistic behavior of NRTIs and NNRTIs in combination therapy contributes to their effectiveness as antivirals and delaying the onset of resistance.

The molecular and mechanistic understanding of this synergy between drugs that inhibit the same enzyme was not acheived until work from our lab and others described a general mechanism in which NNRTIs can inhibit the ATP-mediated removal of NRTIs from primer termini, thus prolonging the effect of chain termination (*20*, *28*−*30*). This was observed in a broad survey of inhibitors, and further strengthened the concept that communication exists between the NRTI active site and NNRTI binding pocket. Our results showed that the ATP-mediated maximum rate of removal was affected by the presence of the NNRTI, suggesting a long-range effect on removal at the active site when an NNRTI is bound.

Since resistance develops to currently approved RT inhibitors, it is important to investigate the impact of mutations on ATP-mediated removal and NRTI/NNRTI synergy. RT mutations Y181C (nevirapine-resistant mutation) and K103N (efavirenz-resistant mutation), and the double mutation K103N/Y181C were studied to examine the effect of NNRTI mutations on removal. Since the NNRTI resistant mutations collectively result in weaker binding and decreased antiviral activity of NNRTIs, we would anticipate that removal would be affected. Conversely, we studied one set of mutations that develop to the widely administered NRTI AZT. Resistance to AZT is due to an increased rate of ATP-mediated removal of AZTMP from primer termini (*19*), and thus its effect on modulating synergy are worth considering.

Along with NNRTIs nevirapine and efavirenz, we further studied the effect of NRTI and NNRTI mutations on removal by performing assays with TMC125, a phase III clinical NNRTI candidate shown to be active against clinically relevant mutations because of its molecular flexibility. Our results with these inhibitors demonstrate that RT mutations modulate chain termination and synergy and provide rationale into new NNRTI design.

MATERIALS AND METHODS

Expression and Purification of HIV-1 RT. The RT^{WT} and RT^{AZTR} clones were generously provided by Stephen Hughes, Paul Boyer, and Andrea Ferris of the Frederick Cancer Research and Development Center, MD. RT^{Y181C}, RT^{K103N}, and $RT^{K103N/Y181C}$ were generated by PCR mutagenesis and conventional molecular biology techniques. C-Terminal histidine-tagged heterodimeric p66/p51 reverse transcriptase was purified as previously published (*31*).

Materials. AZTTP was purchased from Moravek Biochemicals. Nevirapine and efavirenz were acquired from Toronto Research Chemicals Inc., North York, Ontario. NNRTIs were diluted in dimethyl sulfoxide. ATP was purchased from Sigma (Ultrapure grade, minimum 99%) and was treated with thermostable pyrophosphatase (Roche) to degrade contaminating pyrophosphate.

Labeling and Annealing of Oligonucleotides. Primers and templates were synthesized at the Keck Facility at Yale University and purified with 20% polyacrylamide denaturing gel electrophoresis. The sequences of primer and template used in this study are D23 (5′-TCA GGT CCC TGT-TCG GGC GCC AC-3′) and D36 (5′-TCT CTA GCA GTG-GCG CCC GAA CAG GGA CCT GAA AGC-3′). The D23-AZTMP primer (designation for primer chain-terminated with AZT monophosphate) was made following a previously reported method (*32*). The primer/template was labeled and annealed as previously described (*33*).

Pre-Steady-State Removal Assays. ATP-mediated removal was studied as previously described (*19*) by quantitating the disappearance of substrate under single-turnover conditions, in which each reaction mixture contained 250 nM RT, 50 nM primer/template, 10 mM Mg^{2+}, and the appropriate concentration of ATP and NNRTI (see figures). All concentrations represent final concentrations, and enzyme concentration is based on a pre-steady-state active site determination. In removal experiments lacking NNRTIs, DMSO was used as a control (representing less than 2% of total reaction mixture). Samples at each time point were separated by polyacrylamide gel electrophoresis and quantitated on a Bio-Rad Molecular Imager FX.

Data Analysis. Data were quantitated and fit using previously reported methods (*19*).

Determination of IC_{50} Values and Maximum Removal Rates. For each NNRTI, ATP-mediated removal experiments were performed with WT RT at a set ATP concentration of 3 mM, with either no NNRTI or different concentrations of the chosen NNRTI. The IC_{50} value was defined by the concentration of NNRTI that inhibited the initial rate of removal by 50%. For each RT, removal experiments were then performed at different ATP concentrations in the presence of the NNRTI at the IC_{50} value. The rate of removal was plotted versus ATP concentration, and the data were fit to a hyperbola to generate the maximum rate of removal (k_{rem}) and the dissociation constant (K_d) for ATP.

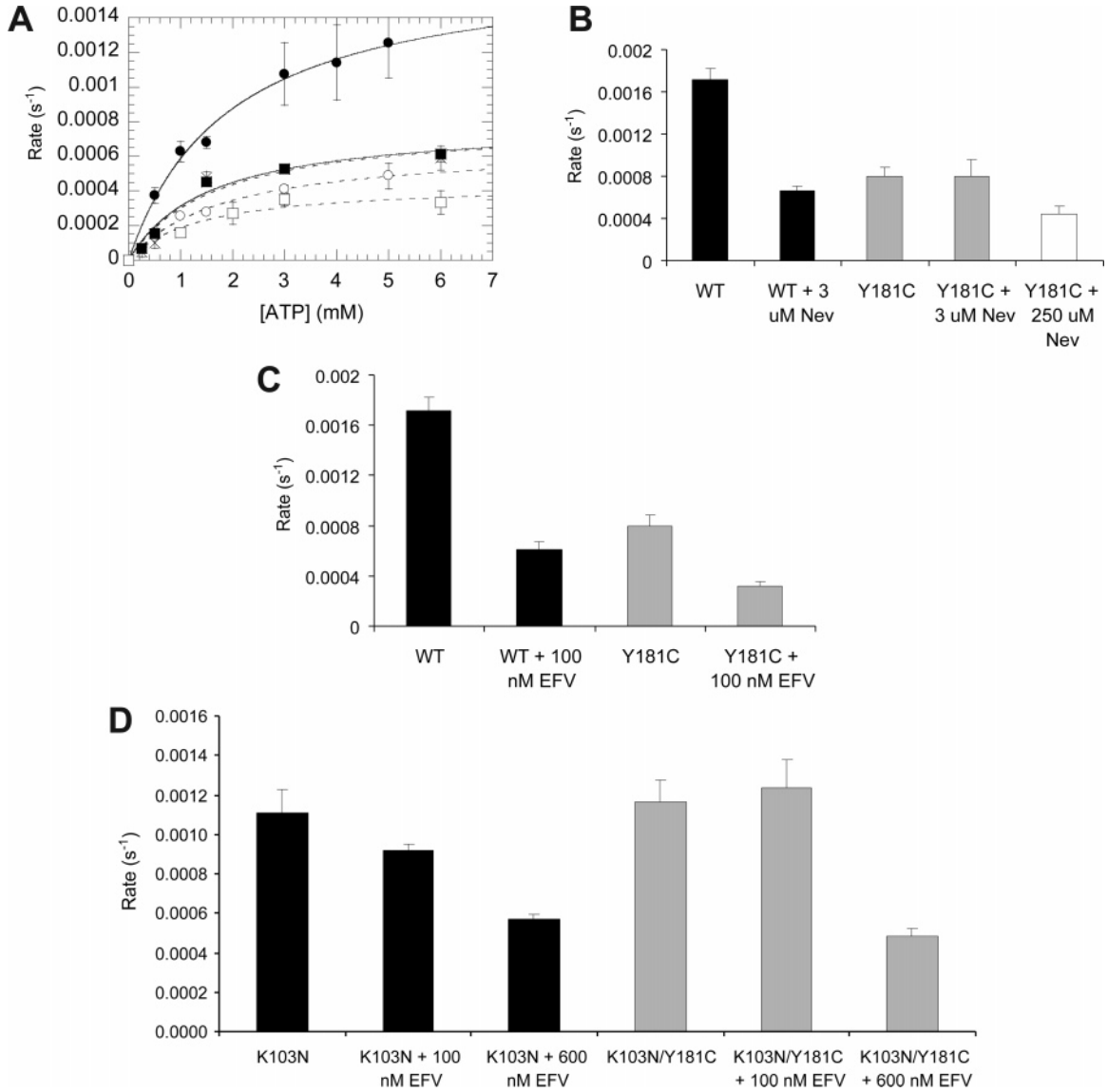

FIGURE 1: Effect of RT Y181C and K103N mutations on NNRTI inhibition of ATP-mediated NRTI removal: (A) K_d curves for AZTMP removal by WT RT in the absence (●, solid line) and presence (○, dotted line) of 3 μM nevirapine and Y181C in the absence of nevirapine (■, solid line), presence of 3 μM nevirapine (×, dotted line), and presence of 250 μM nevirapine (□, dotted line); (B) bar graph of maximum rates of removal from plots in panel A; (C) bar graph of maximum rates of removal by WT and Y181C RTs in the presence of 100 nM efavirenz; (D) bar graph of maximum rates of removal by K103N and K103N/Y181C RTs in the presence of 100 or 600 nM efavirenz.

RESULTS AND DISCUSSION

We previously showed that the ATP-mediated removal of NRTIs can be inhibited by the addition of a nonnucleoside RT inhibitor, by affecting the maximum rate of removal and consequently prolonging the effect of chain termination and potency of NRTIs (*20*). Our results showed this was true for many NRTIs and with the NNRTIs nevirapine and efavirenz. In light of the resistance that develops to NRTIs and NNRTIs, a logical question to ask from our results and others (*28*, *29*) is what effect(s) do RT mutations have on this mechanism. An initial hypothesis to address this question stems from the resistance imposed by mutations to NNRTIs. These mutations are known to decrease the binding affinity of NNRTIs and would be expected to hinder NNRTI inhibition of NRTI removal, therefore affecting chain termination. Rationale for this hypothesis arises from multiple independent studies (*30*, *34*, *35*), including data from Zhu et al., which showed that the combination treatment of AZT and nevirapine was synergistic against a WT viral isolate but not a Y181C isolate (*34*), and work from Maga et al., which showed in vitro that AZT and efavirenz were synergistic against WT but that this synergy was lost when the K103N mutation was introduced (*35*).

Our initial results under pre-steady-state conditions with the Y181C mutation, a frequent mutation occurring during nevirapine treatment (*36*, *37*), supported our hypothesis that NNRTI mutations can attenuate the effect of NNRTIs on removal. We examined this mutant in removal studies in comparison to WT, as shown in Figure 1. It was previously shown that 3 μM nevirapine inhibited AZTMP removal by WT RT by approximately 50% (*20*) (repeated in Figure 1A) by decreasing the maximum rate of removal. As a control, the introduction of the Y181C mutation itself decreases the rate of removal (Figure 1). A priori one might not expect such a result, but this result coincides with work by Selmi et al., which also showed that Y181C decreases removal

Table 1: Observed Fold Resistance for NNRTI Inhibition of NRTI Removal

RT	NNRTI IC_{50} value	fold resistance[a]
WT	3 μM Nev	
	100 nM Efv	
	55 nM TMC125	
Y181C	250 μM Nev	83
	100 nM Efv	1
	55 nM TMC125	1
K103N	600 nM Efv	6
	55 nM TMC125	1
K103N/Y181C	600 nM Efv	6
	55 nM TMC125	1
AZTR[b]	3 μM Nev	<1[c]
	100 nM Efv	<1[c]
	55 nM TMC125	<1[c]

[a] Fold resistance represents change in IC_{50} values for respective NNRTI between mutant and WT RT. [b] AZTR mutations: complex of D67N, K70R, T215Y, and K219Q. [c] NNRTI inhibition of removal by the AZTR mutant showed >50% inhibition at indicated NNRTI concentrations (see Figure 3).

relative to WT and in the context of AZT resistant mutations. Unlike our results, however, Y181C (in the background of AZT resistant mutations) influenced both the maximum rate of removal and the K_m of ATP in their assay (*38*), possibly suggesting that the primer/template sequence may alter removal kinetics (*39*). Y181C and other RT mutations are selected because of drug pressure, and thus we asked what effect Y181C would have on removal with the addition of its respective first-generation inhibitor, nevirapine. The IC_{50} value of 3 μM versus WT imparted no change to the removal of AZTMP by Y181C, supported by results from our lab showing that the K_d of nevirapine increases ~130-fold from nucleotide incorporation studies between RT^{WT} and RT^{Y181C} (*21*). When removal with Y181C and differing concentrations of nevirapine (data not shown) was examined, approximately 250 μM nevirapine inhibited removal by 50%, representing a >80-fold change in IC_{50} values between WT and mutant (Figure 1B, Table 1). As shown in our previous work (*20*), these results show the maximum rate of removal (k_{rem}) was affected with no concomitant change in the K_d (see Supporting Information), suggesting that long-range communication between the NRTI and NNRTI sites does not alter the binding of ATP, but rather the rate of chemistry, even in the context of mutations. The data for all other mutations and NNRTIs tested in this study displayed this same phenomenon, and thus subsequent data is represented by bar graphs comparing rates of removal. Our observations with nevirapine were supported using the second-generation inhibitor efavirenz as a control, because it remains potent against Y181C in vitro in cell culture assays (*37*, *40*−*42*). Our IC_{50} value for efavirenz against WT RT was potent (100 nM) and remained potent versus the Y181C mutant (Figure 1c). The IC_{50} value was 30-fold lower for efavirenz than nevirapine, akin to the difference in potency between these two agents in cell culture (*40*, *43*).

The results with nevirapine and efavirenz presented thus far illustrate the role of Y181C in modulating AZTMP removal; to further demonstrate that NNRTI mutations in general can modulate removal, removal was examined with the efavirenz-resistant mutation K103N and the highly resistant double mutation K103N/Y181C (*44*). We expected that 100 nM efavirenz would have no effect on either mutant, and this was confirmed (Figure 1c). Moreover, unlike Y181C, which shows high resistance to nevirapine, K103N shows moderate resistance to efavirenz. The IC_{50} value determined from dose−response curves (data not shown) was 600 nM (Figure 3), representing only a 6-fold change between mutant and wild type. Between the single and double mutants, we observed no change in IC_{50} values, and we would explain the 600 nM inhibition of both K103N and K103N/Y181C using the argument that since the introduction of Y181C to WT did not affect the IC_{50} value of efavirenz that the addition of Y181C to K103N would likewise not change inhibition by efavirenz. This change in IC_{50} values parallels the change in potencies between nevirapine and efavirenz versus their respective mutations in cell culture assays (*41*−*43*). Altogether, the results show that NNRTI mutants can modulate chain termination and their context in manifesting synergy.

The breadth of anti-HIV clinical, cellular, and biochemical data strongly reinforce that an NNRTI (in addition to other classes) that remains potent against resistance mutations will impart sustained synergistic inhibition in combination therapy. New directions in the NNRTI field have certainly embarked on such inhibitors, such as TMC125 (*40*, *45*−*52*). To further substantiate our mechanism of synergy and the effect that mutations contribute, we hypothesized that a newer generation inhibitor, such as TMC125, would be more active than older generation compounds and remain active against WT and mutations in inhibiting removal. Figure 2 supports this hypothesis. TMC125 proved to be more potent than either nevirapine or efavirenz, with an IC_{50} value of 55 nM, as determined by a dose−response curve (data not shown). The IC_{50} values of 3 μM, 100 nM, and 55 nM for nevirapine, efavirenz, and TMC125, respectively, parallel the activities of these compounds in cell culture. We do note, however, that the absolute values do not match cell culture results, but we would argue that the results reported here do not fully account for the inhibition that each compound can exhibit in the cellular environment, for example, inhibition of dNTP incorporation (*6*, *7*) and differential inhibition in various steps of viral replication (*53*). Using this concentration of TMC125, we examined removal with our various mutants and found ~50% inhibition to occur for all enzymes tested (Figure 2, Table 1), agreeing with published reports of TMC125 activity against resistant mutations (*45*, *47*, *48*, *51*).

We next examined removal in the context of an AZT-resistant mutant RT (with mutations D67N, K70R, T215Y, and K219Q). Published data has shown that the AZTR mutations impart an increased maximum rate of removal (k_{rem}) relative to wild-type RT (*19*), and when removal rates by WT and the AZTR mutant were compared (Figures 1 and 3), these results were confirmed here with the AZTR mutant removing chain-terminated AZTMP four times more rapidly than WT RT (see Supporting Information). The IC_{50} values of nevirapine, efavirenz, and TMC125 versus WT were tested against the AZTR mutant, with the expectation that these concentrations would have a similar effect as seen with wild-type enzyme. Unexpectedly, the addition of 3 μM nevirapine, 100 nM efavirenz, or 55 nM TMC125 significantly decreased (>50%) the rate of removal (Figure 3), suggesting the AZTR mutations themselves impart effect(s) on NNRTI inhibition, by conferring long-range conformational or chemical effects on the NNRTI binding pocket that

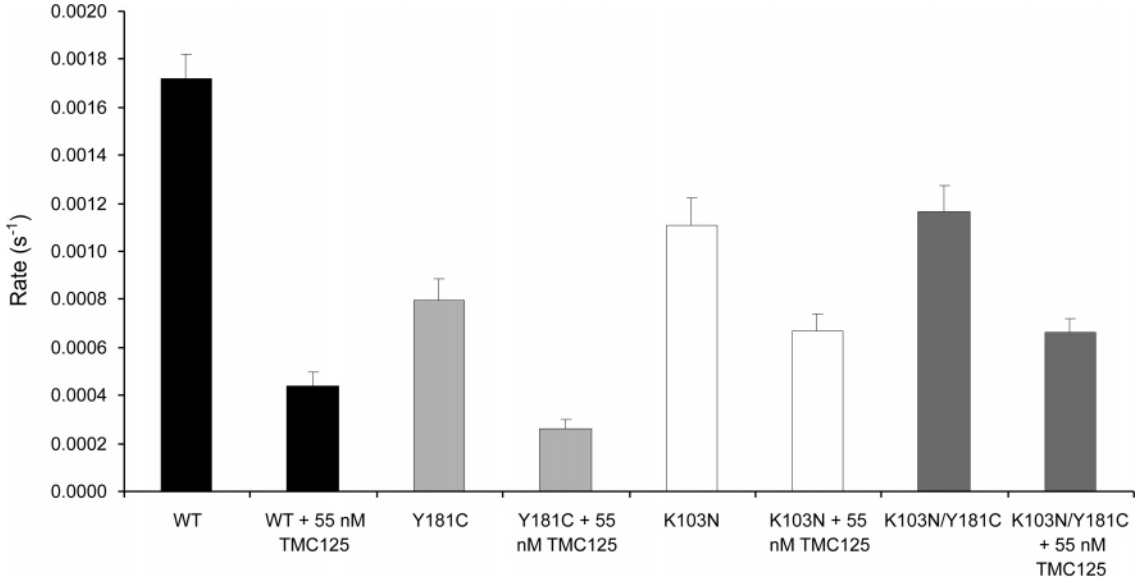

FIGURE 2: TMC125 inhibits ATP-mediated AZTMP removal by NNRTI mutants: bar graph of maximum rates of removal by WT, Y181C, K103N, and K103N/Y181C RTs in the presence of 55 nM TMC125.

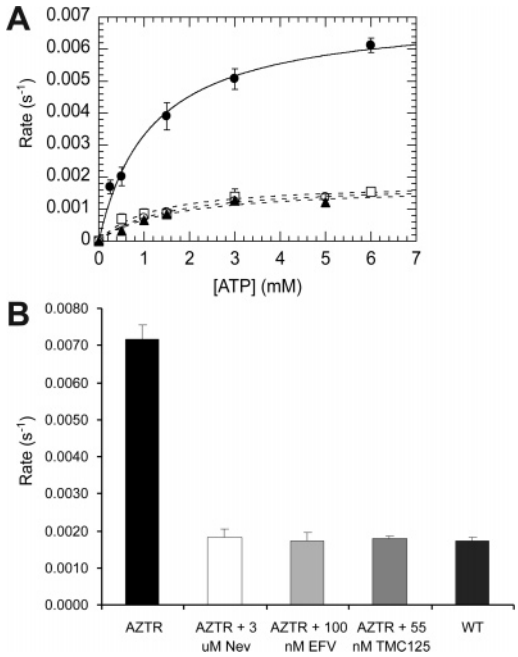

FIGURE 3: The AZTR mutant is hypersensitive to NNRTIs: (A) K_d curve for AZTMP removal by AZTR in the absence of NNRTI (●, solid line), presence of 3 μM nevirapine (□, dotted line), presence of 100 nM efavirenz (▲, dotted line), and presence of 55 nM TMC125 (○, dotted line); (B) bar graph of maximum rates of removal from plots in panel A. Basal rate of removal by WT from Figure 1 is also added for comparison.

increase the potency of NNRTIs. The effect of this inhibition was similar to that of WT alone (Figure 3), illustrating that the advantage that AZT-resistant mutations impart on removal can be scaled back by nonnucleoside inhibition. The results of Zhu et al. support our in vitro observation; the combination of AZT and nevirapine were shown to be synergistic against WT virus and remained synergistic against an AZT-resistant virus (*34*). Similarly, a recent report by Crespan et al. suggests that NRTI mutations D113E, Y115F, Q151E, and Q151N might also affect synergy at the molecular level (*54*), but the steady-state technique used in their experiments should be noted since NNRTIs are known to affect the rate-limiting step in polymerization without affecting the steady-state release of primer/template (*6*, *7*). Together, these results further illustrate that NRTI/NNRTI synergy, and its basis in maintaining chain termination, can be modulated by NRTI mutants.

The results presented here show the interplay between the NRTI and NNRTI pockets. Viral reverse transcriptase has the broad ability to remove NRTIs, but this catalysis can be inhibited by the binding of NNRTIs. The mutations that NRTIs and NNRTIs generate would be expected to modulate the inhibition, and our results support this theory. The results strengthen our molecular mechanism of synergy and the basis to combat HIV-1 with combination treatment. The mechanism presented here with TMC125 may be one basis for the synergy observed between TMC125 and AZT (*48*) and fortifies the notion that flexibility of TMC125 and its congeners offer new avenues in NNRTI design and ultimately NNRTI potency (*46*, *52*).

With the vast research on the clinical utility of NRTIs and NNRTIs, it is no surprise that anti-HIV-1 combination therapies that are efficacious include the combination of two nucleoside analogues and one nonnucleoside inhibitor (*55*, *56*). Our observation that efavirenz, a second-generation NNRTI, is more potent than nevirapine, a first-generation inhibitor, against WT and mutant RTs suggests that NNRTIs under development will be even more beneficial in combination therapy. Agents such as TMC125 have shown nanomolar potency in early clinical trials against WT and a host of mutant viral strains (*40*, *45*). These agents support the present strategy to construct NNRTIs that are more flexible and able to bind to resistant forms of RT. Not only does this afford the development of new therapeutics, but it aids in the synergistic effect that we have further characterized here that exists between nucleoside analogues and nonnucleoside inhibitors.

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SUPPORTING INFORMATION AVAILABLE

A table showing the effect of NNRTIs on ATP-mediated AZTMP removal by RT^{WT} and RT mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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