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N348I in Reverse Transcriptase Provides a Genetic Pathway for HIV-1 to Select TAMs and Mutations Antagonistic to TAMs

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Abstract

Objective—Several nonnucleoside (e.g. Y181C) and nucleoside (e.g. L74V, M184V) resistance mutations in HIV-1 reverse transcriptase (RT) are antagonistic toward thymidine analog mutations (TAMs) that confer zidovudine (AZT) resistance. The N348I mutation in the connection domain of RT also confers AZT resistance however the mechanisms involved are different from TAMs. In this study, we examined whether N348I compensates for the antagonism of the TAM K70R by Y181C, L74V and M184V.

Design and Methods—The AZT-monophosphate (AZT-MP) and ribonuclease H (RNase H) activities of recombinant purified HIV-1 RT containing combinations of K70R, N348I and Y181C, L74V or M184V were assessed using standard biochemical and antiviral assays.

Results—As expected, the introduction of the Y181C, L74V or M184V mutations into K70R HIV-1 RT significantly diminished the ATP-mediated AZT-MP excision activity of the enzyme. However, the N348I mutation compensated for this antagonism on RNA/DNA template/primers by significantly decreasing the frequency of secondary RNase H cleavages that reduce the overall efficiency of the excision reaction.

Conclusion—The acquisition of N348I in HIV-1 RT - which can occur early in therapy, oftentimes before TAMs - may provide a simple genetic pathway that allows the virus to select both TAMs and mutations that are antagonistic toward TAMs.

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Conflicts of Interest: None to declare

Author Contributions

N.S.-C and G. T. designed the study. J.R. and S.H.Y. performed all of the experiments. All authors contributed to data analysis. J.R., G.T., and N.S.-C. wrote the manuscript.

Keywords

HIV-1; reverse transcriptase; resistance; N348I; zidovudine; antagonism

Introduction

HIV-1 reverse transcriptase (RT) is a key target for the development of antiretroviral drugs. To date, the US Food and Drug Administration have approved 12 RT inhibitors that can be classified into two distinct groups: (i) the nucleoside and nucleotide RT inhibitors (NRTI) that, once metabolized to their active triphosphate forms, inhibit viral DNA polymerization by acting as chain-terminators of nucleic acid synthesis [1]; and (ii) the nonnucleoside RT inhibitors (NNRTI) that bind to a hydrophobic pocket in HIV-1 RT and act as allosteric inhibitors of viral reverse transcription [2].

The USA Panel of the International AIDS Society recommends antiretroviral 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 [3]. The rationale for including two NRTIs stems from studies which demonstrated synergy between combinations of NRTIs such as zidovudine (AZT)/didanosine (ddI) or AZT/lamivudine (3TC) [4–8]. From a clinical perspective, the efficacy of these regimens can also be attributed to a higher genetic barrier to resistance due to the ddI and 3TC resistance mutations (L74V and M184V, respectively) antagonizing the AZT-associated resistance mutations (e.g. thymidine analog mutations or TAMs) [9–15]. Similarly, the efficacy of anti-HIV therapies that include both AZT and an NNRTI such as nevirapine may be explained, in part, by the observed synergistic interactions between these two classes of drugs as well the antagonism between their respective resistance mutations [16–20].

Despite the efficacy of combination antiretroviral therapies containing RT inhibitors, multi-drug resistant HIV-1 can still develop, although the current literature suggests that this may require the accumulation of several additional mutations. For example, substitutions at RT codons 44, 118, 207, 208 and 333 have been associated with increased AZT resistance in viruses that carry both TAMs and M184V [21–23]. Recently, we identified the N348I mutation in HIV-1 RT that confers both AZT and NNRTI resistance [24]. N348I appears early in therapy and was found to be highly associated with TAMs, M184V/I and the NNRTI resistance mutations K103N, Y181C/I, and G190A/S. N348I was also found to be significantly associated with therapies that contained AZT and nevirapine. Initially, we hypothesized that N348I was selected early in therapy failure because the mutation decreased susceptibility to both AZT and nevirapine and accordingly provided a simple genetic pathway to resistance that involved only a single nucleotide change [25]. However, recent investigations into the biochemical mechanisms by which N348I in RT confers AZT resistance raised additional questions as to whether this mutation may also compensate for the antagonism between TAMs and Y181C (described below).

HIV-1 RT containing TAMs exhibits an increased capacity to unblock AZT-monophosphate (AZT-MP) terminated primers in the presence of physiological concentrations of ATP by increasing binding affinity of ATP for RT and/or by increasing the kinetic rate of the ATP-mediated excision reaction [26–28]. The Y181C mutation directly antagonizes this ATP-mediated excision activity of RT containing TAMs [17;18]. By contrast, the N348I mutation in HIV-1 RT indirectly increases AZT resistance by decreasing the frequency of secondary ribonuclease H (RNase H) cleavages that significantly reduce the RNA/DNA duplex length of the template/primer (T/P) and diminish the efficiency of AZT-MP excision [24;29;30]. It is not known, however, if the Y181C mutation also antagonizes the N348I AZT resistance

phenotype. Accordingly, in this study our primary goal was to analyze the effects of N348I on the AZT-MP excision phenotype using recombinant purified RTs that contained K70R, Y181C or K70R/Y181C.

Materials and Methods

Reagents

The K70R, L74V, Y181C, M184V and N348I mutations were introduced into the wild-type (WT) p6HRT-Prot prokaryotic expression vector [38] by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene La Jolla, CA). Full-length sequencing of mutant RTs was performed to confirm the presence of the desired mutations and to exclude adventitious mutations introduced during mutagenesis. The WT, K70R, Y181C, N348I, K70R/Y181C, K70R/Y181C/N348I, K70R/L74V, K70R/L74V/N348I, K70R/M184V, K70R/M184V/N348I HIV-1 RTs were purified as described previously [38;39]. The protein concentration of the purified enzymes was determined spectrophotometrically at 280 nm using an extinction coefficient (ϵ_{280}) of $260450 \text{ M}^{-1} \text{ cm}^{-1}$, and by Bradford protein assays (Sigma-Aldrich, St. Louis, MO). The RNA- and DNA-dependent DNA polymerase activities of the purified WT and mutant enzymes were essentially identical (data not shown). AZT-triphosphate (AZT-TP) was purchased from Sierra Bioresearch (Tucson, AZ). ATP, dNTPs, and ddNTPs were purchased from GE Healthcare (Piscataway, NJ), and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was acquired from PerkinElmer Life Sciences (Boston, MA). RNA and DNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

Single-round AZT-MP excision assays

A 26-nucleotide DNA primer (**pr26**; 5'-CCTGTTCTGGGCGCCACTGCTAGAGAT-3') was 5'-radiolabeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and chain-terminated with AZT-MP to generate P_{AZT} as reported previously [20;24;35;36]. P_{AZT} was then annealed to either a 35-nucleotide DNA (T_{DNA} ; 5'-AGAATGGAAAATCTCTAGCAGTGGCGCCCGAACAG-3') or RNA (T_{RNA} ; 5'-AGAAUGGAAAAUCUCUAGCAGUGGCGCCCGAACAG-3') template. ATP-mediated AZT-MP excision assays were carried out by first incubating 20 nM $\text{T}_{\text{RNA}}/\text{P}_{\text{AZT}}$ or $\text{T}_{\text{DNA}}/\text{P}_{\text{AZT}}$ with 3 mM ATP, 10 mM MgCl_2 , 1 μM dTTP and 10 μM ddCTP in a buffer containing 50 mM Tris-HCl (pH 7.5) and 50 mM KCl. Reactions were initiated by the addition of 200 nM WT or mutant RT. Aliquots were removed at defined times, quenched with sample loading buffer (98% deionized formamide, 1 mg/ml each of bromophenol blue and xylene cyanol), denatured at 95 °C for 8 min, and then product was resolved from substrate by denaturing polyacrylamide gel electrophoresis and analyzed, as reported previously [20;24;35;36].

Assay for RT RNase H activity

WT and mutant RT RNase H activity was evaluated using the same AZT-MP chain-terminated RNA/DNA T/P substrate described above, except the 5'-end of the RNA was ^{32}P -end-labelled. Assays were carried out using 20 nM $\text{T}_{\text{RNA}}/\text{P}_{\text{AZT}}$, 3 mM ATP and 10 mM MgCl_2 in a buffer containing 50 mM Tris-HCl (pH 7.5) and 50 mM KCl. Reactions were initiated by the addition of 200 nM WT or mutant HIV-1 RT. Aliquots were removed, quenched at varying times, and analyzed as described above.

RT polymerization products formed under continuous DNA polymerization conditions

Heteropolymeric RNA-dependent or DNA-dependent DNA polymerase T/Ps were prepared as reported previously [35;36]. DNA polymerization reactions were carried out by incubating 20 nM heteropolymeric T/P complex with 1 μM concentration of each dNTP, 2 μM of AZT-TP, 3 mM ATP and 10 mM MgCl_2 in buffer containing 50 mM Tris-HCl (pH 7.5) and 50 mM

KCl. Reactions were initiated by the addition of 200 nM WT or mutant RT. After defined incubation periods, aliquots were removed from the reaction tube and quenched with equal volumes of gel loading dye. Products were separated by denaturing gel electrophoresis and quantified, as described above.

Phenotypic drug susceptibility assays

The appropriate drug resistance mutations were introduced by site directed mutagenesis into the background of WT pNL4.3 infectious molecular clone. HIV-1 was recovered by transfection of 293T cells and drug susceptibility assays were performed in the TZM-bl indicator cell line, as described previously [24] with the exception that HIV-1 replication was determined by measuring luciferase activity using the Steady-Glo Luciferase Assay System according to manufacturer's instructions (Promega). Statistically significant differences in the 50% effective dose (EC₅₀) were determined using the Wilcoxon Rank Sum Test.

Results and Discussion

N348I in HIV-1 RT compensates for the antagonism of K70R by Y181C

The Y181C mutation in RT increases HIV-1 sensitivity to AZT even when multiple TAMs are co-selected on the same genome [17]. Previous biochemical studies suggested that this phenotype was due to the Y181C mutation decreasing the AZT-MP excision activity of both WT and D67N/K70R/T215F/K219Q HIV-1 RT by directly impacting ATP binding and/or the rate of AZT-MP excision [18;31]. Because the mechanism by which N348I in HIV-1 RT confers AZT resistance is different from that conferred by TAMs (described in Introduction) [24;29;30], we hypothesized that Y181C might not antagonize the excision activity of N348I HIV-1 RT and, accordingly, that N348I may compensate for the Y181C-mediated antagonism of TAMs. To investigate this hypothesis, we examined the ability of recombinant HIV-1 RT containing combinations of Y181C, K70R and N348I to excise AZT-MP using established biochemical assay systems. In this study, we focused on a single TAM (i.e. K70R) because we were interested to determine how N348I influenced the interplay between TAMs and Y181C at the onset of resistance development. In this regard, the K70R mutation is one of the first TAMs to appear under AZT drug pressure [32;33]. Furthermore, K70R in HIV-1 RT is associated with a strong ATP-mediated excision phenotype *in vitro* [34].

We first examined the ability of WT, K70R, Y181C, K70R/Y181C, and K70R/Y181C/N348I HIV-1 RT to excise AZT-MP and rescue DNA synthesis from chain-terminated DNA/DNA and RNA/DNA T/Ps (Fig.1). As described previously [18;31], Y181C HIV-1 RT unblocked AZT-MP chain-terminated primers inefficiently on both DNA/DNA and RNA/DNA T/Ps (Fig. 1B, 1C). As expected, Y181C also antagonized the ability of K70R RT to excise AZT-MP and recover DNA synthesis, although this defect was more pronounced on an RNA/DNA T/P than on a DNA/DNA T/P (Fig.1B, 1C). Introduction of the N348I mutation into RT containing K70R/Y181C RT increased the enzymes excision activity on the RNA/DNA T/P but not on the DNA/DNA T/P (Fig.1B, 1C).

Our group, [24] as well as those of Drs. Matthias Götte [29] and Vinay Pathak [30], have demonstrated that the N348I mutation in HIV-1 RT increases AZT resistance by decreasing the frequency of secondary RNase H cleavages that significantly reduce the RNA/DNA duplex length of the T/P and diminish the efficiency of AZT-MP excision. In this regard, we previously delineated the relationship between AZT-MP excision efficiency and RNase H activity on the RNA/DNA T/P substrate used in these experiments [20;35]. These studies showed that the primary polymerase-dependent RNase H cleavage of RT does not impact the enzyme's AZT-MP excision efficiency, but polymerase-independent RNase H cleavages that reduce the RNA/DNA duplex length to less than 12 nucleotides abolish AZT-MP excision activity [20;35]. In

light of these data, we next evaluated the RNase H activity of WT and mutant RT that occurred during the ATP-mediated excision reactions described in Figure 1. As reported previously [24], N348I significantly reduced the frequency of a polymerase-independent cleavage event that decreases the RNA/DNA duplex to 10 nucleotides (Fig. 2B). In comparison with the WT enzyme, the K70R and Y181C mutations had minimal impact on the RNase H activity of RT (Fig. 2). Introduction of the N348I mutation into K70R/Y181C RT, however, significantly reduced the frequency of this polymerase-independent cleavage event (Fig. 2); a finding that is consistent with the notion that N348I in HIV-1 RT impacts the efficiency of the AZT-MP excision reaction by RNase H-dependent mechanism.

In the experiments described above, we evaluated the AZT-MP excision and RNase H cleavage activities of the WT and mutant enzymes on a defined (in terms of sequence and length) RNA/DNA T/P. Because both excision and RNase H activities of RT are likely affected by nucleic acid sequence and length, we next evaluated the ability of WT and mutant enzymes to synthesize DNA in the presence of AZT-TP and ATP using a long heteropolymeric RNA template, corresponding to the HIV-1 sequence used for (–) strong stop DNA synthesis, primed with a DNA oligonucleotide. The 173-nucleotide incorporation events needed to produce full-length DNA product in this assay system allow for multiple AZT-TP incorporation and AZT-MP excision events during the formation of full-length final product [35;36]. In the presence of 3 mM ATP, the N348I and K70R enzymes were significantly more efficient than WT enzyme in synthesizing full-length DNA product (Fig. 3). By contrast, both the Y181C and K70R/Y181C enzymes were less efficient in generating full-length DNA product. Consistent with the data in Fig. 1, the N348I mutation partially compensated for the antagonism between K70R and Y181C: more final DNA synthesis product was evident for the K70R/Y181C/N348I RT compared to the WT, Y181C and K70R/Y181C RTs (Fig. 3).

We also assessed the susceptibility of HIV-1 containing K70R, K70R/Y181C or K70R/Y181C/N348I to AZT in a TZM-bl indicator cell line. HIV-1 containing K70R did not confer significant resistance to AZT in these assays. The EC_{50} value for inhibition of replication of K70R HIV-1 by AZT ($0.27 \pm 0.04 \mu\text{M}$, $n = 6$) was increased only 1.2-fold relative to the WT virus ($EC_{50} = 0.23 \pm 0.04 \mu\text{M}$, $n = 6$). Accordingly, the assay window was not significantly large enough to reproducibly measure AZT resistance, antagonism of K70R by Y181C, and the subsequent recovery of the AZT resistance phenotype by introduction of the N348I mutation. However, in viruses that contained both K70R and T215Y ($EC_{50} = 0.94 \pm 0.13 \mu\text{M}$, $n = 5$; 4.1-fold AZT resistance; $p = 0.009$), the introduction of the N348I mutation clearly compensated for the antagonism of the AZT-resistance phenotype by Y181C: the EC_{50} value for inhibition of replication of K70R/T215Y/Y181C/N348I HIV-1 by AZT ($1.3 \pm 0.5 \mu\text{M}$, $n = 4$) was increased 2.8-fold relative to the K70R/T215Y/Y181C virus ($EC_{50} = 0.46 \pm 0.14 \mu\text{M}$, $n = 5$; $p = 0.032$).

Taken together, these data show that the Y181C mutation does not antagonize the ability of N348I to excise AZT-MP via an RNase H-dependent mechanism in HIV-1 RT containing K70R. Accordingly, N348I in HIV-1 RT compensates for the antagonism between TAMs and Y181C.

N348I in HIV-1 RT also compensates for the antagonism of K70R by L74V and M181V

Previous studies have demonstrated that the NRTI discrimination mutations L74V and M184V antagonize the AZT-MP excision phenotype of RT containing TAMs [9–15]. To determine whether L74V- or M184V-mediated antagonism of TAMs could also be rescued by the N348I mutation, we compared the AZT-MP excision and RNase H activities of K70R/L74V HIV-1 RT with K70R/L74V/N348I HIV-1 RT and K70R/M184V HIV-1 RT with K70R/M184V/N348I HIV-1 RT (Fig. 4, Fig. 5). Consistent with previously published data [12–15], the introduction of either the L74V or the M184V mutations into HIV-1 RT containing K70R

dramatically decreased the ATP-mediated AZT-MP excision activity of HIV-1 RT (Fig.4). However, the N348I mutation completely alleviated the antagonism of K70R by L74V (Fig. 4B) and partially compensated for the antagonism of K70R by M184V (Fig.4A). As expected, N348I decreased the formation of the polymerase-independent RNase H cleavage product that reduced the RNA/DNA duplex to 10 nucleotides in length for both the K70R/M184V (Fig.5A) and K70R/L74V (Fig.5B) RTs.

In the RNase H activity experiments described above, we only assessed the activity of enzymes that contained both K70R and a mutation antagonistic to K70R (i.e. Y181C, M184V or L74V). In Fig. 5C, we show that the observed in the formation of the polymerase-independent RNase H cleavage product that reduced the RNA/DNA duplex to 10 nucleotides by N348I RT relative to the WT RT is not significantly impacted by the introduction of the Y181C, L74V or M184V mutations.

Taken together, these findings demonstrate that neither L74V nor M184V antagonizes the ability of N348I in HIV-1 RT to excise AZT-MP via an RNase H-dependent mechanism. Therefore, N348I in HIV-1 RT can also compensate for the antagonism of TAMs by L74V and M184V.

Conclusions

This study suggests that the acquisition of N348I in HIV-1 RT, which can occur early during therapy oftentimes before TAMs [24], may provide a simple genetic pathway that allows the virus to select both TAMs and mutations that are antagonistic to TAMs (e.g. L74V, Y181C and M184V). This finding is consistent with recent studies that show a strong association between N348I with TAMs, M184V/I and Y181C [24] or that N348I is frequently observed in AZT- and/or ddI-containing therapies [37]. In fact, in the Stanford University HIV Database, N348I is frequently observed in viruses from patients failing combination antiretroviral therapies that contained an NNRTI and either AZT/3TC (frequency of N348I = 22.2%) or AZT/ddI (frequency of N348I = 9.5%). Finally, this study further highlights the complex but potentially important role of mutations in the C-terminal domains of HIV-1 RT in drug resistance.

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Figure 1. ATP-mediated AZT-MP excision activity of WT, K70R, Y181C, K70R/Y181C and K70R/Y181C/N348I HIV-1 RT on RNA/DNA and DNA/DNA T/Ps

A) Schematic representation of the ATP-mediated excision assay used in this study. Experimental details are provided in the *Materials and Methods*. AZT-MP is designated as “Z”

B) Representative autoradiogram of AZT-MP excision reactions carried out by WT and mutant RT on RNA/DNA and DNA/DNA T/Ps. The reaction times were 3, 5, 10, 15, 30, 60 and 90 min.

C) Isotherms of ATP-mediated AZT-MP excision reactions carried out by WT and mutant HIV-1 RT on a DNA/DNA T/P (left-hand curve) and on an RNA/DNA T/P (right-hand curve). Data are the mean $\pm$ standard deviation from at least three independent experiments.

Figure 2. RNase H cleavage activity of WT, K70R, Y181C, N348I, K70R/Y181C and K70R/Y181C/N348I HIV-1 RT that occurs during the AZT-MP excision reaction

A) Schematic illustrating the location of the RNase H cleavage sites in the RNA template.

B) Representative autoradiogram of the RNase H cleavage activity of the WT and mutant HIV-1 RTs. Experiments were carried out as described in the *Materials and Methods*. The reaction times were 1, 3, 5, 10, 20, 30, 45, 60 and 90 min.

Figure 3. Autoradiogram of steady-state DNA synthesis by WT and mutant HIV-1 RT in the presence of AZT-TP and 3 mM ATP

A) Complete autoradiogram indicating primer, final product, and AZT-MP chain-termination sites. Experiments were carried out as described in the *Materials and Methods*. The time points for the reactions were 15, 30, 60, 90 and 120 min.

B) Different contrast of the final product bands from the autoradiogram presented in (A). This figure highlights that the addition of N348I to K70R/Y181C HIV-1 RT compensates for the antagonism of K70R by Y181C.

Figure 4. ATP-mediated AZT-MP excision activities of WT and mutant HIV-1 RT on an RNA/DNA T/P

A) The autoradiogram shows the AZT-MP excision reactions carried out by K70R/M184V and K70R/M184V/N348I HIV-1 RT. The reaction times were 3, 5, 10, 20, 30, 60 and 90 min. The isotherm shows ATP-mediated AZT-MP excision reactions carried out by WT, K70R, K70R/M184V and K70R/M184V/N348I HIV-1 RT. Data are the mean $\pm$ standard deviation from at least three independent experiments.

B) The autoradiogram shows the AZT-MP excision reactions carried out by K70R/L74V and K70R/L74V/N348I HIV-1 RT. The reaction times were 3, 5, 10, 20, 30, 60 and 90 min. The isotherm shows ATP-mediated AZT-MP excision reactions carried out by WT, K70R, K70R/L74V and K70R/L74V/N348I HIV-1 RT. Data are the mean $\pm$ standard deviation from at least three independent experiments.

Figure 5. RNase H cleavage during ATP-mediated AZT-MP excision by WT and mutant HIV-1 RT

A) Representative autoradiogram of the RNase H cleavage activities of K70R/LM184V and K70R/M184V/N348I HIV-1 RT. The reaction times are 1, 3, 5, 10, 20, 30, 40, 60 and 90 min.

B) Representative autoradiogram of the RNase H cleavage activities of K70R/L74V and K70R/L74V/N348I HIV-1 RT. The reaction times are 1, 3, 5, 10, 20, 30, 60 and 90 min.

C) Representative autoradiogram of the RNase H cleavage activities of WT, N348I, Y181C/N348I, L74V/N348I and M184V/N348I HIV-1 RT. The reaction times are 1, 3, 5, 10, 20, 30, 60 and 90 min.