Epidemiological and Biological Evidence for a Compensatory Effect of Connection Domain Mutation N348I on M184V in HIV-1 Reverse Transcriptase

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Background. The connection domain mutation N348I confers resistance to zidovudine (AZT) and is associated with the lamivudine (3TC) mutation M184V. We explored the biochemical and virological influence of N348I in the context of M184V.

Methods. Genotypic resistance data for patients receiving monotherapy or dual therapy with AZT, lamivudine (3TC), or AZT/3TC were analyzed. Rates of N348I emergence were compared between treatment groups. Mutant reverse transcriptases (RTs) containing M184V and/or N348I were generated to study enzymatic and virological properties.

Results. We included 50 AZT-treated, 11 3TC-treated, and 10 AZT/3TC-treated patients. N348I was observed in 3 (6%), 0, and 4 (40%) of these patients, respectively. The rate of N348I emergence was increased by 5-fold in the AZT/3TC group (11.7 instances [95% confidence interval {CI}, 3.2–30.1 instances] per 100 person-years of receipt of AZT), compared with the rate noted for the AZT group (2.3 instances [95% CI, 0.4–6.8 instances] per 100 person-years of receipt of AZT; $P = .04$). Biochemical data show that N348I can partially compensate for the diminution in processive DNA synthesis and the reduction in AZT excision associated with M184V. Furthermore, virological analyses demonstrate that N348I confers low-level resistance to AZT and partly restores the reduced RT activity of the M184V variant.

Conclusion. In vivo selection of N348I is driven by AZT and is further facilitated when 3TC is coadministered. Compensatory interactions between N348I and M184V help to explain these findings.

In human immunodeficiency virus type 1 (HIV-1), the development of resistance to antiretroviral treatment (ART) is induced by mutational changes in the genome,

and many mutations have already been characterized that confer resistance to specific antiretroviral compounds [1]. Because of the mode of action of nucleoside reverse-transcriptase inhibitors (NRTIs), such as zidovudine (AZT), and nonnucleoside reverse-transcriptase inhibitors (NNRTIs), such as nevirapine (NVP), these mutations are predominantly located in the N-terminal region of the p66 subunit of the HIV reverse transcriptase (RT; amino acid residues 1–321). For example, classical mutations conferring resistance to AZT are clustered around the polymerase active site and are referred to as "TAMs" (thymidine analogue–associated mutations). They act by increasing the rate of adenosine triphosphate (ATP)–dependent excision of the AZT-
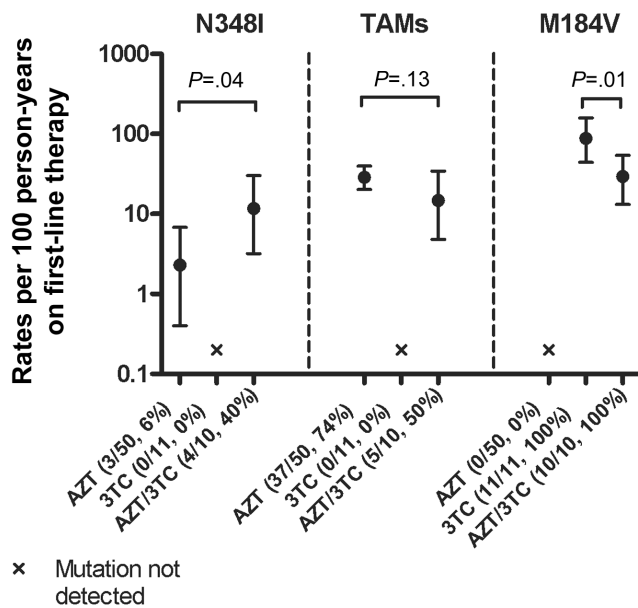
Figure 1. Rates of selection of N348I, thymidine analogue–associated mutations (TAMs), and M184V, according to different first-line nucleoside reverse-transcriptase inhibitor treatments (zidovudine [AZT], AZT/lamivudine [3TC], and 3TC). *P* values were calculated using Poisson regression. Error bars denote 95% confidence intervals.

monophosphate (AZT-MP) from the 3′ end of the primer, whereby ATP acts as a pyrophosphate (PPi) donor [2]. However, several more-recent studies have demonstrated that residues of the C-terminus, including the connection domain (residues 322–440) and residues in the ribonuclease (RNase) H region (residues 441–560) of RT, may also have an effect on viral susceptibility to antiretroviral drugs [3–8].

The connection domain mutation N348I is arguably the best studied example in this context [9–11]. The prevalence of this mutation is very low in ART-naive patients but is high in treatment-experienced patients [9, 11, 12]. The presence of N348I has been associated with increased resistance to both AZT and NVP, making N348I a dual-class resistance mutation [9, 11]. Initial observations indicated that many C-terminus mutations, including N348I, can enhance AZT resistance to a background of TAMs [4, 13, 14]. It was hypothesized that mutations in the C-terminus of RT reduce RNase H activity, thereby delaying the degradation of the RNA template and complex dissociation, which in turn allows more time for AZT-MP excision [4]. Further investigation into the role of N348I in conferring AZT resistance revealed that this mutation reduces RNase H activity by reducing the affinity of the enzymes for the DNA/RNA substrate, specifically in the RNase H–competent complex [10]. An additional observation was that the N348I mutation also increased processive DNA synthesis in enzymes containing TAMs. These findings suggested that N348I contributes to AZT

resistance in both an RNase H–dependent and RNase H–independent manner.

However, the dynamics of N348I emergence in vivo and the clinical relevance of this mutation are poorly understood. In a recent study, Yap et al [9] observed that N348I is preferentially selected during treatment including AZT or NVP and that it appears very early during the course of treatment. The data from that study and the observations of von Wyl et al [15] further indicate that the presence of N348I is strongly associated with the presence of lamivudine (3TC) mutation M184V, although N348I alone does not appear to have a significant effect on 3TC resistance [9]. It has previously been established that the M184V mutation antagonizes AZT resistance in the background of TAMs, leading to AZT resensitization and AZT hypersusceptibility effects when these mutations are present together in the virus [16–19]. Hence, it was hypothesized that the presence of N348I may mediate this antagonistic relationship and allow for simultaneous resistance to AZT and 3TC in the presence of TAMs and M184V. However, cell-based in vitro susceptibility measurements do not support this theory. M184V appears to cause AZT resensitization even when N348I is present in the background of TAMs [5, 9]. Thus, the viral incentive for the early coselection of N348I and M184V remains elusive. In the present study, we aimed to explore the dynamics of the emergence of the N348I mutation in the context of M184V in vivo and in vitro. In particular, we investigated the effects of this coselection on the enzymatic and virological functions of RT.

METHODS

Epidemiological Analysis

We pooled viral sequences from the AIDS Clinical Trials Group (ACTG) 320 trial [20, 21] (found in the Stanford University HIV Drug Resistance Database [22]), the Swiss HIV Cohort Study [23, 24], and a trial of 3TC monotherapy [25]. These sequences span the full protease and the first 400 amino acids of the RT (GenBank accession numbers GQ848100–GQ848156). We selected genotypic tests that were performed while patients were receiving the first course of monotherapy or dual antiretroviral therapy with AZT and/or 3TC. Rates of emergence of mutations (TAMs, M184V, and N348I) were calculated on the basis of the total time that patients had received treatment until genotypic testing was performed. These rates were compared across the different treatments with Poisson regression. Statistical analyses were performed with Stata (version 10.1 SE; Stata Corporation). The level of significance was set at 5%, and all *P* values were 2-sided.

Construction of Recombinant RT pHXB2 Clones

To create a pHXB2 RT deletion clone, a unique *Ngo*MIV restriction site at the end of RT was generated by site-directed
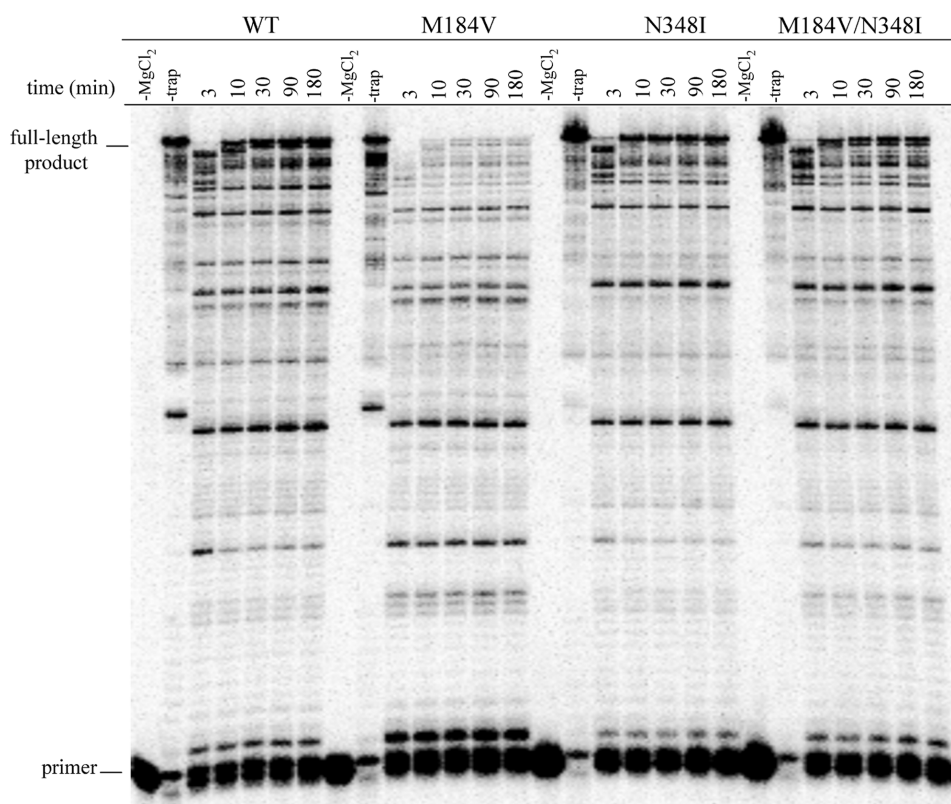
Figure 2. The effect of N348I and M184V mutations on processive DNA synthesis. DNA synthesis was monitored in a time course after the addition of magnesium chloride (MgCl₂) and a heparin trap. The control lanes for each enzyme show that no DNA synthesis occurs in the absence of MgCl₂ and that, in the absence of a heparin trap, all enzymes can generate the full-length product within 1 h.

mutagenesis polymerase chain reaction (PCR). In this construct, the viral RT gene is replaced with a linker sequence after digestion of the plasmid with *Mlu*NI (Roche) and *Ngo*MIV (Roche). The linker sequence containing the unique *Asp*I site was made using the primers 5′-*CCA*__GACGCTGTC__G-3′ and 5′-__CCGGC__**GACAGCGTC***TGG*-3′ (text shown in boldface type denotes the *Asp*I site, text in italicized type denotes the *Mlu*NI site, and text that is underlined denotes the *Ngo*MIV site). These primers contain part of the blunt-end *Mlu*NI restriction site and partly overlap with the pHXB2 *Ngo*MIV restriction site. The primers were incubated at 95°C for 5 min, 55°C for 15 min, and 4°C for correct annealing. Subsequently, the *Asp*I linker sequence was ligated overnight at 4°C by use of T4 ligase (Promega), resulting in pHXBΔRTAsp. By doing so, transformation of relegated vectors can be prevented by digestion with *Asp*I.

To obtain the pHXB2 with the various RT mutations (wild type [WT], M184V, N348I, and M184V/N348I), the pRT6H DNA constructs of WT, M184V, N348I, and M184V/N348I RT were amplified using RTBall 5′-ATGGCCCAAAAGTTAAACA-ATGG-3′ and NgoMIV-INT1rev 5′-TTAGTCAGTGCCGGCA-TCAGGA-3′, by means of the Expand High Fidelity PCR System (Roche), essentially as described by the manufacturer. The PCR

product and the pHXBΔRTAsp were digested with *Ngo*MIV and *Mlu*NI and subsequently were ligated overnight at 4°C with T4 ligase. The ligated product was digested with *Asp*I and purified with the Qiagen PCR Purification Kit (Qiagen). These ligation products were transformed into *Escherichia coli* JM109 High Efficiency Competent Cells (Promega), by means of heat shock at 42°C, and spread on Luria-Bertani agar plates containing 40 µg/mL ampicillin. Colonies were inoculated into 100 mL of Luria-Bertani medium with 40 µg/mL ampicillin. The plasmids were isolated using the Plasmid Midi Kit (Qiagen). All HIV-1 constructs were verified by nucleotide sequencing.

Viral Culture

Cells. MT2 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 with L-glutamine (BioWhittaker) supplemented with 10% fetal bovine serum (Biochrom AG) and 10 µg/mL gentamicin (Gibco). 293T cells were maintained in Dublecco's modified Eagle medium (BioWhittaker) supplemented with 10% fetal bovine serum (Biochrom AG) and 10 µg/mL gentamicin. All cells were passed twice weekly.

Generation of recombinant viruses. To obtain the recombinant viruses, 10 µg of the recombinant plasmids was used to
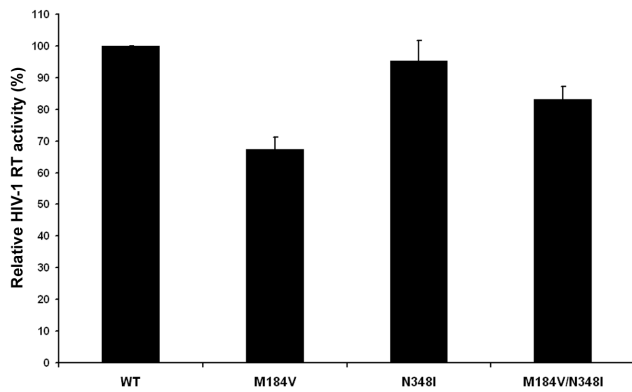
Figure 3. Relative reverse-transcriptase (RT) activity of wild-type (WT) virus and recombinant RT viruses: M184V, N348I, and M184V/N348I. WT RT activity was set as 100%, and the viral RT activity of recombinant strains was plotted against WT activity ($n = 3$). Error bars denote standard errors. HIV-1, human immunodeficiency virus type 1.

transfect 293T cells at 90%–95% confluence. For transfection, Lipofectamine 2000 reagent (Invitrogen) was used in accordance with the manufacturer's protocol. After 48 h, recombinant viruses were harvested, and viral supernatant was obtained for p24 analysis.

RT Activity and Drug Susceptibility Assays

To determine RT activity, 20 ng of p24 from different HXB2 viral mutant strains (N348I, M184V, and the double mutant M184V/N348I) and WT virus was used in the RT activity assay colorimetric (Roche), as described by the manufacturer. The drug susceptibility of recombinant viruses was determined in the multiple-cycle MTT assay [26].

Biochemical Studies

Enzymes and nucleic acids. The HIV-1 RT enzymes were generated and purified as described elsewhere [27]. "TAMs" refers to the AZT-resistant enzyme harboring mutations M41L, T215Y, L210W, and D67N. "WT" RT refers to the HXB2 HIV strain WT enzyme with no mutations. RNA and DNA oligonucleotides were obtained as described elsewhere [10].

Enzyme processivity. A total of 20 nmol/L 5′-radiolabeled DNA primer PBS-28 was annealed to complementary PBS-250 [10]. The RNA/DNA hybrid was then incubated with 400 nmol/L RT and 2 μmol/L of each of the 4 deoxyribonucleotide triphosphates (dNTPs) in a buffer containing 100 μmol/L EDTA, 50 mmol/L NaCl, and Tris-HCl, pH 7.8. As described elsewhere [10], the large excess of RT over the primer/template substrate compensates for putative differences in active site concentrations among the different enzyme preparations. DNA synthesis was initiated at 37°C with the addition of 6 mmol/L magnesium chloride (MgCl$_2$) and 2 mg/mL heparin trap (Bioshop). DNA

synthesis products were isolated and visualized as described elsewhere [10].

Combined AZT-MP incorporation and excision. Inhibition of DNA synthesis was monitored in the presence of 2 μmol/L AZT-triphosphate (TP), under the conditions described above (in the absence of a heparin trap). Excision and the ensuing rescue of chain-terminated DNA synthesis were subsequently studied in the presence of 50 μmol/L PPi and 3 mmol/L ATP, respectively. PPi-mediated DNA synthesis rescue was quantified with ImageQuant software (version 5.2; GE). The fraction of full-length product at 180 min is measured as the ratio of the product over the sum of the product and unextended substrate.

RNase H activity. The radiolabeled RNA template (5′-ggaaaucucuagcaguggcgcccgaacagggacct-3′) was hybridized to an excess of DNA primer (5′-AGGTCCCTGTTCGGGCGCCACT-3′). A total of 100 nmol/L RNA/DNA hybrid was then incubated with a 2-fold molar excess of RT (WT or mutant) in the presence of 100 μmol/L EDTA, 50 mmol/L NaCl, and 50 mmol/L Tris-HCl, pH 7.8. RNase H cleavage was initiated with the addition of 6 mmol/L MgCl$_2$ and monitored over time at 37°C (3, 7, 15, 30, 45, and 60 min). Samples were isolated and visualized on a 12% polyacrylamide gel.

RESULTS

Rates of selection of N348I during therapy with AZT or AZT/3TC. In total, 71 patients (50 receiving AZT, 10 receiving AZT/3TC, and 11 receiving 3TC) had genotypic drug resistance tests performed while they were receiving their first NRTI treatment. As shown in Figure 1, of the 71 samples obtained from patients receiving first-line treatment, 3 (6%) of 50 patients who were receiving AZT harbored viruses with the N348I mutation, compared with 4 (40%) of 10 patients who were receiving AZT/3TC. N348I was not detected in the 11 samples from the 3TC monotherapy group. The duration of treatment for patients who had received thymidine analogues at the time of testing was similar for the AZT and AZT/3TC groups, with a median duration of 1.5 years (interquartile range [IQR], 0.7–4.2 years) and 2.1 years (IQR, 0.5–5.0 years), respectively ($P = .86$, by Mann-Whitney U test). Consequently, the AZT/3TC group had a >5-fold higher rate of N348I emergence (11.7 instances/100 person-years of exposure to thymidine analogues [95% CI, 3.2–30.1 instances/100 person-years of exposure to thymidine analogues]) than the AZT group (2.3 instances/100 person-years of exposure to thymidine analogues [95% CI, 0.4–6.8 instances/100 person-years of exposure to thymidine analogues]; $P = .04$). The fact that no instance of an N348I mutation occurred in the 3TC monotherapy group after a median of 1.2 years (IQR, 0.70–1.4 years) of treatment strongly suggests that N348I is selected by AZT but that selection is greatly enhanced when 3TC is coadministered with AZT.
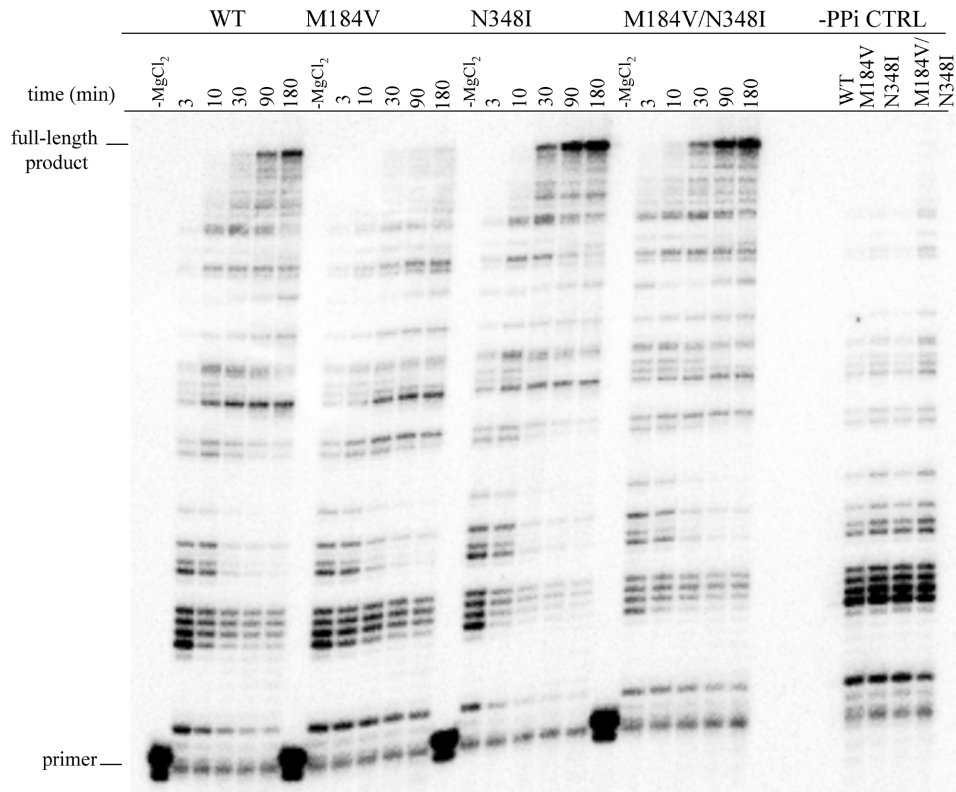
Figure 4. Pyrophosphate (PPi)–mediated excision of zidovudine-monophosphate (AZT-MP) and ensuing DNA synthesis rescue in the context of M184V and N348I. DNA synthesis was monitored in the presence of 2 μmol/L deoxyribonucleotide triphosphates, 2 μmol/L AZT, and 50 μmol/L PPi. Control lanes show the unextended primer in the absence of magnesium chloride (MgCl$_2$). PPi CTRL, DNA synthesis in the absence of PPi. WT, wild type.

N348I and compensation for M184V-induced deficits in processive DNA synthesis. Previous studies have shown that RT enzymes harboring the M184V mutation show deficits in processive DNA synthesis and nucleic acid binding [28, 29]. We have shown elsewhere that N348I can increase processive DNA synthesis in the background of TAMs, although the deficits with regard to processive DNA synthesis with enzymes containing TAMs are, by far, not as pronounced as those seen with M184V [10]. Hence, we aimed to determine whether the N348I mutation can also compensate for M184V-induced polymerization deficits when the 2 mutations are present together in RT. We monitored processive DNA synthesis with enzymes harboring M184V, N348I, or both mutations (Figure 2). DNA synthesis was initiated in the presence of a heparin trap to ensure single-turnover conditions. As expected, the M184V mutant shows reduced processivity when compared with WT RT, as is indicated by reductions in full-length DNA product formation. The N348I mutant, on the other hand, does not appear to be compromised in this regard. Interestingly, when the 2 mutations are present together, processivity is reestablished, suggesting that the presence of N348I mutation does indeed compensate for M184V-introduced deficits in processive DNA synthesis. These findings are consistent with the results

of an activity assay with RT enzymes isolated from virions (Figure 3). Diminished product formation associated with M184V is partially corrected by N348I.

We next asked whether the combined effects of an efficient processive DNA synthesis and diminished RNase H activity associated with N348I may facilitate excision and rescue of DNA synthesis. If correct, we also expect to observe such a phenotype in the absence of TAMs with PPi as the substrate for the excision reaction. To address this problem, we performed a multisite AZT-MP excision assay in the presence of PPi, in which the appearance of full-length DNA product is indicative of rescued DNA synthesis (Figure 4). After 180 min, the M184V mutant showed 24% full-length product formation, compared with 59% for WT RT. This reduction in full-length product formation correlates with reduced DNA processivity of the mutant enzyme and its diminished ability to excise AZT-MP [16]. On the other hand, the enzyme containing the N348I mutation shows even subtle increases in full-length product formation, compared with WT RT (81% vs 59%, respectively). The M184V/N348I mutant shows AZT-MP excision levels comparable to those of the N348I mutant (78% vs 81%, respectively), suggesting that N348I-mediated AZT-MP excision is not largely reduced against a background of M184V. We also
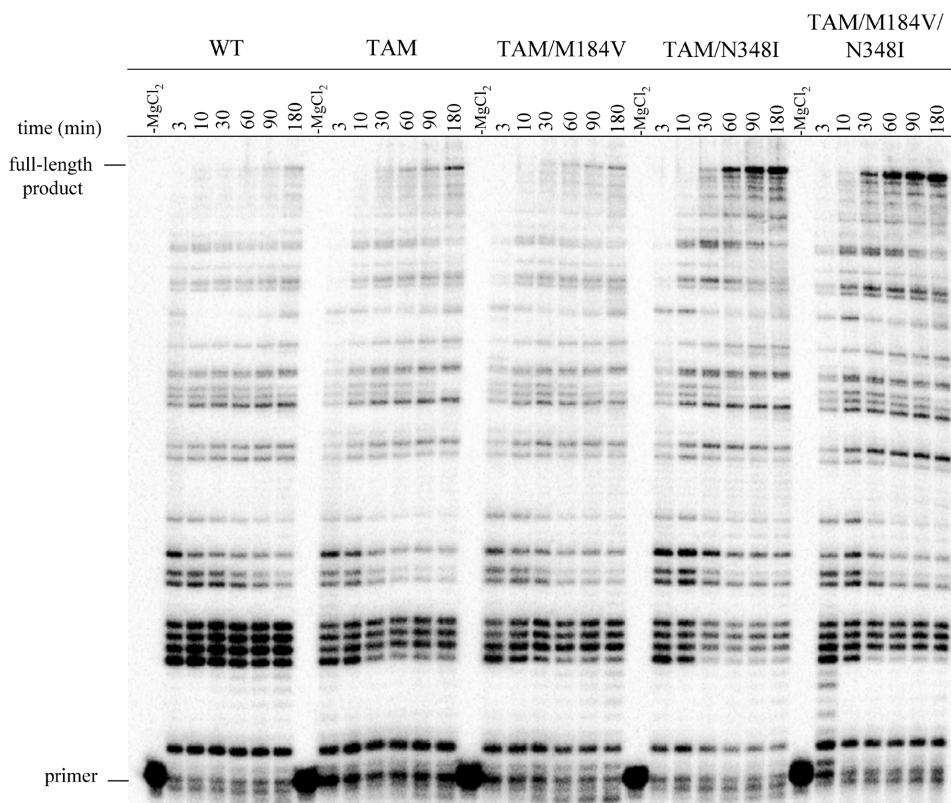
Figure 5. Adenosine triphosphate (ATP)–mediated excision of zidovudine-monophosphate (AZT-MP) and ensuing DNA synthesis rescue in the context of thymidine analogue–associated mutations (TAMs), M184V, and N348I. DNA synthesis was monitored in the presence of 2 μmol/L deoxyribonucleotide triphosphates, 2 μmol/L AZT, and 3.5 mmol/L ATP. Control lanes show the unextended primer in the absence of magnesium chloride (MgCl$_2$).

studied the efficiency of ATP-dependent excision and the ensuing rescue of AZT-terminated DNA synthesis at multiple positions (Figure 5). When M184V is introduced against a background of TAMs, rescue of DNA synthesis is reduced relative to TAMs, which is in agreement with AZT resensitization effects associated with M184V. The mutant enzyme containing TAMs and N348I shows the highest level of DNA synthesis, and the yield of the full-length product remains at similar high levels when the M184V mutation is simultaneously present. These findings suggest that N348I is able to override the negative effect of M184V on the excision reaction and that the N348I phenotype is dominant over M184V in these biochemical assays.

Failure of M184V to compensate for N348I-mediated deficits in RNase H activity. Conversely, we hypothesized that the M184V mutation may be able to counteract the RNase H–related deficits of N348I that contribute, at least in part, to the increased efficiency of the combined excision rescue of DNA synthesis. To address this question, we studied the efficiency of RNase H cleavage in time course experiments (Figure 6). The M184V mutation does not appear to affect RNase H activity, as indicated by high yields of short RNA products that are comparable with WT RT. The N348I mutant shows reduced

RNase H activity, as seen by the reduced production of shorter products. The addition of M184V against the N348I background does not appear to compensate for this deficit.

Drug susceptibility assays. Susceptibility assays for AZT exhibited a 2-fold increase in resistance when N348I was present, which decreased almost to resistance levels noted for the WT in the context of M184V and N348I (Figure 7). Resistance to 3TC was high in M184V and M184V/N348I mutants (>600-fold resistance for both), whereas levels of 3TC resistance in mutants with N348I alone were comparable to those noted in the WT (not shown).

DISCUSSION

Using a multidisciplinary approach, we investigated in vivo dynamics and the biochemical and virological implications of the emergence of the connection domain mutation N348I in the context of M184V. Our in vivo analyses indicated that N348I is selected by thymidine analogues but that selection rate is enhanced by 5-fold when 3TC is used concomitantly (Figure 1). This observation strongly suggests a synergism between M184V and the emergence of N348I. The biochemical experiments yielded possible explanations for the coselection of these
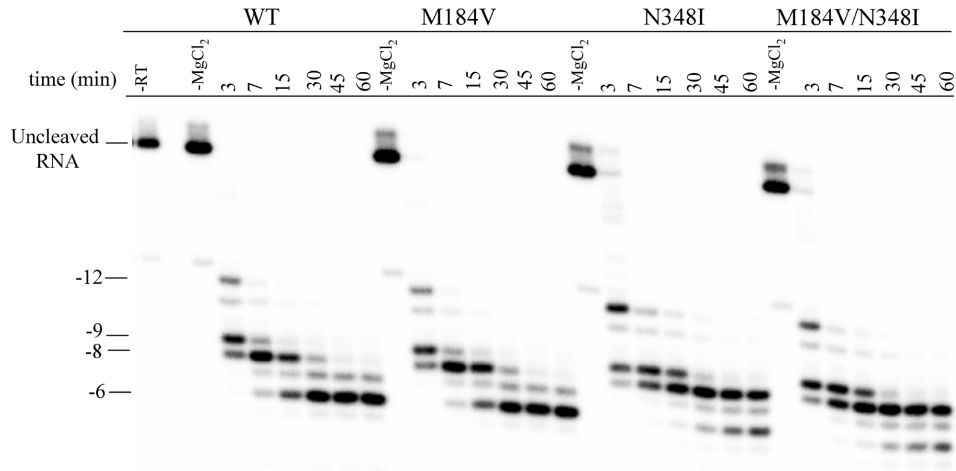
Figure 6. The effect of N348I and M184V mutations on RNA template cleavage. Ribonuclease (RNase) H activity for each reverse-transcriptase (RT) enzyme was monitored in a time course after the addition of magnesium chloride (MgCl₂). Controls indicate that RNA cleavage occurs only in the presence of RT and MgCl₂. The −12, −9, −8 and −6 labels denote the RNA fragment size, measured from the 3′ end of the primer. WT, wild type.

mutations. The M184V mutant shows severe deficits in processive DNA synthesis. Compensation for this phenotype occurs when the N348I mutation is added (Figure 2). These findings were confirmed in an RT assay in which the addition of N348I to the recombinant RT in the context of M184V led to a restoration of RT activity, compared with an RT with M184V as the sole mutation (Figure 3).

We further studied the excision of AZT-MP in a panel of mutant RTs with N348I, M184V (Figure 4), and TAMs (Figure 5). In this study, we found that RT harboring M184V alone reduces PPi-mediated AZT-MP excision, whereas the presence of N348I alone shows a subtle increase in the excision rate relative to WT. Moreover, we observed that the introduction of N348I mutation in a background of M184V fully restores AZT-MP removal and DNA synthesis rescue (Figure 4). When we studied M184V- and N348I-mediated alterations in the efficiency of ATP-dependent AZT-MP excision in the context of TAMs, we found that N348I is able to further enhance the excision rate in the presence of TAMs and to override the negative effect of M184V on the reaction (Figure 5). This effect, although more subtle, was confirmed in a phenotypic drug resistance assay (Figure 7).

RNase H activity assays further confirm that the presence of N348I leads to reduced RNase H activity, whereas M184V has no effect in this regard (Figure 6). In addition, no increase in RNase H activity was observed when M184V was introduced in the background of N348I, suggesting that M184V cannot compensate for RNase H–mediated deficiencies introduced by N348I.

Although recent studies suggested that N348I may not coun-

teract the antagonism between M184V and TAMs [5, 9], our own data point to a subtle reduction in AZT susceptibility when the N348I/M184V double mutant was compared with the recombinant virus containing M184V. Moreover, DNA product formation in the absence of inhibitors is likewise increased with the double mutant. In light of our findings that N348I hardly ever occurs alone and that M184V is preceding the emergence of N348I, these combined data are indeed consistent with a compensatory role of N348I under selective pressure by AZT.

To summarize, in conjunction with in vivo clinical data, our findings suggest that N348I may arise in association with failure of treatment involving thymidine analogues, particularly in com-
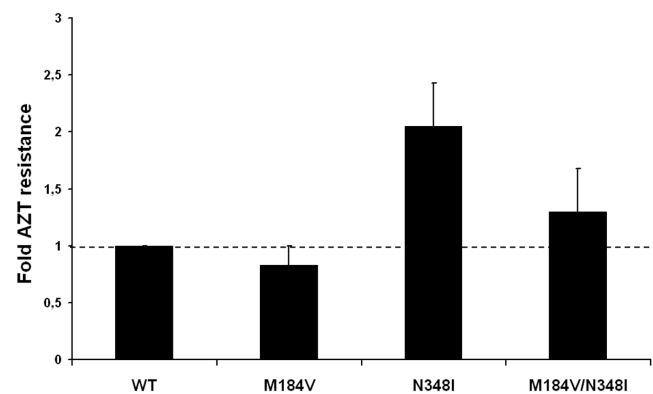

Figure 7. Fold resistance to zidovudine (AZT) for wild-type (WT) virus and recombinant reverse-transcriptase (RT) viruses: M184V, N348I, and M184V/N348I. Fold resistance of recombinant strains was plotted against WT ($n \geq 4$). Error bars denote the standard error.

bination with 3TC, because N348I may compensate for M184V-mediated deficits with regard to DNA polymerization and the combined excision and rescue of AZT-terminated DNA synthesis. The clinical effect of the N348I connection domain mutation, with regard to treatment responses to NRTIs and NNRTIs to date, is not known. A preliminary analysis within the Swiss HIV Cohort Study did not reveal an effect of N348I on treatment response; however, the sample size was limited (data not shown). Additional clinical studies are clearly warranted.

SWISS HIV COHORT STUDY MEMBERS

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