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Combinations of Mutations in the Connection Domain of Human Immunodeficiency Virus Type 1 Reverse Transcriptase: Assessing the Impact on Nucleoside and Nonnucleoside Reverse Transcriptase Inhibitor Resistance▽

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Recent reports have described the effect of mutations in the connection and RNase H domains of reverse transcriptase (RT) on nucleoside and nonnucleoside reverse transcriptase inhibitor (NRTI and NNRTI, respectively) resistance in the presence of thymidine analog resistance mutations (TAMs) and NNRTI mutations (J. H. Brehm, D. Koontz, J. D. Meteer, V. Pathak, N. Sluis-Cremer, and J. W. Mellors, J. Virol. 81:7852-7859, 2007; K. A. Delviks-Frankenberry, G. N. Nikolenko, R. Barr, and V. K. Pathak, J. Virol. 81:6837-6845, 2007; G. N. Nikolenko, K. A. Delviks-Frankenberry, S. Palmer, F. Maldarelli, M. J. Fivash, Jr., J. M. Coffin, and V. K. Pathak, Proc. Natl. Acad. Sci. U. S. A. 104:317-322, 2007; G. N. Nikolenko, S. Palmer, F. Maldarelli, J. W. Mellors, J. M. Coffin, and V. K. Pathak, Proc. Natl. Acad. Sci. U. S. A. 102:2093-2098, 2005; and S. H. Yap, C. W. Sheen, J. Fahey, M. Zanin, D. Tyssen, V. D. Lima, B. Wynhoven, M. Kuiper, N. Sluis-Cremer, P. R. Harrigan, and G. Tachedjian, PLoS Med. 4:e335, 2007). In the present study, novel mutations in the connection domain of RT (T369I/V), first identified in patient-derived viruses, were characterized, and their effects on NNRTI and NNRTI susceptibility were determined. Furthermore, the effect of N348I on NRTI and NNRTI resistance was confirmed. HIV-1 with either N348I or T369I/V demonstrated reduced susceptibility to nevirapine (NVP), efavirenz (EFV), delaviridine (DLV), and zidovudine (ZDV) compared to wild-type HIV-1. However, HIV-1 with T369I and N348I demonstrated 10- to 60-fold resistance to these same drugs. In clinical samples, these two connection domain RT mutations were predominantly observed in viruses containing TAMs and NNRTI mutations and did not alter the susceptible-resistant classifications of these samples. Introduction of T369I, N348I, or T369I/N348I also reduced replication capacity (RC). These observations suggest that it may be of scientific interest to test these mutations against new NNRTI candidates.

Human immunodeficiency type 1 (HIV-1) reverse transcriptase (RT) catalyzes the conversion of single-stranded genomic RNA into double-stranded DNA, a process that involves the RNA-dependent and DNA-dependent DNA polymerase and RNase H activities of the enzyme (10). HIV-1 RT is an asymmetric heterodimer consisting of p66 and p51 polypeptides (13). The p66 subunit contains both the DNA polymerase and RNase H active sites and is composed of three domains: the N-terminal polymerase domain (residues 1 to 318), the connection domain (residues 319 to 426), and the C-terminal RNase H domain (residues 427 to 560). The p51 subunit, although it lacks the C-terminal RNase H domain, contains identical N-terminal sequences and is thought to primarily serve a structural role that provides RT stability (34).

HIV-1 RT is an important therapeutic target for HIV-AIDS. Two distinct classes of RT inhibitors are in clinical use: nucleoside-nucleotide RT inhibitors (NRTI), which include zidovudine (3′-azido-3′-dideoxythymidine [ZDV]), lamivudine (3TC), emtricitabine (FTC), zalcitabine (ddC), didanosine (ddI), stavudine (d4T), abacavir (ABC), and tenofovir (TNV), and nonnucleoside inhibitors (NNRTI), which include nevirapine (NVP), delavirdine (DLV), efavirenz (EFV), and etravirine (ETR). NRTIs inhibit HIV-1 replication by competing with the natural deoxynucleoside triphosphates (dNTPs) for incorporation into newly synthesized viral DNA and subsequently causing premature termination of the nascent DNA strand. In contrast, NNRTIs act noncompetitively by binding to a hydrophobic pocket adjacent to, but distinct from, the polymerase active site and mediating allosteric changes that inhibit RT activity (14, 31, 35).

Suboptimal treatment with NNRTI-containing regimens frequently results in the rapid emergence of drug-resistant virus containing amino acid changes within the NNRTI-binding pocket that diminish or eliminate drug binding (25, 26, 31, 33). The most frequently observed amino acid changes associated with NNRTI resistance are located at positions 100 to 110, 180 to 190, and 220 to 240. NRTI-associated resistance mutations can be broadly categorized into two groups depending on their mechanism of resistance, only one of which affects drug binding (16, 30). The polymerase domain mutations M41L, D67N, K70R, L210W, T215F/Y, and K219Q/E, typically referred to as thymidine analog resistance mutations (TAMs), increase the ability of HIV-1 RT to excise a newly incorporated NRTI-monophosphate (NRTI-MP), thereby reversing premature chain termination (1, 4, 17). This mechanism is termed NRTI excision. By comparison, the polymerase domain mutations

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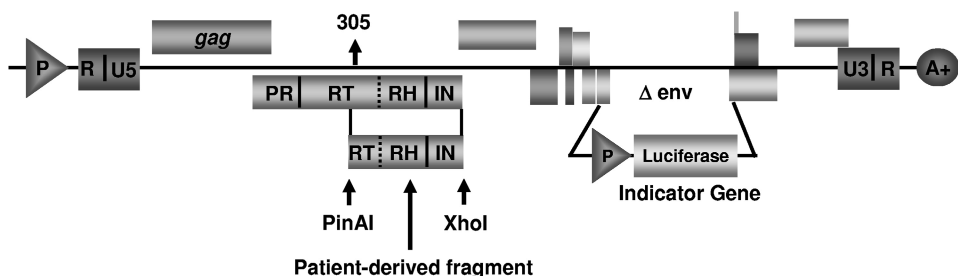

FIG. 1. Schematic representation of RH/IN resistance test vectors. RH/IN RTVs were assembled by cloning *pol* sequences encoding the C terminus of RT (codons 317 to 426), RNase H (RH), and IN from individual patient viruses into a HIV-1 (NL4-3) vector containing a luciferase expression cassette inserted into the *env* region.

K65R, K70E, L74V, Q151M (in complex with A62V, V75I, F77L, and F116Y), and M184V increase the selectivity of RT for incorporation of natural dNTP substrates over the NRTI-triphosphate (NRTI-TP) (15, 28). This mechanism is termed NRTI discrimination.

Most NNRTI and NRTI resistance mutations are located in the DNA polymerase domain of HIV-1 RT. However, several recent reports have described mutations in the connection and RNase H domains of RT that reduce NRTI and NNRTI susceptibility (5, 7, 11, 12, 19, 36). Nikolenko et al. reported that, in the context of TAMs, mutations D549N and H539N in the RNase H domain of RT reduced susceptibility to ZDV and decreased RNase H activity (19). A subsequent report by the same authors described eight amino acid substitutions (E312Q, G335C/D, N348I, A360I/V, V365I, and A376S) in the RT connection domain of viruses isolated from treatment-experienced patients, but not treatment-naive patients, which also reduced ZDV susceptibility and decreased RNase H activity (18). Brehm et al. also reported that two novel mutations identified *in vitro* under ZDV selective pressure, A371V in the connection domain and Q509L in the RNase H domain, together conferred a 90-fold reduction in ZDV susceptibility in the presence of TAMs but had a minimal effect in the absence of TAMs (5). More recently, Yap et al. demonstrated that N348I in the connection domain of RT conferred large reductions in ZDV and NVP susceptibility. N348I appeared early in therapy and was highly associated with NRTI (M41L, T215Y/F, M184V/I) and NNRTI (K103N, Y181C/I) resistance mutations (36).

In this study we identified novel mutations (T369I/V) in the connection domain of RT and evaluated their effects on NNRTI and NRTI susceptibility. In addition, we identified patient viruses containing the N348I mutation and confirmed the effect of this mutation on ZDV and NNRTI resistance as previously reported. We confirmed our observations with patient viruses by constructing and testing site-directed mutants (SDMs) containing the N348I and/or T369I/V mutations in the RT sequence of a well-characterized laboratory strain of HIV-1 (NL4-3). We also interrogated a large phenotypic-genotypic database to evaluate the prevalence of these mutations in treatment-experienced and treatment-naïve patient populations and to assess their impact on NRTI and NNRTI susceptibility and virus replication capacity (RC).

MATERIALS AND METHODS

Antiviral drugs. The following is a list of the study drugs and their sources: zidovudine (ZDV, AZT), didanosine (ddI), stavudine (d4T), and zalcitabine (ddC) (Sigma Chemical, St. Louis, MO); lamivudine (3TC) (Moravek Chemical, Brea, CA); nevirapine (NVP) (Roxanne Laboratories, Redding, CT); delavirdine (DLV) (Pharmacia-Upjohn, Kalamazoo, MI); and efavirenz (EFV) (DuPont Pharmaceuticals, Wilmington, Del.).

Construction of resistance test vectors. PhenoSense resistance test vectors (RTVs) containing patient-derived HIV-1 *pol* sequences encoding protease (PR) and the first 305 amino acids of reverse transcriptase (RT), collectively referred to as PR/RT, were constructed as previously described (23). A modification of this method was used to construct RTVs containing *pol* sequences encoding the C terminus of RT (codons 305 to 426 [codons 306 to 316 span the 5′ primer and are identical to NL4-3 sequence; codons 317 to 426 are derived from patient virus]) and RNase H and IN, collectively referred to as RH/IN. RH/IN RTVs were generated by inserting RH/IN amplification products flanked by 5′ PinAI and 3′ XhoI restriction sites into an HIV-1 (NL4-3) genomic vector adapted with PinAI and XhoI RH/IN sequence acceptor sites and containing a luciferase reporter cassette that partly replaced the *env* gene (Fig. 1).

Site-directed mutants and chimeric viruses. Single or double mutations were introduced into the HIV-1 genomic vector described above, using the megaprimer method of site-directed mutagenesis (29). Specifically, mutations in the connection domain of RT (at amino acids 369 and/or 348) were introduced in the RH/IN vector whereas mutations in the polymerase domain of RT (K103N, Y181C, G190A, G190S, or L100I) were introduced in the PR/RT vector. The RH/IN fragments were then transferred into the PR/RT RTV as PinAI-XhoI fragments.

Similarly, RTVs containing chimeric *pol* sequences composed of PR/RT fragments containing well-characterized NRTI and NNRTI mutations together with RH/IN fragments containing 3′ RT mutations at amino acids 348 and/or 369 were constructed by replacing, at the clonal level, the "wild-type" (WT) PR/RT regions of RH/IN RTVs with the "NRTI and NNRTI drug-resistant" PR/RT regions of PR/RT RTVs that were initially derived from patient viruses or by site-directed mutagenesis.

Phenotypic drug susceptibility testing. NRTI and NNRTI susceptibility was measured in a single replication cycle assay as previously described (23). Pseudotyped viruses were produced by cotransfecting 293 (human embryonic kidney) cell cultures with RTV plasmid DNA plus an expression plasmid encoding the Env protein of amphotropic murine leukemia virus. High-titer virus stocks were harvested 48 h after transfection and used to infect fresh 293 target cells. Serial dilutions of NNRTI and NRTI drugs were added to cells prior to infection. Approximately 72 h after infection, target cells were lysed and luciferase activity was measured to assess virus replication in the presence or absence of drug. The percentage of inhibition at each drug concentration was calculated as follows: [1 − (luciferase activity in the presence of the drug/luciferase activity in the absence of the drug)] × 100. The data are displayed by graphic comparisons showing percent inhibition of luciferase activity versus the $\log_{10}$ drug concentration. The concentration of drug required to inhibit virus replication by 50% (EC_{50}) was calculated from the inhibition curves. The fold change (FC) in susceptibility is defined as the ratio of the EC_{50} of the test sample to the EC_{50} of a drug-sensitive reference virus (NL4-3) that was tested in the same experiment. The PhenoSense assays are validated to reproducibly measure less-than-2-fold changes in EC_{50} FC values (23).

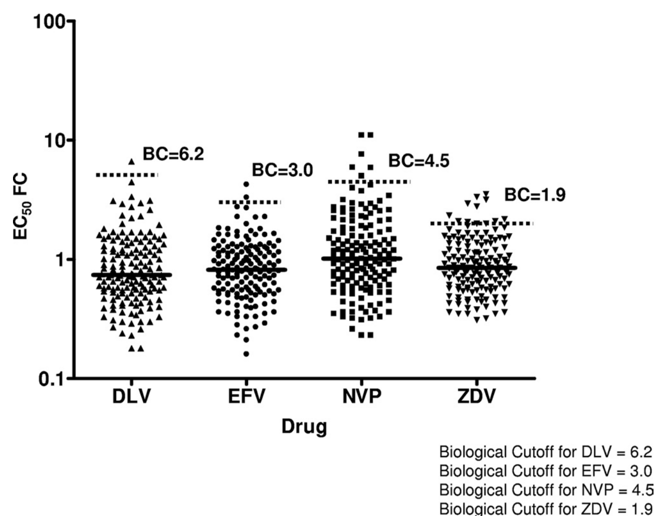

FIG. 2. NNRTI and ZDV susceptibility of RH/IN RTVs derived from 154 patient viruses. The distribution of EC_{50} FCs of 154 RTVs containing patient-derived RH/IN sequences is shown for EFV, NVP, DLV, and ZDV. One, 2, 6, and 13 of the 154 samples demonstrated reduced susceptibility to DLV, EFV, NVP, and ZDV, respectively. The horizontal dotted lines represent the biological cutoff for susceptibility to each drug (22).

RTV *pol* replication capacity. RTV *pol* replication capacity was measured by using a modification of the single-replication-cycle phenotype assay described above (23). Briefly, RTV pseudovirus stocks were used to infect 293 cells in the absence of drug. The numbers of relative luciferase units (RLU) produced by cells infected with RTVs containing drug-susceptible and drug-resistant *pol* sequences were measured at 72 h postinfection. The amount of luciferase activity detected in the infected cells was used as a direct measure of "*pol*-mediated infectivity" or "replication capacity," i.e., the ability of the virus to complete a single round of replication. Replication capacity was calculated as follows: $[1 - (\text{infection } RLU_{\text{test virus}} NF_{\text{TXN}})/\text{infection } RLU_{\text{control virus}}] \times 100$. NF_{TXN} is the normalization factor for transfection efficiency and is calculated by the following equation: $NF_{\text{TXN}} = \text{transfection } RLU_{\text{test virus}}/\text{transfection } RLU_{\text{control virus}}$. Replication capacity is expressed as a percentage of the replication capacity of the reference virus.

Genotypic analysis of RTV *pol* sequences. The amino acid sequences of RT and IN were derived from the DNA sequences of the RTV pools prepared as described above. Sequence analysis was performed by using a thermocycling method using fluorescent dye-labeled dideoxynucleotide chain terminator chemistry (ABI, Foster City, CA). Sequencing reaction products were resolved by using a 96 parallel capillary gel electrophoresis system (ABI 3700). Base calling and amino acid sequence derivations were performed by using customized Sequencher software (GeneCodes, Ann Arbor, MI) that identifies and reports amino acid substitutions relative to a reference sequence (NL4-3; GenBank accession number M19921).

Patient cohorts utilized to generate datasets. A genotypic data set comprising RH/IN and PR/RT nucleotide sequences from 251 unique patient viruses submitted for commercial HIV genotypic resistance testing was utilized to calculate the prevalence of connection domain mutations. Out of these 251 patient viruses, 171 were subtype B and 80 were non-B samples. Of the 171 subtype B samples, 37 lacked NRTI and NNRTI mutations (as defined by U.S. International AIDS Society guidelines) whereas 134 contained mutations in RT. Similarly, 12 of the 80 non-B samples lacked RT mutations whereas 68 contained RT mutations.

NRTI and NNRTI phenotypic susceptibility data were available for only a subset (154) of the 251 RH/IN RTVs. This subset had percentages of RT-experienced patients similar to the percentages seen with the larger set of 251 patients and a similar diversity of subtypes (i.e., 80% of 154 samples contained RT resistance mutations, and 60% of the 154 samples were subtype B). This RH/IN data set comprising 154 matched genotypes and phenotypes was utilized to conduct initial analysis of mutations in the connection domain of RT associated with reduced NNRTI and ZDV susceptibility.

Statistical analysis. The matched genotypic and phenotypic data set derived from the 154 RH/IN RTVs described above was utilized to perform statistical analyses. All amino acid positions in the connection domain of RT that were mutated relative to NL4-3 and were present in at least two samples were selected and analyzed for association with reduced NVP susceptibility. Reduced susceptibility was defined as a 2-fold reduction in the NVP EC_{50} FC tested with the RH/IN RTVs relative to the NVP EC_{50} FC of the WT NL4-3 reference virus. Each amino acid position (including mixtures) was classified as WT or non-WT. Covariation with other RT resistance mutations was also examined for TAMs (M41L, D67N, K70R, L210W, T215F/Y, K219Q/E), nucleotide analog resistance mutations (NAMs) (M41L, K65R, D67N, T69insert, K70R/E, L74V, V75A/M/S/T, Y115F, Q151M, M184V/I, L210W, T215F/Y, K219Q/E), and NNRTI mutations (A98G, L100I, K101E, K101P, K103N, K103S, V106A, V106M, Y181C/I, Y188L/C/H, G190A/S, P225H, F227L, M230L, P236L). Values for significance of association were calculated using Fisher's exact test as implemented in the fisher.test function in R-package version 2.7.2. *P* values were corrected for multiple testing using the Benjamini-Hochberg method.

RESULTS

Identification of mutations in the connection domain of RT associated with reduced NNRTI and ZDV susceptibility. Resistance test vectors (RTVs) containing *pol* sequences encoding the C-terminal end of reverse transcriptase (RT), RNase H (RH), and integrase (IN), collectively termed RH/IN, from 154 patient viruses were constructed (Fig. 1) to assess susceptibility to NNRTIs and NRTIs. In the vast majority of cases, the pseudoviruses produced from these RH/IN RTVs were susceptible to inhibition; however, in 1 case (patient 384), 2 cases (patients 384 and 396), 6 cases (patients 384, 396, 471, 440, 62, and 477), and 13 cases (patients 384, 396, 471, 440, 477, 429, 58, 138, 87, 474, 249, 439, and 442), the pseudoviruses demonstrated reduced susceptibility to DLV, EFV, NVP, and ZDV, respectively (Fig. 2, Table 1). Reduced susceptibility was defined as an EC_{50} FC that exceeded the established biological cutoff (BC) for each drug (22). Genotypic analysis of the RH/IN regions of the six samples demonstrating reduced susceptibility to NVP (EC_{50} FC BC = 4.5) revealed mutations at RT amino acid position 348 or 369 (Table 2). All 14 viruses contained well-characterized NRTI and NNRTI resistance mutations in the corresponding PR/RT regions of these samples, indicating that they were derived from NRTI- and NNRTI-experienced patients (Table 3).

TABLE 1. RT amino acid substitutions in patients with reduced NNRTI or ZDV susceptibility

Patient sample	EC_{50} FC results obtained using RH/IN RTVs[a]			
	DLV	EFV	NVP	ZDV
396[b,c]	2.8	**3.3**	**5.0**	**3.2**
471[b,c]	2.9	1.8	**5.9**	**2.8**
440[b,c]	3.1	2.3	**5.9**	**3.3**
62[b]	3.4	1.8	**7.6**	1.3
477[b,c]	4.5	2.8	**11.0**	**1.9**
384[b,c]	**6.7**	**4.2**	**11.0**	**3.0**
429[c]	1.1	1.3	1.2	**2.1**
58[c]	1.5	0.9	1.7	**2.3**
138[c]	1.7	1.1	1.8	**2.0**
87[c]	1.3	1.3	1.9	**2.1**
474[c]	1.6	1.3	3.0	**2.1**
249[c]	1.3	1.3	3.1	**2.0**
439[c]	3.1	2.7	3.7	**2.2**
442[c]	2.6	2.0	4.0	**3.5**

[a] Values in boldface indicate FC results above the biological cutoffs for the corresponding drugs.
[b] Patient with reduced susceptibility to DLV, EFV, or NVP.
[c] Patient with reduced susceptibility to ZDV.

TABLE 2. RT amino acid substitutions in the RT connection domain derived from the RH/IN RTVs

Patient sample	Genotype of connection domain of RT
396[a,b]	D324D/E, Q334P, **N348I**, R356K, M357T, K358R, E370G, T377R, E399D, E404D
471[a,b]	I326V, G335D, P345Q, F346Y, K347K/Q, **N348N/I**, R356K, M357M/R, K358K/R, G359S, K366R, A371A/V, T377Q, S379C, K390R, A400T, E404D
440[a,b]	D324E, R356K, M357I/T, K358R, G359S, K366R, **T369V**, A371T, K390R, A400T
62[a]	S322A, N348N/I, K358R, **T369T/I**, T377M, S379C, A400T, T403I, E404D/N
477[a,b]	N348I, K358R, K390R, E396D, E399D, A400L
384[a,b]	Y319H, G335D, R356K, M357K, K358R, G359S, K366R, **T369V**, A371V, I375V, A376V, T386A, K395K/R, A400T, T403M, W410Y
429[b]	D324P, G333E, Q334L, R356K, M357I, K358R, A360A/T, K390R, A400T
58[b]	V317V/A, I329L, R356K, K358R, A360V, E399D, A400T
138[b]	Q334H, G335S, T338S, R356K, K358R, Q394L, E399D, A400T
87[b]	I341F, **N348I**, M357T, K358R, T377M, K385R, P387P/S, T397T/I, A400T
474[b]	G335D, P345Q, F346Y, **N348I**, R356K, M357T, K358R, T377R, S379C, T386I, K390R, A400I/L
249[b]	V317V/A, D324E, I329L, Q334D, G335S, R356K, K358R, G359T, V365I, **T369V**, T377M, K390R, E396D, E399D, E404D
439[b]	Q334Q/L, M357T, K358R, A360T, Q394T, E399D, A400T
442[b]	V317A, G333E, Q334I/V, R356R/K, K358R, G359S, K366R, A371V, K388K/R, K390R, E399D, A400T

[a] Patient with reduced susceptibility to DLV, EFV, or NVP.
[b] Patient with reduced susceptibility to ZDV.

Each position in the connection domain of RT of these 154 RH/IN RTVs was also analyzed using Fisher's exact test to identify amino acid substitutions that were significantly associated with 2-fold or greater reductions in NVP susceptibility relative to the wild-type (WT) reference. Two positions in the connection domain of RT (N348 and T369) were significantly associated with reduced susceptibility to NVP (corrected P value = 0.049). Mutations at N348 and T369 were present in 10% and 17.5% of the 154 viruses evaluated. These findings suggested that mutations at positions 348 and 369 in RT confer reduced susceptibility to NVP and possibly other NNRTIs and ZDV.

Mutations at N348 and T369 in the connection domain of RT reduce susceptibility to NNRTIs and ZDV. Site-directed mutagenesis was used to construct the N348I and T369I/V single mutants and the N348I/T369I double mutant to directly assess the effect of these mutations on NNRTI and ZDV susceptibility (Table 4) (29). N348I and T369I/V, in isolation, caused less than 10-fold increase in resistance to all three NNRTIs (FC NVP > FC DLV > FC EFV). More substantial (10 to 60-fold) reductions in NNRTI susceptibility were observed when the N348I and T369I mutations were present in combination. Similarly, a N348I or T369I/V mutation alone conferred 2-fold reductions in ZDV susceptibility, whereas N348I and T369I mutations together caused 5-fold resistance to ZDV.

TABLE 3. Resistance-associated mutations in the RT polymerase domain derived from paired PR/RT RTVs

Patient sample	RT mutation(s)
396[a,b]	M41L, M184M/V, T215Y, K219R
471[a,b]	M41L, D67D/N, Y181C, M184V, L210W, T215Y
440[a,b]	M41L, M184V, L210W, T215Y
62[a]	K103N, M184V, T215Y, P225P/H
477[a,b]	M184V, T215Y
384[a,b]	M184V
429[b]	M41L, D67N, K70R, T215F, K219Q
58[b]	M41L, A98A/G, K103N, Y181Y/C, G190G/A, L210W, T215Y
138[b]	M41M/L, D67D/N, T215Y
87[b]	M41L, L210W
474[b]	M41L, D67N, K101E, Y181C, M184V, G190A, L210W, T215Y, K219N
249[b]	M41L, D67N, M184V, G190G/A, T215H/Y, K219N
439[b]	M41L, D67N, M184V, L210W, T215Y
442[b]	M41L, D67N, T69D, L100I, K103N, M184V, L210W, T215Y, K219N

[a] Patients with reduced susceptibility to DLV, EFV or NVP.
[b] Patients with reduced susceptibility to ZDV.

We also used site-directed mutagenesis to construct and evaluate the NNRTI and ZDV susceptibility of viruses containing the N348I, T369I, and N348I/T369I RT connection domain mutations together with well-characterized NNRTI resistance mutations in the RT polymerase domain, i.e., K103N, Y181C, G190A, G190S, and L100I (Table 4). Generally, the addition of one or both connection domain mutations to well-characterized polymerase domain mutations was consistently associated with further reductions in NNRTI susceptibility. These losses were notable even in cases where the polymerase domain mutation conferred little or no reduction in NNRTI susceptibility (e.g., EFV [Y181C], NVP [L00I]) or in fact conferred NNRTI hypersusceptibility (e.g., DLV [G190A/S]).

To directly evaluate the effect of RT connection domain mutations in the context of patient-derived viruses, the N348I and T369I mutations were introduced, at the clonal level, into four different patient-derived PR/RT RTVs containing well-characterized NNRTI and/or NRTI resistance mutations within the RT polymerase domain. Overall, the results of our evaluations using RTVs containing patient virus-derived PR/RT sequences (Table 5) were highly concordant with the results we obtained using RTVs engineered by site-directed mutagenesis to contain mutations in the connection and polymerase domains of RT (Table 4). Based on these data, we estimate that the addition of either T369I or N348I reduces NNRTI and ZDV susceptibility roughly 5- to 10-fold, although the magnitude may differ depending on the particular profile

TABLE 4. Impact of RT connection domain mutations (N348I and T369I/V) on the NNRTI and ZDV susceptibility of viruses lacking or containing well-characterized RT polymerase domain mutations K103N, Y181C, G190A/S, or L100I

Mutation(s) in RT	Phenotypic susceptibility data: EC_{50} fold change (SD)[a]			
	DLV	EFV	NVP	ZDV
T369I	5.2 (0.50)	2.4 (0.40)	8.4 (0.50)	2.7 (0.80)
T369V[b]	5.5	3.1	9.2	2.1
N348I	3.4 (0.30)	1.9 (0.30)	4.1 (0.10)	2.4 (0.90)
T369I/N348I	29.8 (1.70)	8.1 (3.10)	58.6 (2.20)	5.4 (1.20)
K103N	75.7 (16.80)	21.3 (9.50)	81 (41.4)	1.1 (0.30)
K103N/N348I	>250 (NA[c])	115 (41.2)	>400 (NA)	2.6 (1)
K103N/T369I	>250 (NA)	164.1 (77.40)	>400 (NA)	2.3 (0.70)
K103N/T369I/N348I	>250 (NA)	>700 (NA)	>400 (NA)	7.9 (0)
Y181C	44.7 (6.70)	1.7 (0.10)	317 (143.8)	0.3 (0.10)
Y181C/N348I	>250 (NA)	3.2 (0.20)	>400 (NA)	0.7 (0.10)
Y181C/T369I	>250 (NA)	4 (0.8)	>400 (NA)	0.9 (0.30)
Y181C/T369I/N348I	>250 (NA)	17 (6.2)	>400 (NA)	2.2 (0.60)
G190A	0.3 (0.10)	5.2 (1.50)	127.7 (30.10)	1.1 (0.20)
G190A/N348I[b]	1.1	26.0	>400	2.0
G190A/T369I	1.2 (0.10)	33.7 (9)	>400 (NA)	1.7 (0.20)
G190A/T369I/N348I	5.9 (0.80)	>700 (NA)	>400 (NA)	4.2 (2.10)
G190S	0.3 (0.04)	63 (9.9)	145 (65.1)	0.8 (0.02)
G190S/N348I	0.9 (0.20)	>700 (NA)	>400 (NA)	1.7 (0.40)
G190S/T369I	0.6 (0.10)	>700 (NA)	>400 (NA)	0.7 (0.20)
G190S/T369I/N348I	6.9 (0.30)	>700 (NA)	>400 (NA)	5.8 (0.30)
L100I	34 (0)	9 (0.1)	2.1 (0)	0.1 (0)
L100I/N348I	90.7 (7.20)	16 (4.4)	5.3 (0.60)	0.3 (0.20)
L100I/T369I	49.7 (9.60)	13.6 (4.80)	4.8 (0.50)	0.2 (0.10)
L100I/T369I/N348I	>250 (NA)	137.7 (50.50)	56.3 (4.20)	0.5 (0.20)

[a] The data represent mean fold changes (± standard deviations) obtained from the results of three independent experiments. Changes in phenotypic susceptibility are expressed as the fold change in EC_{50} based on the EC_{50} of a drug-susceptible reference virus (EC_{50} of sample/EC_{50} of reference).
[b] Results are from single experiments testing for these two viruses.
[c] NA, standard deviations could not be calculated, as the EC_{50}s were greater than the highest drug concentrations tested.

of NRTI and/or NNRTI resistance mutations within the polymerase domain.

RT connection domain mutations impair *pol*-mediated replication capacity. The replication capacity (RC) of pseudovirions derived from RTVs containing connection domain mutations was characterized by using the single-cycle replication assay. Compared to the reference virus (NL4-3) RC, the N348I mutant RC was slightly diminished (83%) whereas the RCs of the T369I mutant (53%) and N348I/T369I double mutant (11%) were significantly more impaired (Fig. 3).

The effect of connection domain mutations on the RC of pseudovirions derived from RTVs containing well-characterized NNRTI resistance mutations (G190A/S, K103N, Y181C, L100I) within the polymerase domain was also evaluated (Fig.

TABLE 5. Influence of RT connection domain mutations (N348I and T369I) on NNRTI and ZDV susceptibility in patient-derived viruses containing well-known NNRTI and NRTI resistance mutations in the RT polymerase domain

Patient sample (mutation[s] in RT) + C terminus RT mutations	Phenotypic susceptibility data: EC_{50} fold change (SD)[a]			
	DLV	EFV	NVP	ZDV
50 (K103N)	63.3 (12.60)	22.3 (3.80)	65.3 (7.51)	1.0 (0.20)
50 + T369I	>250 (NA[b])	>700 (NA)	>400 (NA)	3.3 (1.30)
50 + N348I	>250 (NA)	>700 (NA)	>400 (NA)	2.7 (1)
58 (M41L, K103N, V118I, Y181Y/C, G190G/A, L210W, T215Y)	128 (19.3)	23.7 (3.20)	>400 (NA)	643.7 (140.6)
58 + T369I	>250 (NA)	>700 (NA)	>400 (NA)	>2,400 (NA)
58 + N348I	>250 (NA)	>700 (NA)	>400 (NA)	>2,400 (NA)
62 (K103N, M184V, T215Y)	12.7 (3.10)	17.7 (2.50)	55.7 (9.71)	2.0 (0.14)
62 + T369I	112 (16)	146.7 (91.60)	>400 (NA)	11.8 (2)
62 + N348I	79.7 (4.50)	93 (26.1)	>400 (NA)	7.1 (2.30)
45 (M41L, D67N, V118I, M184M/V, L210W, T215Y)	0.1 (0.02)	0.17 (0.06)	0.3 (0.01)	89.7 (14.50)
45 + T369I	0.3 (0.03)	0.6 (0.06)	2.1 (0.01)	>2,400 (NA)
45 + N348I	0.2 (0.02)	0.47 (0.06)	0.81 (0.11)	>2,400 (NA)

[a] The data shown represent mean fold changes (± standard deviations) obtained from the results of three independent experiments. Changes in phenotypic susceptibility are expressed as the fold change in EC_{50} based on the EC_{50} of a drug-susceptible reference virus (EC_{50} of sample/EC_{50} of reference).
[b] NA, standard deviations could not be calculated as the EC_{50}s were greater than the highest drug concentrations tested.

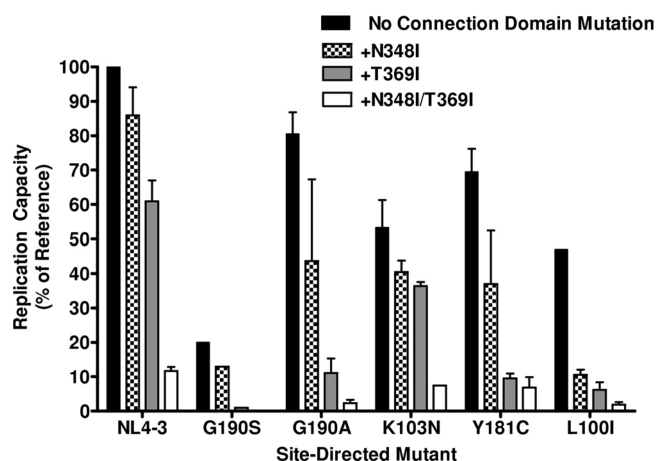

FIG. 3. Replication capacity of RH/IN RTVs containing site-directed mutations in the C terminus of RT. The data are shown as mean values obtained from the results of three independent experiments, and the error bars represent the standard errors of measurement (SEM). Replication capacity is expressed as a percentage of that of the wild-type reference virus (defined as 100%).

3). In all cases evaluated, connection domain mutations further reduced the RC of viruses with well-characterized NNRTI mutations in the polymerase domain. Generally, the T369I mutation conferred larger reductions in RC than the N348I mutation, while the largest reductions in RC were observed when both connection domain mutations were present. From these observations, we conclude that the reductions in susceptibility conferred by connection domain mutations are associated with reductions in RC as measured in the absence of drug.

Connection domain mutations are infrequent in clinical samples. A genotypic data set comprising RH/IN and PR/RT nucleotide sequences from 251 unique patient viruses submitted for commercial HIV resistance testing was utilized to calculate the prevalence of connection domain mutations. Out of these 251 patient viruses, 171 were subtype B and 80 were non-B samples. Based on our survey of the 171 subtype B viruses, connection domain mutations at positions 369 and 348 were present in 5% and 0% of the viruses lacking NRTI and NNRTI mutations ($n = 37$), respectively, and in 15% and 10% of the viruses containing NRTI and/or NNRTI mutations ($n = 134$). The two mutations (T369I/N348I) were not detected concurrently in viruses lacking NRTI and NNRTI mutations and were present in 1.5% of the viruses containing NRTI and/or NNRTI mutations. Connection domain mutations were not detected in the 80 non-subtype B HIV viruses evaluated, of which 12 samples lacked NRTI and/or NNRTI mutations and 68 contained RTI mutations.

A survey of the 394 treatment-naïve viruses within the Los Alamos National Laboratories database comprising 17 different HIV-1 clades identified substitutions at position 348 in 0.2% and at position 369 in 9.2% of samples. At position 369, T-to-I substitutions were not observed at all, T-to-A substitutions were observed in 8.4% of the samples, and T-to-V substitutions were observed in 0.8% of the samples. Thus, N348I is rarely present in treatment-naïve HIV viruses. Similarly, T369I and T369V are rarely found in treatment-naïve viruses, while T369A is present as a polymorphism in a small percentage of naïve patient viruses.

Covariation of connection domain mutations with known RT resistance mutations. Covariation of mutations at positions 348 and 369 with RT resistance mutations was also examined for TAMs, NAMs, and NNRTI mutations by using the genotypic data set comprising 171 subtype B samples with matching RH/IN and PR/RT genotypes. Unfortunately, the analysis was limited due to low prevalence of connection domain mutations in this data set. In this limited data set, mutations at position 369 were significantly associated with Y115F, M184V, F227L, and K219N. However, we did not observe any significant association of mutations at position 348 with known RT resistance mutations. Others have reported that N348I is highly associated with NRTI (M41L, T215Y/F, M184V/I) and NNRTI (K103N, Y181C/I) resistance mutations (36), and the lack of statistically significant association in our data set may be due to the limited number of samples.

DISCUSSION

Currently, assays that are used for routine assessments of reverse transcriptase inhibitor (RTI) susceptibility (PhenoSense, Antivirogram, and Phenoscript) do not capture the C-terminal region of RT comprising the connection and RNase H domains. Hence, the identities, prevalences, and potential effects on RTI susceptibility of amino acid substitutions in these regions remain largely undefined. Using the C-terminal regions of RT (codons 317 to 426) derived from 154 patient virus samples submitted for routine drug resistance testing, we show that this region alone has minor influences on NNRTI and NRTI susceptibility. However, a small subset of viruses that exhibited modest reductions in NNRTI and NRTI susceptibility enabled the identification of several novel mutations in the C-terminal region of RT and the characterization of their effects on NNRTI and NRTI susceptibility. By performing a simple statistical analysis of all positions in the C terminus of RT, we were able to associate amino acid substitutions at positions 348 and 369 with subtle (>2-fold) reductions in NVP susceptibility. These associations were confirmed experimentally by introducing the mutations into the RT sequences of a wild-type reference virus and measuring modest reductions in NNRTI and ZDV susceptibility. The characterization of these mutations was extended by introducing these mutations into the RT sequences of patient-derived viruses containing well-characterized NNRTI and NRTI mutations in the polymerase domain. We drew several conclusions from this series of experiments. First, in the absence of NNRTI and NRTI resistance mutations in the polymerase domain, the T369I and N348I mutations independently confer modest (<10-fold) reductions in NNRTI and ZDV susceptibility. Second, the T369I and N348I mutations independently reduce NNRTI and ZDV susceptibility of viruses containing NNRTI and/or NRTI mutations. Notably, in cases where the NNRTI mutation confers reductions in NNRTI susceptibility, the presence of one or more connection domain mutations provides further reductions in susceptibility (i.e., ~10-fold). In cases where the NNRTI mutation confers NNRTI hypersusceptibility (e.g., G190A/S [DLV]), the presence of one or more connection domain mutations diminishes or abolishes NNRTI hypersus-

ceptibility. Finally, when the T369I and N348I mutations occur together, their effects on NNRTI and ZDV susceptibility are greater (>10-fold) than those seen with either mutation alone (<10-fold), irrespective of the presence or absence of NNRTI and/or NRTI mutations.

Several retrospective statistical analyses of clinical datasets have identified substitutions in the connection and RNase H domains of RT that are more prevalent in samples from antiretroviral-experienced patients than in those from antiretroviral-naïve patients (6, 9, 24, 27). It is noteworthy that in our survey of subtype B viruses, substitutions at positions 369 and 348 were more prevalent in viruses from treatment-experienced patients than in viruses from treatment-naïve individuals. This observation is in agreement with recent publications reporting a higher prevalence of N348I in RTI-experienced patients, the association of N348I with NRTI (M184V/I [TAMs]) and NNRTI (K103N, Y181C) resistance mutations, and the selection of N348I during NVP and ZDV treatment (20, 36). Furthermore, Nikolenko et al. and Brehm et al. have reported that novel mutations in the connection and RNase H domain of RT conferred reductions in susceptibility to ZDV and emerged during virus passage in the presence of ZDV *in vitro* (5, 19). In our survey, mutations at 369 and 348 were not observed in non-subtype B viruses.

Apart from a single case in which a Q145M mutation was reported to confer broad resistance to several NRTIs and NNRTIs (21) and data suggesting that Y181C/I may confer resistance to stavudine in addition to NVP (2, 3), the T369I/A/V and N348I connection domain mutations represent examples of *in vivo*-selected mutations with the rather unique ability to confer decreased susceptibility to two distinct classes of RT inhibitors. Recent studies have demonstrated that RT mutations that decrease RNase H activity and reduce ZDV susceptibility do so by enhancing the ability of RT to excise incorporated ZDV and thereby slowing down the rate at which the RNA template strand is degraded (19). This result was further corroborated by Yap et al., who showed that the presence of an N348I mutation, alone and in combination with TAMs, decreased the RNase H activity (36). Moreover, Ehteshami et al. recently reported that the A360V mutation, located close to the RT DNA primer, also affected ZDV excision by indirectly affecting RNase H cleavage (8).

The structural mechanism by which T369I/A/V or N348I confers reductions in NNRTI susceptibility is unknown. Neither amino acid 369 nor 348 is located close to the NNRTI-binding pocket; therefore, it is highly unlikely that these mutations function like the well-characterized NNRTI mutations that have been mapped to the NNRTI binding pocket of the polymerase domain and that act by preventing NNRTI binding. Since amino acid 369 is located close to the RT heterodimer interface of the p66 and p51 subunits, it is possible that T369I/A/V influences NNRTI susceptibility through alterations in p66/p51 dimerization. Evidence that NNRTIs act as enhancers of RT dimerization has been previously reported, although the mechanism has not been clearly demonstrated (32). Thus, we can hypothesize that connection domain mutations, such as T369I/A/V, indirectly affect NNRTI susceptibility by altering RT dimerization.

In summary, we have identified amino acid substitutions in the connection domain of HIV-1 RT that reduce susceptibility to NNRTIs and ZDV. These mutations are rarely observed in the viruses of antiretroviral (ARV)-naïve individuals, but their prevalence is increased in the viruses of ARV-experienced subjects that contain well-characterized NRTI and/or NNRTI mutations in the RT polymerase domain. Furthermore, in the clinical samples that we tested, N348I and T369I were detected in viruses that already exhibited reduced susceptibility to NRTIs and NNRTIs as shown by the use of our standard PR/RT vector from the same patients. Additionally, viruses containing connection domain mutations have reduced RC, which suggests that the greater reduction in susceptibility that they provide carries an added fitness cost for the virus. This observation was supported by the findings of Hachiya et al., who observed that N348I impaired viral replication in a cell-type-dependent manner (11). Thus, although the current routine drug resistance assays do not evaluate the C-terminal regions of RT, the clinical impact of mutations in this region appears low. The fact that these substitutions are observed in only a small percentage of viruses, coupled with the fact that most of the viruses bearing these mutations also contain polymerase domain mutations that would result in a drug resistance classification in routine assays, implies that reengineering of the current assays to include this region is not a clinical urgency. However, with the advent of novel RT and, eventually, RNase H inhibitors, continued study of these mutations and their impact on drug resistance is of great scientific value. Moreover, an increased understanding of the mechanism of action of C-terminal RT mutations on RT resistance will undoubtedly aid in the design of novel antiretrovirals, especially those targeting the connection domain of RT.

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