Selection of Mutations in the Connection and RNase H Domains of Human Immunodeficiency Virus Type 1 Reverse Transcriptase That Increase Resistance to 3'-Azido-3'-Dideoxythymidine[▽]

Jessica H. Brehm,¹ Dianna Koontz,¹ Jeffrey D. Meteer,¹ Vinay Pathak,²
Nicolas Sluis-Cremer,¹ and John W. Mellors^{1*}

Division of Infectious Diseases, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261,¹ and National Cancer Institute, Frederick, Maryland 21702²

Received 6 October 2006/Accepted 5 May 2007

Recent work indicates that mutations in the C-terminal domains of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) increase 3'-azido-3'-dideoxythymidine (AZT) resistance. Because it is not known whether AZT selects for mutations outside of the polymerase domain of RT, we carried out *in vitro* experiments in which HIV-1_{LAI} or AZT-resistant HIV-1_{LAI} (M41L/L210W/T215Y) was passaged in MT-2 cells in increasing concentrations of AZT. The first resistance mutations to appear in HIV-1_{LAI} were two polymerase domain thymidine analog mutations (TAMs), D67N and K70R, and two novel mutations, A371V in the connection domain and Q509L in the RNase H domain, that together conferred up to 90-fold AZT resistance. Thereafter, the T215I mutation appeared but was later replaced by T215F, resulting in a large increase in AZT resistance (~16,000-fold). Mutations in the connection and RNase H domains were not selected starting with AZT-resistant virus (M41L/L210W/T215Y). The roles of A371V and Q509L in AZT resistance were confirmed by site-directed mutagenesis: A371V and Q509L together increased AZT resistance ~10- to 50-fold in combination with TAMs (M41L/L210W/T215Y or D67N/K70R/T215F) but had a minimal effect without TAMs (1.7-fold). A371V and Q509L also increased cross-resistance with TAMs to lamivudine and abacavir, but not stavudine or didanosine. These results provide the first evidence that mutations in the connection and RNase H domains of RT can be selected *in vitro* by AZT and confer greater AZT resistance and cross-resistance to nucleoside RT inhibitors in combination with TAMs in the polymerase domain.

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is responsible for the conversion of the single-stranded viral RNA genome into double-stranded DNA. The enzyme is multifunctional and exhibits both RNA- and DNA-dependent DNA polymerase activity and RNase H activity (6, 8, 34). HIV-1 RT is a heterodimer composed of a 560-amino-acid, 66-kDa subunit (p66) and a p66-derived 440-amino-acid, 51-kDa subunit (p51) (12, 16). 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). By contrast, the p51 subunit is composed of only the polymerase and connection domains and may largely play a structural role in RT heterodimer stability (35).

HIV-1 RT is an important therapeutic target, and two distinct groups of RT inhibitors have been identified. They are the nucleoside or nucleotide RT inhibitors (NRTI), which include zidovudine (3'-azido-3'-dideoxythymidine [AZT]), lamivudine (3TC), emtricitabine, zalcitabine, didanosine (ddI), stavudine (d4T), abacavir (ABC), and tenofovir (TNV), and the non-nucleoside inhibitors, which include nevirapine, delavirdine, and efavirenz. NRTI inhibit HIV-1 replication by competing

with the natural deoxynucleoside triphosphate (dNTP) substrate for binding and incorporation into the nascent DNA chain. Once incorporated, NRTI act as chain terminators of viral DNA synthesis. By contrast, nonnucleoside inhibitors bind to RT in a location distinct from the polymerase active site and act as allosteric inhibitors of HIV-1 reverse transcription. Although combination therapies that contain two or more RT inhibitors have profoundly reduced morbidity and mortality from HIV-1 infection, their long-term efficacy is limited by the selection of drug-resistant HIV-1.

Mutations that confer resistance to NRTI have been identified by *in vitro* passage experiments and from sequences amplified from patients experiencing virologic failure on NRTI therapy. In general, NRTI-associated resistance mutations can be broadly categorized into two groups, depending on their mechanism of resistance (19, 32). The polymerase domain mutations M41L, D67N, K70R, L210W, T215F/Y, and K219Q/E are typically referred to as thymidine analog mutations (TAMs). These mutations increase the ability of HIV-1 RT to excise a chain-terminating NRTI-monophosphate (NRTI-MP) from a prematurely terminated DNA chain (1, 3, 20, 28). This resistance mechanism has been termed NRTI excision. By comparison, the polymerase domain mutations K65R, K70E, L74V, Q151M (in complex with A62V, V75I, F77L, and F116Y), and M184V increase the selectivity of RT for incorporation of natural dNTP substrate versus the NRTI-triphosphate (11, 17, 29, 31a). This resistance mechanism has been termed NRTI discrimination.

All the NRTI mutations included in the most widely used

* Corresponding author. Mailing address: University of Pittsburgh School of Medicine, S818 Scaife Hall, 3550 Terrace Street, Pittsburgh, PA 15261. Phone: (412) 624-8512. Fax: (412) 383-7982. E-mail: mellors@dom.pitt.edu.

[▽] Published ahead of print on 16 May 2007.

resistance tables, such as that from the International AIDS Society-USA expert panel (13), are located in the DNA polymerase domain of HIV-1 RT. This is the case, in part, because most commercial genotype assays do not analyze the complete connection and RNase H domains of RT. Recently, Nikolenko et al. reported that the mutations D549N and H539N, which decrease RT RNase H activity, also increase resistance to AZT (22). Specifically, the D549N and H539N mutations increased AZT resistance by 12-fold and 180-fold, respectively, and reduced d4T susceptibility by 2.4-fold and 10-fold, respectively (22). Furthermore, when D549N was present with the TAMs D67N, K70R, T215Y, and K219Q, AZT and d4T resistances increased 1,230-fold and 12.5-fold, respectively. The mutations had no effect on susceptibility to efavirenz or to ddI and 3TC. The authors proposed that mutations in the RNase H domain that decrease RNase H activity also reduce RNA template degradation, thereby increasing the time for AZT-MP to be excised from the terminated primer and for polymerization to resume on an intact template.

It is not known, however, whether mutations in the RNase H domain of RT are selected for by AZT. Therefore, we carried out *in vitro* selections of AZT-resistant HIV-1, sequenced the entire coding region of RT to identify all drug resistance-related mutations, and characterized the effects of these mutations using site-directed recombinant viruses.

MATERIALS AND METHODS

NRTI. AZT and ddI were obtained from Sigma Chemical Corporation (St. Louis, MO). 3TC and d4T were provided by Raymond Schinazi (Emory University). TNV was provided by Gilead Sciences (Foster City, CA) and ABC by GlaxoSmithKline (Research Triangle Park, NC). NRTI were prepared as 10 mM or 30 mM stock solutions in dimethyl sulfoxide or sterile water and stored at -20°C . The compounds were diluted immediately before use to the desired concentrations in Dulbecco's modified Eagle medium, phenol red free (Gibco-BRL, Grand Island, NY).

Cells and viruses. MT-2 cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health) were cultured in RPMI 1640 (Whittaker MA Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES buffer, 50 IU/ml of penicillin, and 50 mg/ml of streptomycin (referred to as R10). The P4/R5 reporter cell line (provided by Nathaniel Landau, Salk Institute, La Jolla, CA), which expresses the β -galactosidase gene under the control of the HIV long terminal repeat promoter that is transactivated by HIV-1 tat, was maintained in Dulbecco's modified Eagle medium, phenol red free, supplemented with 10% fetal bovine serum, 50 IU/ml of penicillin, 50 $\mu\text{g}/\text{ml}$ of streptomycin, and 0.5 $\mu\text{g}/\text{ml}$ of puromycin (Clontech, Palo Alto, CA). Stock viruses were prepared in MT-2 cells as described previously (23). Briefly, 5 to 10 μg of plasmid DNA was electroporated into 1.3×10^7 MT-2 cells. Cell-free supernatants were collected 7 days after transfection at peak cytopathic effect (CPE) and stored at -80°C . The infectivity of the virus stocks was determined by a threefold endpoint dilution in P4/R5 cells, and the 50% tissue culture infectivity dose was calculated using the Reed and Muench equation (25). To confirm the genotype of the stock viruses, viral RNA was extracted from cell supernatants and treated with 1 U/ μl of DNase I for 2 h. Codons 1 to 560 of RT were amplified using the following primers: RT forward, 5'-AAGCTATAGGTACAGTATTA GTAGGACTAC-3', and RT reverse, 5'-TGCTCTCCAATTACTGTGATAT TTCTCA-3'. The PCR products were purified (Wizard PCR purification system; Promega, Madison, WI) and sequenced using a Big Dye terminator kit (v.3.1) on an ABI 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA). The ratios of mutant to wild type at specific nucleotide positions were estimated by comparison of peak heights, as previously described (10).

Selection of AZT-resistant viruses. Resistant virus was selected in two independent experiments by the passage of wild-type HIV_{LAI} or HIV_{LAI} containing the M41L/L210W/T215Y mutations (AZT-resistant HIV_{LAI}) in MT-2 cells in increasing concentrations of AZT. To initiate each selection experiment, MT-2 cells (1×10^6) were pretreated for 2 hours with 0.5 μM and 25 μM AZT for

wild-type HIV_{LAI} and AZT-resistant HIV_{LAI}, respectively, before virus was added. Viral replication was monitored by CPE. At +3/4 CPE (three or four syncytia per field at $\times 100$ magnification), the cell supernatant was harvested and 0.1 ml of supernatant was added to fresh MT-2 cells to initiate a new passage. The concentration of AZT was doubled every three passages. The selection pressure was increased from an initial AZT concentration of 0.5 μM to a final concentration of 32 μM for wild-type HIV_{LAI} and from 25 μM to 150 μM for the AZT-resistant HIV_{LAI}. The concentration of drug required to inhibit viral replication by 50% (IC_{50}) was calculated every five passages to identify changes in AZT susceptibility, and the resistance (*n*-fold) was determined by dividing the IC_{50} of the mutant virus by the IC_{50} of wild-type HIV-1_{LAI}. The genotype of the passaged virus was determined as described above.

Drug susceptibility assays. NRTI susceptibility was determined in P4/R5 cells as described previously (23). Briefly, two- or threefold dilutions of inhibitor were added to P4/R5 cells in triplicate, and the cells were infected with an amount of virus that produced 100 relative light units (RLU) in no-drug virus control wells. After 48 h, the cells were lysed (Gal-Screen; Tropix/Applied Biosystems, Foster City, CA) and the RLU were measured using a ThermoLabSystems luminometer (Waltham, MA). The IC_{50} and resistance were calculated as described above. IC_{50} values from three to nine experiments were $\log_{10}$ transformed and compared for statistically significant differences using the two-sample Student's *t* test.

Clonal analysis of HIV-1 RT for mutation linkage. The entire sequence of HIV-1 RT from passaged viruses was RT-PCR amplified using RT forward and RT reverse primers (defined above), and the PCR product was cloned into the TOPO TA cloning vector (Invitrogen, Carlsbad, CA). After transformation into *Escherichia coli* TOP10 competent cells, clones containing the correct insert were identified through blue-white screening. DNA from clones was purified and sequenced as described above.

Construction of mutant recombinant HIV-1. Mutant recombinant plasmid clones of virus were generated by oligonucleotide site-directed mutagenesis as described previously (31), using the p6HRT-MO plasmid. p6HRT-MO contains the entire RT and protease coding sequence as previously described (18) and four silent restriction sites (XmaI, MluI, XbaI, and NgoMIV from the 5' to 3' end of RT at codons 14, 358, 490, and 554, respectively). After site-directed mutagenesis (QIAamp kit; QIAGEN, Valencia, CA), the mutated RT was ligated into pxxHIV-1_{LAI} MO, which contains the entire genome of HIV-1_{LAI} and the same silent restriction sites as p6HRT-MO. Infectious virus was generated by electroporating the mutated xxHIV-1_{LAI} MO plasmid into MT-2 cells as described above. All mutations in recombinant viruses were confirmed by full-length sequencing of the entire RT coding region.

Assays of replication capacity and replication kinetics. The p24 (ng/ml) of each viral stock was determined by enzyme-linked immunosorbent assay (Alliance HIV-1 p24 ELISA kit; Perkin-Elmer, Wellesley, MA), and the single-cycle replication capacity was measured by adding 10 ng of viral p24 to 5×10^4 P4/R5 cells in a 96-well plate (6 wells per virus). After 48 h, the cells were lysed and the RLU were measured as described above. Mean RLU from three independent experiments were compared for statistically significant differences using the two-sample Student's *t* test. The multiple-cycle replication kinetics was determined in MT-2 cells. Virus (10 ng of p24) was added to 5×10^6 MT-2 cells. After 2 h, R10 was added to the infected MT-2 cells to give a final concentration of 1×10^6 cells/ml. An initial aliquot was taken after the 2-h infection as background, and 0.5-ml samples were collected every day for 7 days. The cultures were replenished with 0.5 ml of R10 after each aliquot was harvested. The p24 (ng/ml) concentration of each aliquot was measured, and values from three independent experiments were compared for statistically significant differences using the two-sample Student's *t* test.

Visualization of the three-dimensional structure of HIV-1 RT. The Molecular Operating Environment (Chemical Computing Group Inc., Montreal, Quebec, Canada) was used to visualize structural images of RT bound to an RNA/DNA template/primer (T/P) (Protein Data Bank access number 1HYS) (30).

RESULTS

Selection of AZT-resistant virus. Two independent AZT selection experiments were conducted. One started with wild-type HIV-1_{LAI}, and the second started with AZT-resistant HIV-1_{LAI} (M41L/L210W/T215Y). Both viruses were serially passaged in MT-2 cells in increasing concentrations of AZT. After every five passages, AZT susceptibility was measured in a single-cycle viral-replication assay in P4/R5 cells (described in Materials and Methods). Viral RNA was extracted and

TABLE 1. Selection of AZT-resistant virus starting with wild-type HIV-1_{LAI} or HIV_{LAI} encoding M41L/ L210W/T215Y (AZT resistant)

Passage	AZT concn (μM)	IC ₅₀ (μM)	Resistance (n-fold) ^a	Mutation(s)
HIV-1_{LAI}				
1	0.5	0.2	1.5	ND ^b
10	0.5	0.3	1.6	ND
20	0.5	0.7	5.1	ND
25	1.0	0.7	4.1	D67D/N, K70K/R
30	2.0	3.0	26	D67D/N, K70K/R, A371A/V, Q509Q/L
35	2.0	10	66	D67N, K70R, A371A/V, Q509Q/L
40	4.0	10	86	D67N, K70R, A371A/V, Q509Q/L
55	16	39	255	D67N, K70R, T215T/I, A371A/V, Q509Q/L
60	32	56	489	D67N, K70R, T215I, A371A/V, Q509L
65	32	810	>16,200	D67N, K70R, T215I/F, A371A/V, Q509L
AZT^R				
1	25	21	6.0	ND
10	50	34	15	ND
25	100	126	39	M41L, D67D/N, L210W, L214F, T215Y
30	150	181	62	M41L, D67D/N, L210W, L214F, T215Y
35	150	>810	>1,332	M41L, D67N, L210W, L214F, T215Y

^a Resistance compared to wild-type HIV-1_{LAI} passaged in parallel without AZT.^b ND, not done.

converted to cDNA, and the entire coding region of RT (residues 1 to 560) was PCR amplified and sequenced to monitor the appearance of mutations. In the selection experiment starting with HIV-1_{LAI}, AZT susceptibility was reduced 4.1-fold by passage 25, and two polymerase domain TAMs were identified as a 1:6 mutant–wild-type mixture of D67N and K70R (Table 1). By passage 30, the virus was 26-fold resistant to AZT, with an increase in the mutant/wild-type ratio of D67N and K70R to 1:3 and 1:2, respectively, in conjunction with the appearance of two novel mutations in RT, A371V (1:8 mutant–wild-type mixture) and Q509L (1:7 mixture), in the connection and RNase H domains of RT, respectively. Between passages 30 and 55, AZT resistance increased up to 255-fold as the D67N, K70R, A371V, and Q509L mutants became dominant in the population. By passage 55, the T215I mutation appeared but was replaced by T215F by passage 65, increasing AZT resistance >16,200-fold.

In the selection experiment starting with AZT-resistant HIV-1_{LAI} (M41L/L210W/T215Y), AZT resistance increased from 6-fold to >1,000-fold by passage 35 (Table 1). This increase in AZT resistance was associated with the acquisition of two additional mutations in the polymerase domain of RT: D67N and L214F. Mutations in the connection or RNase H domains of RT were not detected. Because each passage experiment was carried out once, the reproducibility of the observed mutant selection is unknown.

Linkage analysis of mutations. To evaluate whether D67N, K70R, T215F, A371V, and Q509L were selected on the same viral genome, the RT coding region from passage 65 virus was amplified by RT-PCR and cloned, and the full-length RT coding regions of 12 clones were sequenced (Table 2). All 12 clones contained D67N, K70R, and Q509L. Six of the clones had all five mutations, and three clones contained T215I with D67N, K70R, A371V, and Q509L. The remaining three clones contained D67N, K70R, Q509L, and either T215I or T215F, but not A371V. Additional mutations that were identified included R358K in four clones and F416Y in three clones (Table 2).

Drug susceptibilities of recombinant viruses. To confirm the roles of A371V and Q509L in AZT resistance, recombinant mutant viruses were generated by site-directed mutagenesis. Five mutant viruses were constructed to represent the appearance of mutations in the AZT selection experiment at passages 25, 35, 60, and 65 (Table 1). An additional 10 mutant viruses were generated to delineate the roles of A371V and Q509L alone and together with different combinations of TAMs (Table 3). The A371V and Q509L mutations, alone or together, did not confer significant AZT resistance in the absence of TAMs. When the A371V mutation alone was added to viruses that contained different combinations of TAMs, AZT resistance was only marginally increased (1.2- to 2-fold). By contrast, viruses that contained Q509L and different combinations of TAMs exhibited significantly greater resistance to AZT (3.0- to 11-fold). When both A371V and Q509L were combined with TAMs, AZT resistance was also significantly greater (9- to 52-fold) than those of viruses that contained only one of the mutations or neither of them. It was also of interest to determine whether A371V and Q509L increased AZT resistance in a M41L/L210W/T215Y background, because A371V and Q509L were not selected in viruses having these TAMs. The addition of A371V and Q509L to M41L/L210W/T215Y did significantly increase AZT resistance (10-fold) (Table 3).

Of note, there was only a small difference in AZT resistance between the D67N/K70R/A371V/Q509L mutant (39-fold) and the D67N/K70R/T215I/A371V/Q509L mutant (41-fold) (Table 3). Thus, the selective advantage of T215I was not obvious from these drug susceptibility analyses. In addition, clonal analysis of passage 65 virus also identified R358K and F416Y mutants in the selected virus population (Table 2). Susceptibility testing of site-directed mutants showed that R358K in the background of D67N/K70R/T215F (TAMs) did not increase AZT resistance compared to TAMs alone (data not shown). However, F416Y and R358K/F416Y in the background of TAMs increased AZT resistance two- and fourfold, respectively (data not shown).

TABLE 2. D67N, K70R, T215I/F, A371V, and Q509L are linked on the same genome

HIV _{LAI} residue	Residue in clone no.												% of clones
	1	2	3	4	5	6	7	8	9	10	11	12	
G18				D									8
L26											S		8
T39				P									8
D67	N	N	N	N	N	N	N	N	N	N	N	N	100
K70	R	R	R	R	R	R	R	R	R	R	R	R	100
L73								S					8
I94				T									8
G99								E					8
K104					N		E						8 (N), 8 (E)
S117					L								8
V118				I									8
D123		G											8
G190						Q							8
S191			T										8
I202						T					T		16
T215	F	I	I	I	F	F	F	F	I	F	F	I	42 (I), 58 (F)
E122									G				8
Q242											R		8
I288								S					8
W337					R								8
R358						K	K			K		K	33
A371	V	V	V		V	V		V	V	V	V		75
K385					E								8
I393			L										8
F416	Y								Y	Y			25
I434					M								8
A445						V							8
L491				R									8
R461					K								8
N494									S				8
A502	T												8
Q509	L	L	L	L	L	L	L	L	L	L	L	L	100
L517											S		8
N519									S				8
A534						T							8
D549			N										8
A554			T										8
G555	Q												8
G555			K										8

Cross-resistance to other NRTI. The effect of A371V and Q509L in combination with TAMs on cross-resistance to other NRTI was also analyzed (Table 4). Statistically significant increases in cross-resistance to 3TC ($P = 0.047$ and 0.014 for D67N/K70R/A371V/Q509L and D67N/K70R/T215F/A371V/Q509L, respectively) and ABC ($P = 0.020$ and 0.23 , respectively) were noted in viruses that contained A371V and Q509L in combination with TAMs compared with those that contained only TAMs. Viruses that contained TAMs and A371V/Q509L also exhibited a trend toward greater cross-resistance to TNV ($P = 0.10$ and 0.058 , respectively), but not to d4T or ddI (Table 4).

Replication capacity and replication kinetics of mutant viruses. Since the selective advantage of T215I was not evident from the drug susceptibility analyses (Table 3), we next assessed the replication capacities and kinetics of the four recombinant viruses with RT sequences identical to those in viruses from passages 25, 35, 60, and 65. Replication capacity was assessed in a single-cycle assay in P4/R5 cells, and replication kinetics was assessed using a multiple-cycle assay in MT-2 cells. Cells were infected with a standard inoculum (10

ng of p24) of each virus. Figure 1A shows that the replication capacity of the D67N/K70R/A371V/Q509L mutant was reduced to 48% of that of the wild-type virus. This loss in replication capacity, however, was restored to wild-type levels by the addition of the T215I mutation. By contrast, the T215F mutation reduced the replication capacity of the D67N/K70R/T215F/A371V/Q509L virus to 20% of that of the wild-type virus. This reduction in replication capacity from the T215F mutation was associated with significantly greater AZT resistance (Table 3), illustrating a trade-off between replication capacity and resistance. Similar results were observed in replication kinetics assays carried out in MT-2 cells over a 7-day period (Fig. 1B). Specifically, the replication of the D67N/K70R and D67N/K70R/A371V/Q509L mutant viruses was reduced 46% and 37%, respectively, on day 6 compared with wild-type virus. The impaired replication of the D67N/K70R/A371V/Q509L virus was restored to levels similar to that of the wild type by the T215I mutation. As described above, the T215F mutation markedly impaired viral replication.

Locations of residues A371 and Q509 in RT. Analysis of the crystal structure of HIV-1 RT in complex with an RNA/DNA

TABLE 3. AZT susceptibilities of site-directed mutants

Mutation	IC ₅₀ (μM) ^a	Resistance (n-fold) ^b	P value ^c
Wild type	0.2 ± 0.1	0.75	0.75
A371V	0.2 ± 0.04	0.7	0.2
Q509L	0.3 ± 0.2	1.3	0.6
A371V/Q509L	0.3 ± 0.06	1.7	0.4
D67N/K70R ^d	1.1 ± 0.6	4.6	<0.001
D67N/K70R/A371V	1.4 ± 0.5	6.4	<0.001
D67N/K70R/Q509L	3.0 ± 1.0	14	<0.001
D67N/K70R/A371V/Q509L ^d	9.1 ± 5.2	39	<0.001
D67N/K70R/T215I	0.3 ± 0.2	1.3	0.5
D67N/K70R/T215I/A371V	0.6 ± 0.3	2.6	0.07
D67N/K70R/T215I/Q509L	3.0 ± 2.2	14	0.004
D67N/K70R/T215I/A371V/Q509L ^d	9.4 ± 6.7	41	<0.001
D67N/K70R/T215F	3.8 ± 2.1	18	0.002
D67N/K70R/T215F/A371V	4.9 ± 2.9	22	<0.001
D67N/K70R/T215F/Q509L ^d	28 ± 17	128	<0.001
D67N/K70R/T215F/A371V/Q509L ^d	203 ± 40	934	<0.001
M41L/L210W/T215Y	6.3 ± 1.9	22	<0.001
M41L/L210W/T215Y/A371V/Q509L	85 ± 40	291	<0.001

^a Mean ± standard deviation from three to nine experiments.^b Resistance of mutant compared to wild type.^c Statistical significance compared to wild-type HIV_{LAI}.^d Mutation combination selected in vitro by AZT.

polypurine tract T/P substrate revealed that both A371 and Q509 are located near the T/P DNA binding tract (Fig. 2). A371 is 2.8 Å from K374, the side chain of which interacts with the phosphate backbone of the RNA template strand through a hydrogen bond (Fig. 2B). Q509 is close to the RNase H primer grip (segment of RT that aligns the RNA template with the RNase H active site through positioning of the DNA primer [30]), in particular, residue I505. The RNase H primer grip of HIV-1 RT contacts the DNA primer strand and positions the template strand near the RNase H active site, influencing RNase H cleavage efficiency and specificity (30).

DISCUSSION

In this study, we showed that AZT selects novel mutations in RT, specifically A371V in the connection domain and Q509L in the RNase H domain, that increase AZT resistance up to 50-fold when combined with the TAMs in the polymerase domain. This provides the first clear virologic evidence that mutations in both the connection and RNase H domains of RT can be selected by AZT. In addition, we show that these mutations, when combined with TAMs, confer greater cross-re-

sistance to 3TC and ABC, with a trend toward greater TNV resistance.

The only mutations that arose during the selection that started with AZT-resistant virus encoding the TAMs M41L, L210W, and T215Y were D67N and L214F in the polymerase domain (Table 1), which increased AZT resistance to >1,000-fold at passage 35. No mutations were detected in the connection or RNase H domains. This indicates that very high-level AZT resistance is possible with mutations restricted to the polymerase domain. Nevertheless, we determined the effects of A371V and Q509L mutations on the AZT susceptibility of virus with M41L/L210W/T215Y. AZT resistance was increased 10-fold by the addition of A371V and Q509L to the M41L/L210W/T215Y background, indicating that the phenotypic effects of A371V/Q509L are not specific for a single TAM pattern.

Other evidence suggests that mutations outside of the polymerase domain of HIV-1 RT are involved in resistance to NRTI. For example, Nikolenko et al. recently demonstrated that mutations that reduce RNase H activity, such as D549N and H539N, increase AZT resistance (22), but these mutations have not been identified in viruses from antiretroviral-experi-

TABLE 4. Cross-resistance of site-directed mutants to NRTI

Mutation in HIV _{LAI} RT	IC ₅₀ (resistance) ^a				
	3TC	ABC	TNV	d4T	ddI
Wild type	0.5 ± 0.1	6.4 ± 0.5	3.3 ± 0.9	7.2 ± 0.6	4.2 ± 0.9
D67N/K70R	1.0 ± 0.3 (1.9) ^c	7.9 ± 0.4 (1.2) ^c	4.9 ± 1.5 (1.5)	10 ± 3.1 (1.4)	5.0 ± 0.9 (1.2)
D67N/K70R/A371V/Q509L	2.7 ± 1.4 (5.2) ^{c,d}	13 ± 2.5 (2.0) ^{c,d}	7.3 ± 1.3 (2.2) ^c	9.6 ± 2.9 (1.3)	4.9 ± 0.9 (1.2)
D67N/K70R/T215F	3.8 ± 0.59 (7.0) ^b	15 ± 1.5 (2.4) ^b	4.9 ± 0.7 (1.5)	17 ± 4.6 (2.4) ^c	6.8 ± 0.3 (1.6) ^c
D67N/K70R/T215F/A371V/Q509L	7.5 ± 1.8 (15) ^{b,e}	19 ± 4.5 (3.0) ^c	9.0 ± 3.5 (2.7) ^c	14 ± 4.4 (2.0) ^c	6.6 ± 0.4 (1.6) ^c

^a Mean ± standard deviation from three experiments. Resistance (n-fold) compared to the wild type is in parentheses.^b IC₅₀ is significantly different from wild type; *P* < 0.001.^c IC₅₀ is significantly different from wild type; *P* < 0.05.^d IC₅₀ is significantly different from D67N/K70R; *P* < 0.05.^e IC₅₀ is significantly different from D67N/K70R/T215F; *P* < 0.5.

FIG. 1. Single-cycle and multiple-cycle replication assays of recombinant HIV_{LAI} containing the A371V and Q509L mutations. (A) Single-cycle replication was measured in P4/R5 cells infected with 10 ng p24 in a 96-well plate. After 48 h, cells were lysed and viral replication was measured using RLU. (B) Multiple-cycle replication was measured in MT-2 cells infected with 10 ng p24. p24 was measured from cell supernatant harvested daily for 7 days. The recombinant viruses analyzed were wild-type HIV_{LAI} (closed circle), D67N/K70R (open square), D67N/K70R/A371V/Q509L (closed square), D67N/K70R/T215I/A371V/Q509L (open circle), and D67N/K70R/T215F/A371V/Q509L (closed triangle). The data represent the means and standard deviations from three independent experiments. *, *P* values of <0.05 were considered significant compared to the wild-type control (HIV_{LAI}).

enced patients, nor have they been selected for by AZT *in vitro*. Initial analyses of clinical samples, however, have identified mutations in the connection and RNase H domains of RT that can increase AZT resistance (7, 9, 21). For example, mutations G335C, N348I, and A360I reduce AZT susceptibility 30-, 35-, and 30-fold, respectively, when present with TAMs (9, 21). In addition, a polymorphism at RT amino acid 333 (G to E) has been observed in samples from patients on combination therapy with AZT and 3TC (15). The G333E polymorphism counteracts the increase in AZT sensitivity of virus with the 3TC resistance mutation, M184V (33).

Several retrospective statistical analyses of clinical-genotype databases have identified other mutations in the connection and RNase H domains of RT that appear more frequently in samples from antiretroviral-experienced patients than antiretroviral-naïve patients (5, 7, 26, 27). However, the roles of these mutations in NRTI resistance have not been proven. The A371V mutation has been identified in patient genotypes in the Stanford HIV Drug Resistance Database (26), and our preliminary analysis of this database revealed that patients treated with AZT showed an increase in the frequencies of several mutations in the C terminus of RT (amino acids 350 to 560). For example, A371V was detected in 5.6% of 160 samples from treatment-naïve individuals and in 10.9% of 91 samples from patients treated with AZT monotherapy. Another mutation at codon 371 (A to T) was also seen at 2.1% frequency in AZT monotherapy samples. In addition, A371V was associated with mutations at T215 (Y/F/I/S) in 77% of the AZT monotherapy samples and with 46%, 23%, 31%, 23%, and 15% of the samples with M41L, D67N, K70R, L210W, and K219Q, respectively. Only 16 full-length sequences (to codon 560) from AZT monotherapy samples are available in the

Stanford database, and none of these have mutations at codon 509. Additional full-length RT sequences from patients who have received AZT therapy are being generated to examine the RNase H domain, including codon 509.

Two phenotypic mechanisms of NRTI resistance have been proposed. The first is NRTI discrimination, and it involves mutations in RT (such as K65R, K70E, L74V, Q151M, and M184V) that enable RT to preferentially incorporate the natural dNTP substrate versus the NRTI-triphosphate (2, 4, 29, 31a). The second mechanism has been termed NRTI excision associated with TAMs. The available biochemical evidence suggests that TAMs increase the ability of HIV-1 RT to phosphorylolytically excise AZT-MP from the chain-terminated T/P (3, 28). Because A371V and Q509L were selected in combination with TAMs and do not confer resistance to AZT alone, we hypothesize that these mutations enhance the RT-mediated excision reaction.

Analysis of the crystal structure of RT bound to an RNA/DNA T/P showed that A371V and Q509L reside close to the DNA binding tract in RT (Fig. 2). This suggests that the mutations may affect either T/P interactions (in the case of A371V and Q509L) or RNase H activity (in the case of Q509L). With regard to the latter, several studies have clearly demonstrated that mutations in the RNase primer grip can significantly impact the rates and efficiency of RNase H cleavage (14, 24). Mechanistic studies are currently under way to define the biochemical mechanisms by which A371V and Q509L increase AZT resistance.

Because there was only a small difference between the IC₅₀ values of viruses with D67N/K70R/A371V/Q509L and D67N/K70R/T215I/A371V/Q509L (Table 3), replication capacity and kinetics assays were performed to determine whether the

FIG. 2. Structural representation of AZT-selected mutations in the p66 subunit of RT. (A) Locations of residues A371 and Q509 in relation to TAMs D67, K70, and T215, the polymerase active site, and the RNase H active site. (B) Both A371 and Q509 are located near the T/P DNA binding tract. A371 is 2.8 Å from K374, whose side chain interacts with the phosphate backbone of the RNA template strand (white line). Q509 is in proximity to I505, a residue that makes up the RNase H primer grip. p66 subunit of RT, cartoon; DNA primer, green ribbon; RNA template, purple ribbon. The structure was drawn using the Molecular Operating Environment, based on coordinates from Sarafianos et al. (30). Protein Data Bank access number 1HYS.

T215I mutation affected viral replication capacity/kinetics. Single-cycle and multiple-cycle replication assays clearly showed that the T215I mutation restored the replication capacity and kinetics of the D67N/K70R/T215I/A371V/Q509L mutant to wild-type levels (Fig. 1). This likely explains why the T215I mutant emerged without having a significant impact on AZT resistance. The T215I mutation was subsequently replaced by T215F at higher AZT selective concentrations. This replacement is likely explained by the fact that the T215F mutation

conferred ~25-fold-greater AZT resistance than T215I, but at the cost of reduced replication capacity and kinetics in the absence of AZT.

In summary, we have selected mutations in vitro in the 3' region of RT that increase AZT resistance and cross-resistance to other nucleoside analog RT inhibitors. Biochemical analyses are in progress to define the mechanisms involved, and additional studies of clinical isolates are planned to define the occurrence and clinical significance of the mutations.

ACKNOWLEDGMENTS

This work was supported by grants from the National Cancer Institute (SAIC contract 20XS190A) and the National Institute of Allergy and Infectious Diseases (Virology Support Subcontract from the AACTG Central Group Grant U01AI38858).

REFERENCES

- Arion, D., N. Kaushik, S. McCormick, G. Borkow, and M. A. Parniak. 1998. Phenotypic mechanism of HIV-1 resistance to 3'-azido-3'-deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. *Biochemistry* **37**: 15908–15917.
- Boyer, P. L., S. G. Sarafianos, E. Arnold, and S. H. Hughes. 2002. The M184V mutation reduces the selective excision of zidovudine 5'-monophosphate (AZTMP) by the reverse transcriptase of human immunodeficiency virus type 1. *J. Virol.* **76**:3248–3256.
- Boyer, P. L., S. G. Sarafianos, E. Arnold, and S. H. Hughes. 2001. Selective excision of AZTMP by drug-resistant human immunodeficiency virus reverse transcriptase. *J. Virol.* **75**:4832–4842.
- Boyer, P. L., C. Tantillo, A. Jacobo-Molina, R. G. Nanni, J. Ding, E. Arnold, and S. H. Hughes. 1994. Sensitivity of wild-type human immunodeficiency virus type 1 reverse transcriptase to dideoxynucleotides depends on template length; the sensitivity of drug-resistant mutants does not. *Proc. Natl. Acad. Sci. USA* **91**:4882–4886.
- Chen, L., A. Perlina, and C. J. Lee. 2004. Positive selection detection in 40,000 human immunodeficiency virus (HIV) type 1 sequences automatically identifies drug resistance and positive fitness mutations in HIV protease and reverse transcriptase. *J. Virol.* **78**:3722–3732.
- Furine, E. S., and J. E. Reardon. 1991. Reverse transcriptase RNase H from the human immunodeficiency virus. Relationship of the DNA polymerase and RNA hydrolysis activities. *J. Biol. Chem.* **266**:406–412.
- Galli, R., B. Wynhoven, B. Sattha, G. Tachedjian, and P. Harrigan. 2004. Beyond codon 240: mutations in the HIV-1 reverse transcriptase selected after exposure to antiretrovirals, abstr. WePeB5710. Poster exhibition, XV International AIDS Conference, Bangkok, Thailand, 11 to 16 July 2004.
- Ghosh, M., K. J. Howard, C. E. Cameron, S. J. Benkovic, S. H. Hughes, and S. F. Le Grice. 1995. Truncating alpha-helix E' of p66 human immunodeficiency virus reverse transcriptase modulates RNase H function and impairs DNA strand transfer. *J. Biol. Chem.* **270**:7068–7076.
- Gupta, S., S. Fransen, E. E. Paxinos, W. Huang, E. Stawiski, C. J. Petropoulos, and N. T. Parkin. 2006. Infrequent occurrence of mutations in the C-terminal region of reverse transcriptase modulates susceptibility to RT inhibitors. *Antivir. Ther.* **11**:S143.
- Halvas, E. K., G. M. Aldrovandi, P. Balfe, I. A. Beck, V. F. Boltz, J. M. Coffin, L. M. Frenkel, J. D. Hazelwood, V. A. Johnson, M. Kearney, A. Kovacs, D. R. Kuritzkes, K. J. Metzner, D. V. Nissley, M. Nowicki, S. Palmer, R. Ziermann, R. Y. Zhao, C. L. Jennings, J. Bremer, D. Brambilla, and J. W. Mellors. 2006. Blinded, multicenter comparison of methods to detect a drug-resistant mutant of human immunodeficiency virus type 1 at low frequency. *J. Clin. Microbiol.* **44**:2612–2614.
- Huang, H., R. Chopra, G. L. Verdine, and S. C. Harrison. 1998. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* **282**:1669–1675.
- Jacobo-Molina, A., J. Ding, R. G. Nanni, A. D. Clark, Jr., X. Lu, C. Tantillo, R. L. Williams, G. Kamer, A. L. Ferris, P. Clark, A. Hizi, S. H. Hughes, and E. Arnold. 1993. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. *Proc. Natl. Acad. Sci. USA* **90**:6320–6324.
- Johnson, V. A., F. Brun-Vezinet, B. Clotet, B. Conway, D. R. Kuritzkes, D. Pillay, J. M. Schapiro, A. Telenti, and D. D. Richman. 2005. Update of the drug resistance mutations in HIV-1: fall 2005. *Top. HIV Med.* **13**:125–131.
- Julias, J. G., M. J. McWilliams, S. G. Sarafianos, W. G. Alvord, E. Arnold, and S. H. Hughes. 2003. Mutation of amino acids in the connection domain of human immunodeficiency virus type 1 reverse transcriptase that contain the template-primer affects RNase H activity. *J. Virol.* **77**:8548–8554.
- Kemp, S. D., C. Shi, S. Bloor, P. R. Harrigan, J. W. Mellors, and B. A. Larder. 1998. A novel polymorphism at codon 333 of human immunodeficiency virus type 1 reverse transcriptase can facilitate dual resistance to zidovudine and L-2',3'-dideoxy-3'-thiacytidine. *J. Virol.* **72**:5093–5098.
- Kohlstaedt, L. A., J. Wang, J. M. Friedman, P. A. Rice, and T. A. Steitz. 1992. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**:1783–1790.
- Krebs, R., U. Immendorfer, S. H. Thrall, B. M. Wohrl, and R. S. Goody. 1997. Single-step kinetics of HIV-1 reverse transcriptase mutants responsible for virus resistance to nucleoside inhibitors zidovudine and 3-TC. *Biochemistry* **36**:10292–10300.
- Le Grice, S. F., and F. Gruninger-Leitch. 1990. Rapid purification of homodimer and heterodimer HIV-1 reverse transcriptase by metal chelate affinity chromatography. *Eur. J. Biochem.* **187**:307–314.
- Marcelin, A. G., C. Delaunay, M. Wirden, P. Viegas, A. Simon, C. Katlama, and V. Calvez. 2004. Thymidine analogue reverse transcriptase inhibitors resistance mutations profiles and association to other nucleoside reverse transcriptase inhibitors resistance mutations observed in the context of virological failure. *J. Med. Virol.* **72**:162–165.
- Meyer, P. R., S. E. Matsuura, A. M. Mian, A. G. So, and W. A. Scott. 1999. A mechanism of AZT resistance: an increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase. *Mol. Cell* **4**:35–43.
- Nikolenko, G. N., K. A. Franenberry, S. Palmer, F. Maldarelli, J. W. Mellors, J. M. Coffin, and V. K. Pathak. 2006. The HIV-1 reverse transcriptase connection domain from treatment-experienced patients contributes to AZT resistance. *Antivir. Ther.* **11**:S142.
- Nikolenko, G. N., S. Palmer, F. Maldarelli, J. W. Mellors, J. M. Coffin, and V. K. Pathak. 2005. Mechanism for nucleoside analog-mediated abrogation of HIV-1 replication: balance between RNase H activity and nucleotide excision. *Proc. Natl. Acad. Sci. USA* **102**:2093–2098.
- Parikh, U. M., D. L. Koontz, C. K. Chu, R. F. Schinazi, and J. W. Mellors. 2005. In vitro activity of structurally diverse nucleoside analogs against human immunodeficiency virus type 1 with the K65R mutation in reverse transcriptase. *Antimicrob. Agents Chemother.* **49**:1139–1144.
- Rausch, J. W., D. Lener, J. T. Miller, J. G. Julias, S. H. Hughes, and S. F. Le Grice. 2002. Altering the RNase H primer grip of human immunodeficiency virus reverse transcriptase modifies cleavage specificity. *Biochemistry* **41**:4856–4865.
- Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* **27**:493–497.
- Rhee, S. Y., M. J. Gonzales, R. Kantor, B. J. Betts, J. Ravela, and R. W. Shafer. 2003. Human immunodeficiency virus reverse transcriptase and protease sequence database. *Nucleic Acids Res.* **31**:298–303.
- Roquebert, B., M. Wirden, A. Simon, J. Deval, C. Katlama, V. Calvez, and A. G. Marcelin. 2007. Relationship between mutations in HIV-1 RNase H domain and nucleoside reverse transcriptase inhibitors resistance mutations in naive and pre-treated HIV infected patients. *J. Med. Virol.* **79**:207–211.
- Sarafianos, S. G., A. D. Clark, Jr., K. Das, S. Tuske, J. J. Birktoft, P. Ilankumaran, A. R. Ramesha, J. M. Sayer, D. M. Jerina, P. L. Boyer, S. H. Hughes, and E. Arnold. 2002. Structures of HIV-1 reverse transcriptase with pre- and post-translocation AZTMP-terminated DNA. *EMBO J.* **21**:6614–6624.
- Sarafianos, S. G., K. Das, A. D. Clark, Jr., J. Ding, P. L. Boyer, S. H. Hughes, and E. Arnold. 1999. Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with beta-branched amino acids. *Proc. Natl. Acad. Sci. USA* **96**:10027–10032.
- Sarafianos, S. G., K. Das, C. Tantillo, A. D. Clark, Jr., J. Ding, J. M. Whitcomb, P. L. Boyer, S. H. Hughes, and E. Arnold. 2001. Crystal structure of HIV-1 reverse transcriptase in complex with a polypurine tract RNA: DNA. *EMBO J.* **20**:1449–1461.
- Shi, C., and J. W. Mellors. 1997. A recombinant retroviral system for rapid in vivo analysis of human immunodeficiency virus type 1 susceptibility to reverse transcriptase inhibitors. *Antimicrob. Agents Chemother.* **41**:2781–2785.
- Sluis-Cremer, N., C.-W. Sheen, S. Zelina, P. S. A. Torres, U. M. Parikh, and J. W. Mellors. 2007. Molecular mechanism by which the K70E mutation in human immunodeficiency virus type 1 reverse transcriptase confers resistance to nucleoside reverse transcriptase inhibitors. *Antimicrob. Agents Chemother.* **51**:48–53.
- Sluis-Cremer, N., D. Arion, and M. A. Parniak. 2000. Molecular mechanisms of HIV-1 resistance to nucleoside reverse transcriptase inhibitors (NRTIs). *Cell Mol. Life Sci.* **57**:1408–1422.
- Tisdale, M., S. D. Kemp, N. R. Parry, and B. A. Larder. 1993. Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3'-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **90**:5653–5656.
- Vaccaro, J. A., H. A. Singh, and K. S. Anderson. 1999. Initiation of minus-strand DNA synthesis by human immunodeficiency virus type 1 reverse transcriptase. *Biochemistry* **38**:15978–15985.
- Wang, J., S. J. Smerdon, J. Jager, L. A. Kohlstaedt, P. A. Rice, J. M. Friedman, and T. A. Steitz. 1994. Structural basis of asymmetry in the human immunodeficiency virus type 1 reverse transcriptase heterodimer. *Proc. Natl. Acad. Sci. USA* **91**:7242–7246.