HIV-1 Subtype C Reverse Transcriptase and Protease Genotypes in Zimbabwean Patients Failing Antiretroviral Therapy

RAMI KANTOR[1], **LYNN S. ZIJENAH**[2], **ROBERT W. SHAFER**[1], **SOLOMON MUTETWA**[3], **ELIZABETH JOHNSTON**[1], **ROBERT LLOYD**[4], **ANDREA VON LIEVEN**[5], **DENNIS ISRAELSKI**[1], and **DAVID A. KATZENSTEIN**[1]

1*Division of Infectious Diseases and AIDS Research, Stanford University, Stanford, California 94305*

2*Department of Immunology, University of Zimbabwe Medical School, Harare, Zimbabwe*

3*Department of Medical Microbiology, University of Zimbabwe Medical School, Harare, Zimbabwe*

4*Visible Genetics, Suwanee, Georgia 30024*

5*Department of Community Medicine, University of Zimbabwe Medical School, Harare, Zimbabwe*

Abstract

HIV-1 drug resistance mutations have been identified and characterized mostly in subtype B HIV-1 infection. The extent to which antiretroviral drugs select for drug resistance mutations in non-subtype B HIV-1 is not known. We obtained HIV-1 reverse transcriptase (RT) and protease sequences from 21 Zimbabwean patients failing antiretroviral drug therapy. We compared these sequences with 56 published RT and protease subtype C sequences from untreated patients, 990 RT and 1140 protease subtype B sequences from treated patients, and 340 RT and 907 protease subtype B sequences from untreated patients and identified four mutation categories of subtype C HIV-1. Seventeen of the 21 patients (81%) had known drug resistance mutations. Mutations at 15 RT and 11 protease positions were more common in subtype C isolates than in subtype B isolates. HIV-1 subtype C-infected individuals receiving antiretroviral therapy develop many of the known subtype B drug resistance mutations. Comparison of subtype C RT and protease sequences with a large database of subtype B sequences identified subtype C-specific polymorphisms and candidate drug resistance mutations.

Dʀᴜɢ ʀᴇꜱɪꜱᴛᴀɴᴄᴇ is a major obstacle to achieving and maintaining virus suppression. Most studies of drug resistance, as well as interpretations of genotypic changes in HIV-1 reverse transcriptase (RT) and protease, are based on sequence data from HIV-1 subtype B viruses. Worldwide, however, non-B isolates are far more common than subtype B isolates, and subtype C isolates account for the majority of infections globally.[1] Identification of mutations in subtype C isolates associated with antiretroviral therapy may alter the interpretation of drug resistance testing and surveillance.

Five previous studies included 183 patients infected with subtype C virus and receiving antiretroviral therapy.[2-6] However, precisely how the sequences from these individuals were related to the antiretroviral treatment has not been published. In this article we describe RT and protease genotypes of subtype C HIV-1 isolates from 21 Zimbabwean individuals receiving

---

antiretroviral therapy. By comparing these sequences with subtype B sequences in a large database, we identify subtype C-specific polymorphisms and potential treatment-related mutations in the RT and protease.

The study population included persons infected with HIV-1 and attending the Centre, an HIV treatment and support clinic in Harare, Zimbabwe. Patients were receiving medications through an access program by which donated drugs were provided through an association of their physicians. Recruitment for the study was performed on two clinic days in 2001. Samples from patients who had received antiretroviral therapy for at least 2 months and who consented to participate were included. The study was approved by the Stanford University (Stanford, CA) administrative panel on human subjects in medical research.

For each sample, sequences corresponding to RT amino acids 1-240 and protease amino acids 1-99 were obtained. Manual RNA extraction was performed with the TRUPREP viral RNA kit (Visible Genetics, Suwanee, GA), with alternate protocols supplied by the manufacturer for concentrating virus. Genotyping of extracted vial RNA was accomplished with the TRUGENE HIV-1 genotyping kit (Visible Genetics) and sequencing platform with modifications. Briefly, modified RTpolymerase chain reactions (RT-PCRs) were accomplished by substitution of kit primers for Research Use Only version 1.5 RT-PCR primers (Visible Genetics, cat. no. VG 30231) and modified thermocycling conditions. Bidirectional DNA CLIP sequencing was performed according to standardized kit chemistry and manufacturer protocols.

The Roche Amplicor Monitor test (version 1.5; Roche Diagnostics, Branchburg, NJ) was used to determine the plasma HIV-1 RNA concentration. Samples were separated and frozen within 6 hr of collection in the Department of Immunology of the University of Zimbabwe (Harare, Zimbabwe). The limit of detection with this assay was 400 copies/ml. CD4[1] cell counts were performed by flow cytometry (Becton Dickinson, Mountain View, CA) in the University of Zimbabwe Public Health Hematology Laboratory.

Mutations were defined as amino acid differences from consensus B RT and protease sequences. Polymorphisms were defined as mutations that occurred in at least 1% of sequences from untreated persons. Treatment-related mutations were defined as mutations that occurred at statistically significant higher rates in sequences from treated versus untreated persons. Drug resistance mutations were defined as mutations known to cause drug resistance based on published experimental data.[7]

Nucleoside RT inhibitor (NRTI) drug resistance mutations were defined as mutations at RT positions 41, 44, 62, 65, 67, 69, 70, 74, 75, 77, 115, 116, 118, 151, 184, 210, 215, and 219. Nonnucleoside RT inhibitor (NNRTI) drug resistance mutations were defined as mutations at RT positions 98, 100, 101, 103, 106, 108, 179, 181, 188, 190, 225, 230, and 236. Primary protease inhibitor (PI) drug resistance mutations were defined as mutations at protease positions 30, 48, 50, 82, 84, and 90. Secondary PI drug resistance mutations were defined as mutations at protease positions 10, 20, 24, 32, 33, 46, 47, 53, 54, 71, 73, 77, and 88. Mutations at protease positions 36, 63, and 93 were not categorized as drug resistance mutations, because although mutations at these positions may contribute to resistance in subtype B isolates, these positions are highly polymorphic (>50%) in subtype C isolates.[5,6,8]

To characterize mutations as possible subtype-specific polymorphisms or treatment-related mutations, isolates from patients in this study were compared with publicly available sequences from (1) 990 RT and 1140 protease subtype B isolates from treated patients, (2) 340 RT and 907 protease subtype B isolates from untreated patients, and (3) 56 RT and protease subtype C isolates from untreated patients.[9] At each amino acid position, a x^2 test comparing the mutation rate of sequences in this study and those in groups 1-3 and between isolates in groups

1-3 was performed. A *p* value of <0.05 was considered statistically significant. Results were not corrected for multiple comparisons.

On the basis of these comparisons, four categories of mutations were defined.

> *Subtype C polymorphisms:* Mutations that were significantly more common in subtype C isolates than in subtype B isolates, but that were present at similar frequencies in treated and untreated persons.

> *Subtype C polymorphism/subtype B treatment-related mutations:* Subtype C polymorphisms that occurred at significantly higher frequencies in treated persons with subtype B virus than in untreated persons with subtype B virus.

> *Subtype C polymorphism and treatment-related mutations:* Subtype C polymorphisms that were present at higher rates in treated compared with untreated subtype C isolates.

> *Subtype B/C treatment-related mutations:* Mutations present at higher rates in treated compared with untreated persons with subtype B and C virus.

Twenty-one patients (84%) had RNA levels >400 copies/ml, the lower limit of quantification; four patients (16%) had undetectable plasma HIV-1 RNA levels. Seventytwo percent (18 of 25) of the patients were males. The median age was 41 years (range, 31-61 years). The median viral load was 4.0 $\log_{10}$ copies/ml (range, <2.6-6.0 $\log_{10}$ copies/ml), and the median CD4[1] cell count was 95×10^6 cells/liter (range, $1-321 \times 10^6$ cells/liter).

Among the 21 patients with detectable viremia, 21 had received NRTIs, 18 had received PIs, and 5 had received NNRTIs. Eleven patients were receiving their first antiretroviral drug regimen, and 10 had received one or more previous antiretroviral regimens. Four of 21 patients (19%) received a regimen with one drug class, 16 of 21 patients (76%) received a regimen with two drug classes, and 1 patient received a regimen with three drug classes. The most commonly used PI was saquinavir (SQV; 10 patients), followed by nelfinavir (NFV; 3 patients), indinavir (IDV; 2 patients), and amprenavir (APV; 2 patients). The most commonly used NRTI was lamivudine (3TC; 15 patients), followed by zidovudine (AZT; 13 patients) and stavudine (d4T; 4 patients). Two patients had received efavirenz and one patient had received nevirapine. The median duration of the most recent antiretroviral regimen was 4 months (range, 2-32 months), and the median duration of total antiretroviral drug therapy was 11 months (range, 3-37 months). The most recent regimen, as well as a list of drugs received before the most recent regimen, are shown in Table 1.

RT and protease sequences were obtained from isolates from each of the 21 patients with detectable viremia. In phylogenetic analyses using TREECON version 1.3b,[10] all 21 sequences clustered with subtype C reference sequences C2220 and 92BR025 (http://hiv-web.lanl.gov).

Differences from consensus B were identified at 58 RT and 37 protease positions with a mean of 15.6 differences per RT isolate and 10.7 differences per protease isolate. Table 1 shows the known drug resistance mutations in the isolates from the Zimbabwean patients. Excluding mutations at positions 36, 63, and 93, which are the consensus amino acids at these positions (>50%) in subtype C isolates, there were 1.0 PI resistance mutation, 2.4 NRTI resistance mutations, and 1.2 NNRTI resistance mutations per isolate. Seventeen isolates (81%) had at least one known drug resistance mutation. Seven isolates (33%) had mutations associated with resistance to just one drug class, eight isolates (38%) had mutations associated with resistance to two drug classes, and two isolates (10%) had mutations associated with resistance to all three drug classes.

NRTI resistance mutations were identified at positions 41, 67, 69, 70, 74, 151, 184, 215, 219 (14/21 patients receiving NRTIs). NNRTI resistance mutations were identified at positions 98, 103, 179, 181 (3/5 patients receiving NNRTIs). PI resistance mutations were identified at positions 10, 20, 36, 54, 63, 71, 73, 82, 90, 93. Seven of the isolates from patients receiving a PI had primary PI resistance mutations; nine had secondary resistance mutations.

The most common NRTI-related drug resistance mutations were M184V (11 patients), T215Y/I/S/F (8 patients), K70R (7 patients), M41L (6 patients), and D67N/G (6 patients). Mutations at position 67, 70, and 215 with or without a mutation at position 219 occurred in six patients. The most common NNRTI-related drug resistance mutation was Y181C (two patients). Mutations at primary PI resistance-related positions included L90M (four patients) and V82I (three patients). The most common mutations at secondary PI resistance-related positions were K20R (4 of 21 patients), I71V (3 of 11 patients), and L10I and G73S (2 of 21 patients each).

Figures 1 and 2 show the 34 RT and 21 protease mutations that were present in 2 or more of the 21 Zimbabwean isolates. Figures 1 and 2 compare the prevalence of mutation in the study patients with the prevalence of mutation in subtype C isolates from untreated persons and with subtype B isolates from treated and untreated persons.

Mutations at RT positions 36, 48, 173, and 177 and at protease positions 12, 15, 19, 41, and 69 were subtype C polymorphisms. Mutations at RT positions 35, 39, 43, 60, 90, 200, 207, and 211 and at protease positions 20, 36, 60, 82, 89, and 93 were subtype C polymorphisms that occurred more commonly in treated than in untreated subtype B isolates. Mutations at RT positions 53, 123, and 174 were subtype C polymorphisms that occurred at a higher rate in treated than in untreated persons with subtype C viruses. Mutations at RT positions 41, 67, 70, 77, 181, 184, 203, 214, 215, 219, and 228 and at protease positions 73, 74, and 90 were non-polymorphic mutations that occurred at a higher rate in subtype B and C isolates from treated patients compared with untreated patients.

Eighty-one percent of patient isolates (17 of 21) had known RT and protease drug resistance mutations associated with at least one of the NRTIs, NNRTIs, or PIs. The primary protease-inhibitor resistance mutation L90M was seen in 4 of 10 patients (40%) receiving SQV, a rate similar to that seen in patients infected with subtype B and receiving SQV (75 of 149, or 50%). The amino acid isoleucine (I) at position 82 in the protease substrate cleft is not one of the common HIV-1 subtype B mutations associated with PI resistance, and occurs at a rate of 1% in treated and untreated individuals. In non-B isolates V82I is more common, occurring in 7% of isolates from untreated patients infected with subtype C and in as high as 72% of isolates from untreated patients infected with subtype G. Four (19%) of our study cohort patients had V82I. Decreased susceptibility of isolates with this mutation to PIs has been suggested,[11] and its contribution to PI resistance in non-B subtypes still needs to be determined.

M184V occurred in 11 of 15 patients (73%) receiving 3T3, a rate similar to that seen in patients infected with subtype B and receiving lamivudine (606 of 819, or 74%). The multiNRTI-resistant Q151M mutation, associated with changes at codons 62, 75, 77, and 116,[12] is reported here for the first time in an HIV-1 subtype C isolate. The patient with this isolate was treated with didanosine (ddI) for 6 months and with AZT and ddI for an additional 8 months. Mutations at RT positions 203 and 228 and at protease position 74 are not known to be associated with drug resistance; however, they were each seen at high rates (5-17%) in isolates from treated patients infected with subtypes B and C compared with untreated patients (0-1%).

Comparison of sequences from treated and untreated persons infected with different HIV-1 subtypes enabled us to identify mutation categories on the basis of the prevalence of mutations in subtypes B and C, with or without drug selection. This approach tests the null hypothesis that mutations associated with treatment are independent of subtype. Subtype C-specific

polymorphisms that occur at equal rates among treated and untreated persons would not be expected to influence the response to antiretroviral therapy. In contrast, the subtype C-specific mutations that occur at a higher rate among treated persons (whether subtype B or C) may have implications for response to therapy even if they do not include any of the experimentally characterized drug resistance mutations.

Comparison of sequences from treated and untreated subjects with subtype C HIV-1 identified three possible RT mutations that warrant further study. These subtype C treatment-related mutations, at RT positions 53, 123, and 174, were present at higher rates in treated persons infected with subtype C, suggesting an association with drug resistance in subtype C isolates. RT position 53 is situated in the "fingers" subdomain of the enzyme and is close to the region that is associated with deoxynucleotide triphosphate (dNTP) binding. RT position 174 is located in the region associated with the template-primer complex interaction.[14]

Of the 95 differences from consensus B that were identified in the RT and protease of the study cohort, one group, consisting of subtype C polymorphisms, associated or not associated with treatment in subtype B, has been previously described.[5,6,13] However, within that group, RT positions 43, 60, and 90 and protease positions 12 and 60 are noted here for the first time.

In summary, as antiretroviral therapy becomes available in Zimbabwe and other areas where subtype C predominates, there is a need to identify similarities and differences in the sequences of the molecular targets of therapy among divergent HIV-1 subtypes. Despite study limitations, including small sample size, patient adherence issues, and the impact of different/suboptimal therapeutic regimens on the resistance patterns, our findings suggest that similar selection of drug resistance in nonsubtype B viruses is anticipated. However, subtype-associated polymorphisms and preexisting resistance-associated mutations may render some drug treatment strategies more or less effective. Clinical trials and surveillance efforts, including treatment histories and determination of sequences and *in vitro* phenotypic resistance of non-subtype B viruses, are needed to identify significant differences in drug susceptibility and resistance among HIV-1 subtypes.

ACKNOWLEDGMENTS

REFERENCES

1. Osmanov S, Pattou C, Walker N, et al. Estimated global distribution and regional spread of HIV-1 genetic subtypes in the year 2000. J Acquir Immune Defic Syndr 2002;29:184–190. [PubMed: 11832690]

2. Yahi N, Fantini J, Tourres C, et al. Use of drug resistance sequence data for the systematic detection of non-B human immunodeficiency virus type 1 (HIV-1) subtypes: How to create a sentinel site for monitoring the genetic diversity of HIV-1 at a country scale. J Infect Dis 2001;183:1311–1317. [PubMed: 11294661]

3. Barlow KL, Tatt ID, Cane PA, et al. Recombinant strains of HIV type 1 in the United Kingdom. AIDS Res Hum Retroviruses 2001;17:467–474. [PubMed: 11282016]

4. Cane PA, De Ruiter A, Rice PA, et al. Resistance-associated mutations in subtype C HIV-1 protease from protease inhibitor-treated and untreated patients. Antivir Ther 2001;6:114.

5. Frater AJ, Beardall A, Ariyoshi K, et al. Impact of baseline polymorphisms in RT and protease on outcome of highly active antiretroviral therapy in HIV-1-infected African patients. AIDS 2001;15:1493–1502. [PubMed: 11504981]

6. Grossman Z, Vardinon N, Chemtob D, et al. Genotypic variation of HIV-1 reverse transcriptase and protease: comparative analysis of clade C and clade B. AIDS 2001;15:1453–1460. [PubMed: 11504976]

7. Hirsch MS, Brun-Vezinet F, D'Aquila RT, et al. Antiretroviral drug resistance testing in adult HIV-1 infection: Recommendations of an International AIDS Society—USA Panel. JAMA 2000;283:2417–2426. [PubMed: 10815085]

8. Shafer, RW.; Winters, MA.; Dupnik, K., et al. A guide to HIV reverse transcriptase and protease sequencing for drug resistance studies. 2001 [Accessed June 3, 2002]. Los Alamos National Laboratory WebsiteAvailable at: http://hiv-web.lanl.gov/content/hiv-db/COMPENDIUM/2001/part1/Shafer.pdf

9. Kantor R, Machekano R, Gonzales MJ, et al. Human immunodeficiency virus reverse transcriptase and protease sequence database: An expanded model integrating natural language text and sequence analysis. Nucleic Acids Res 2001;29:296–299. [PubMed: 11125118]

10. Van de Peer Y, De Wachter R. Construction of evolutionary distance trees with TREECON for Windows: Accounting for variation in nucleotide substitution rate among sites. Comput Appl Biosci 1997;13:227–230. [PubMed: 9183524]

11. Descamps D, Apetrei C, Collin G, et al. Naturally occurring decreased susceptibility of HIV-1 subtype G to protease inhibitors. AIDS 1998;12:1109–1111. [PubMed: 9662212]

12. Shafer RW, Kozal MJ, Winters MA, et al. Combination therapy with zidovudine and didanosine selects for drug-resistant human immunodeficiency virus type 1 strains with unique patterns of *pol* gene mutations. J Infect Dis 1994;169:722–729. [PubMed: 8133086]

13. Loveday C. International perspectives on antiretroviral resistance: Nucleoside reverse transcriptase inhibitor resistance. J Acquir Immune Defic Syndr 2001;26(Suppl 1):S10–S24. [PubMed: 11264998]

14. Gonzales MJ, Machekano RN, Shafer RW. Human immunodeficiency virus type 1 reverse-transcriptase and protease subtypes: Classification, amino acid mutation patterns, and prevalence in a northern California clinic-based population. J Infect Dis 2001;184:998–1006. [PubMed: 11574914]
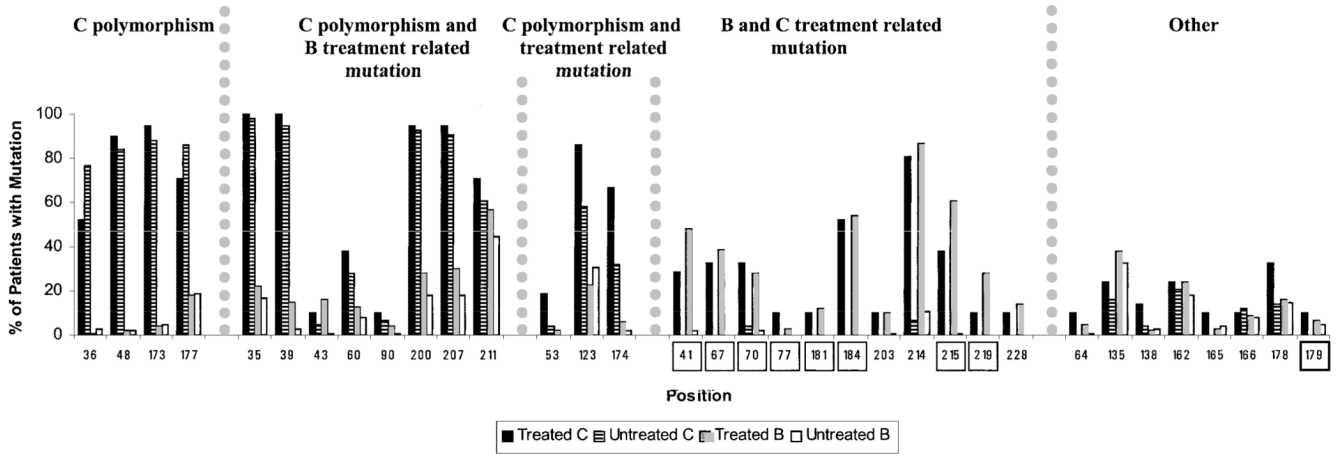
FIG. 1.
Prevalence of HIV-1 RT mutations in 21 subtype C isolates from patients receiving antiretroviral therapy: Comparison with subtype C isolates from untreated patients and subtype B isolates from treated and untreated patients. Shown are only those positions at which two or more mutations were observed in study patients; boxes mark positions associated with drug resistance in subtype B; RT isolates from study patients were compared with 990 isolates from treated patients infected with subtype B, 340 isolates from untreated patients infected with subtype B, and 56 isolates from untreated patients infected with subtype C.
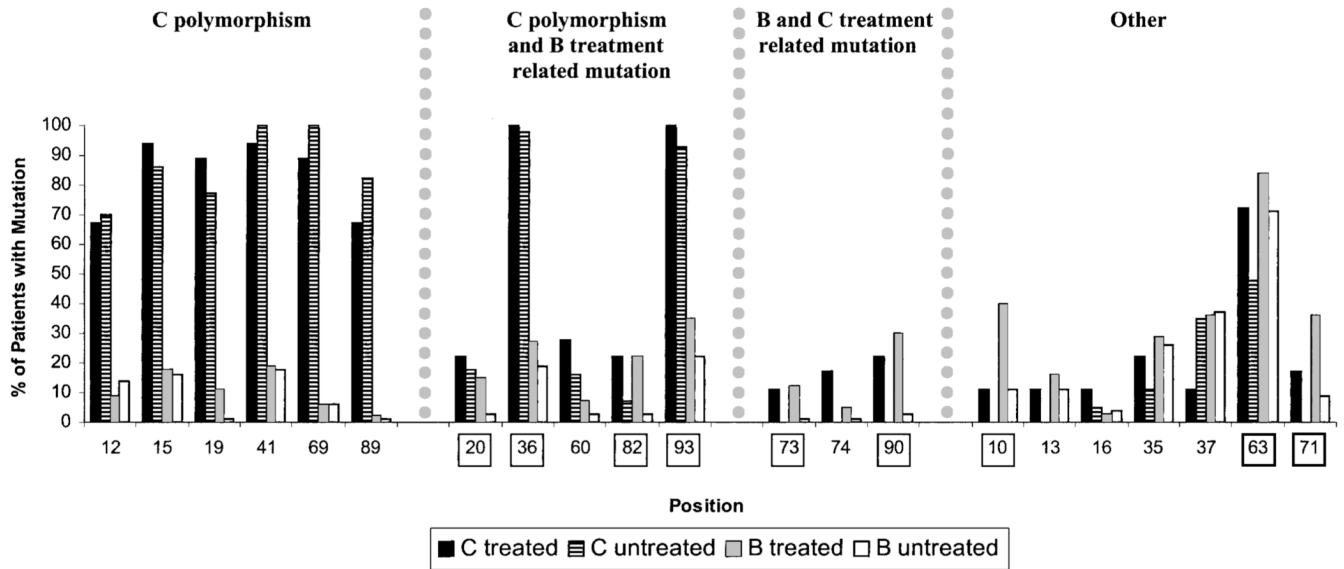
FIG. 2.
Prevalence of HIV-1 protease mutations in 18 subtype C isolates from patients receiving antiretroviral therapy: Comparison with subtype C isolates from untreated patients and subtype B isolates from treated and untreated patients. Shown are only those positions at which two or more mutations were observed in study patients; boxes mark positions associated with drug resistance in subtype B; protease isolates from study patients were compared with 1140 isolates from treated patients infected with subtype B, 907 isolates from untreated patients infected with subtype B, and 56 isolates from untreated patients infected with subtype C.

Table 1

Treatment History and RT and Protease Drug Resistance Mutations in 21 Zimbabwean Patients

Patient ID	Recent regimen	Previous drug exposure	Total Rx duration (months)	RT mutations at drug resistance positions NRTI	NNRTI	Protease mutations at drug-resistance positions
1	AZT + 3TC + SQV	ddI	19	None	None	M36I, L63ALPV, V82I, I93L
2	3TC + d4T + SQV	AZT	8	M184V	V179IV	M36I, L63T, I93L
3	3TC + d4T + SQV		9	M41L, M184V	None	M36I, L63P, A71V, L90M, I93L
4	AZT + 3TC + NFV	ddC, IDV	22	M41L, K70KR, M184V, T215Y	None	M36I, L63AP, G73S, I93L
5	AZT + 3TC + IDV	d4T, ddI	33	None	None	K20R, M36I, I93L
6	IDV + SQV + NVP	AZT, 3TC, ddI	18	M41L, M184V, T215Y	K103N	M36I, L63A, I93L
7	AZT + 3TC + SQV		37	M41L, D67DN, K70KR, M184V, T215Y	A98AG	K20R, M36I, I93L
8	AZT + 3TC + SQV		32	D67N, K70R, V118I, M184V, K219Q	V179IV	K20KR, M36I, L63P, A71AV, L90M, I93L
9	D4T + 3TC + NFV	AZT, NVP	20	M184V	None	K2OR, M36I, L63I, V82I, I93L
10	D4T + 3TC + APV		11	M41L, D67N, L74V, T215Y	Y181I	L10I, M36I, I54FL, L63P, I93L
11	ABC + APV + EFV	d4T, NFV, NVP	9	None	None	M36I, L63P, I93L
12	AZT + 3TC + RTV	EFV	17	M184V	None	M36I, L63V, I93L
13	AZT + ddC + SQV		11	D67G, K70R, K219Q	None	M36I, L63P, V82I, L90M, I93L
14	AZT + ddI		12	D67N, T69N, K70R, F77FL, Q151M, T215T, K219EQ	None	L10I, M36I, I93IL
15	AZT + 3TC	NFV	3	M184V	None	M36I, I93L
16	AZT + 3TC + NFV		8	None	None	M36I, L63P, A71V, G73GS, L90M, I93L
17	AZT + 3TC + SQV		11	D67N, K70R, M184V, T215IT, K219Q	None	M36I, I93L
18	ddI		6	None	None	M36I, I93L
19	AZT + 3TC		5	M41LM, D67DN, K70KR, M184V, T215FIST	None	M36I, I93L
20	AZT + 3TC + SQV		11	None	None	M36I, L63V, V82I, I93L
21	SQV + RTV + EFV	d4T, IDV, NVP	11	None	None	M36I, L63LP, I93L

Abbreviations: Rx, treatment; AZT, zidovudine; 3TC, lamivudine; SQV, saquinavir; ddI, didanosine; d4T, stavudine; NFV, nelfinavir; ddC, zalcitabine; IDV, indinavir; NVP, nevirapine; APV, amprenavir; ABC, abacavir; EFV, efavirenz; RTV, ritonavir.