Antiviral Chemistry & Chemotherapy 15:189–200

Mutations and polymorphisms associated with antiretroviral drugs in HIV-1C-infected African patients

Florence Doualla-Bell[1,2], Simane Gaseitsiwe[1], Thumbi Ndung'u[1], Musetsanagepe Modukanele[1], Trevor Peter[1], Vladimir Novitsky[3], Ndwapi Ndwapi[4], Gaolathe Tendani[4], Ava Avalos[1], William Wester[1], Hermann Bussmann[1], Peter Cardiello[1], Richard Marlink[3], Howard Moffat[4], Ibou Thior[1], Mark A Wainberg[2] and Max Essex[3]*

[1]Botswana-Harvard AIDS Institute Partnership for HIV Research and Education, Gaborone, Botswana
[2]McGill AIDS Center-McGill University, Montreal, Que., Canada
[3]Harvard School of Public Health, Department of Immunology and Infectious Diseases, Boston, Mass., USA
[4]Infectious Diseases Care Clinic, Princess Marina Hospital, Gaborone, Botswana

*Corresponding author: Tel: +1 617 432 2334; Fax: +1 617 739 8348; E-mail: messex@hsph.harvard.edu

To detect and characterize polymerase gene (*pol*) polymorphisms and mutation patterns in HIV-1C-infected Batswana patients treated with reverse transcriptase inhibitors, samples from AIDS patients treated with highly active antiretroviral therapy (HAART) were sequenced for the region encompassing the entire HIV-1 protease (PR) and the first 335 amino acids of reverse transcriptase (RT). Amongst the 16 patients treated with antiretroviral (ARV) drugs, eight started HAART regimens containing didanosine, stavudine and nevirapine (ddI/d4T/NVP) or efavirenz (EFV) (arm A) while the others started with zidovudine (AZT) and lamivudine (3TC) given together as combivir (CBV) with either NVP or EFV as arm B. Arm B is the first line regimen currently provided by the Botswana ARV national programme. Greater efficacy, in terms of treatment duration, was observed in patients in arm B (14 months) as compared with patients in arm A (9 months); $P<0.05$, $n=8$. Appearance of the M184V mutation in the arm B patients coincided with a rebound of viral load (VL) (4.3 ±0.1 $\log_{10}$ RNA copies/ml) and a significantly improved immunological parameter (ΔCD4=207.0 ±48.1 cells/μl; $P<0.05$). Interestingly, patients developing the M184V mutation preferentially harboured polymorphisms Q174K and/or I178L located in close proximity to *pol* position 184. The M184V mutation occurred following a clear clinical benefit consisting of increased CD4 cell counts and lower plasma viral loads. Primary mutations known to be associated with NNRTI and NRTI resistance for HIV-1B were observed in 10 of the 16 treated patients.

Keywords: Botswana, HIV-1C, *pol* mutations, M184V

Introduction

HIV-1 subtype C is responsible for half of all new cases of HIV infection worldwide (Essex & Mboup, 2002). This subtype is predominant in sub-Saharan Africa, with a prevalence above 30% for adults in Botswana (UNAIDS, 2002). Investigating the mechanisms associated with the development of drug resistance, which may account for differential outcomes in treatment, are critical as African countries prepare to provide public ARV treatment. Although the impact of natural polymorphisms on the outcome of ARV therapy is not fully understood, genotypic divergence in the *pol* gene may lead to different drug responses in non-B subtypes. It is possible that drug responses will be different for non-B viral strains. However, few studies have investigated differences among HIV-1 subtypes in regard to ARV responses (Frater *et al.*, 2001; Vergne *et al.*, 2000; Grossman *et al.*, 2001; Loemba *et al.*, 2002; Petrella *et al.*, 2001). Studies on prevention of mother-to-child transmission reported that a higher proportion of Ugandan women infected with HIV-1 subtype D, or South African and Zimbabwean women infected with HIV-1 subtype C, developed NVP resistance compared with women infected with subtype A (Eshleman *et al.*, 2001). Drug resistance may develop at a varying rate depending on the subtypes or genotypes prevailing in a particular geographical region (Kantor & Katzenstein, 2004). Specific polymorphisms exist in baseline (prior to drug exposure) viral sequences of HIV-1 subtype C (Loemba *et al.*, 2002). Moreover, the HIV-1C subtype contains a valine codon 106 polymorphism that facilitates a V106M transition after selection with EFV whereas, in HIV-1 subtype B, the V106A

mutation is a NVP-specific mutation (Brenner *et al.*, 2003). Since January 2002, HIV-infected Batswana have begun to have access to HAART within the public health system. In this study, we retrospectively characterized *pol* polymorphisms and mutation patterns in six drug-naive and 16 ARV-treated Batswana patients.

Materials and methods

Study subjects and specimens

The Infectious Disease Care Clinic (IDCC) at Princess Marina Hospital provides care to HIV-1-infected patients in Gaborone. Blood specimens from 22 patients (six ARV-naive and 16 treated patients), who consented according to the guidelines of the Institutional Review Boards of the Ministry of Health of Botswana and were followed at IDCC between May 2001 and March 2003, were retrospectively analysed. The first-line regimen contained two combinations, ddI/d4T/NVP or EFV (arm A, eight patients) and CBV/NVP or EFV (arm B, eight patients). Treated patients were followed until regimen switch. Switching was mainly due to virological failure and/or the development of opportunistic infections and toxicities. Among treated patients, four experienced virological failure (three in arm A and one in arm B).

Reverse transcriptase (RT) and protease (PR) genotyping

Plasma samples were collected at baseline and at different time-points until the end-point of follow-up or a switch in therapy. Treatment-naive patients were sampled at baseline and at the end of follow-up. Viral genotyping was performed using the ViroSeq HIV-1 Genotyping System (Celera Diagnostics, Alameda, Calif., USA), according to the manufacturer's instructions. Briefly, RNA was extracted from viral particles pelleted from plasma by isopropanol/ethanol precipitation, converted to cDNA and amplified in PCR. The amplicon (~1500 bp) represented the HIV-1 *pol* region, spanning all the protease and the first 335 codons of RT. Both strands of the PCR product were sequenced using six different primers and Big Dye chemistry on an ABI 3100 Genetic Analyser. ViroSeq HIV Analysis software was used for editing and assembling. Generated sequences were compared with previously reported HIV-1C *pol* sequences (Novitsky *et al.*, 2002) and with subtype B strains obtained from the Stanford HIV database (http://hivdb. stanford. edu/).

Phylogenetic analysis

To confirm that patients were infected with HIV-1 subtype C, the 22 nucleotide sequences were aligned using Clustal X-version 1.81 (Thompson *et al.*, 1997). Reference subtype strains were obtained from the Los Alamos HIV sequence database at: http://www.hiv.lanl.gov/content/hiv-db/mainpage.html The neighbour-joining method was employed for phylogenetic tree-building using the program NJplot. To obtain a statistical estimate of the reliability of particular groupings, a bootstrapping was used with 100 replicates and resampling of all characters in the alignment.

Statistical analysis

To detect changes in virological and immunological parameters over time, analyses were performed with the SPSS statistical analysis software program (version 2.03) using Kruskal-Wallis one-way analysis of variance test. The unpaired *t*-test was used to compare differences between the groups. Data are shown as means plus or minus standard error of mean (SEM). A P value less than 0.05 was considered statistically significant.

Results

Clinical characteristics

All patients were followed at IDCC, between May 2001 and March 2003. Treated patients were separated into two groups according to their first line regimen: ddI/d4T/NVP or EFV (arm A, n=8) and CBV/NVP or EFV (arm B, n=8). Five of eight patients in arm A had to switch from their first line regimen an average of 9 months after HAART initiation (8.6 ±1.2 months) while patients in arm B maintained the same regimen for an average of 13 months (13.6 ±2.4 months). Over the entire follow-up period of ARV-naive patients (17.6 ±3.8 months), their CD4 count dropped from 339.3 ±100.5 to 242.0 ±42.5, while $\log_{10}$ of plasma viral load increased from 5.21 ±0.16 to 5.42 ±0.24. The baseline CD4 counts (cells/μl) for patients on arm A and B were 66.4 ±27.4 and 114.6 ±29.5, respectively. The baseline plasma viral load ($\log_{10}$ copies/ml) was 5.33 ±0.30 and 5.73 ±0.11 for patients on arm A and B, respectively. A significant benefit of therapy was observed, in terms of CD4 increase, within the first months of therapy in both arms A and B regimens. Patients in arm A displayed higher viral loads (4.46 ±0.46 $\log_{10}$) at the end-point of follow-up or just before switching regimens than patients in arm B (4.08 ±0.38 $\log_{10}$). Table 1 summarizes the immunological and virological characteristics of the 22 patients analysed in this study.

HIV-1 RT and PR mutations and polymorphisms

Phylogenetic analysis revealed that all 22 sequences in this study clustered within the HIV-1C reference sequences in both the PR (Figure 1A) and RT (Figure 1B) genes, with bootstrap values over 85%. No primary mutations associated with resistance to RT inhibitors were observed in treatment-naive patients (Table 2). Secondary mutations related to PR inhibitors were observed in treatment-naive

Table 1. Patient clinical parameters

		Naive (*n*=6)	Treated (*n*=16)	
HAART regimen		N/A	Arm A: ddI/d4T/NVP or EFV	Arm B: CBV/NVP or EFV
Mean time follow-up or switch from first-line regimen (months)		17.6 ±3.77*	8.62 ±1.16	13.62 ±2.43†
CD4 count (cells/μl)	Baseline (or before treatment)	339.3 ±100.5	66.4 ±27.4 (0–169)	114.6 ±29.5 (6–232)
	After 1–3 months treatment		221.0 ±100.3 (0–434)	177.8 ±22.3 (103–225)
	After 4–6 months treatment		255.8 ±94.6 (25–466)	227 ±18 (155–287)
	End-point (or before regimen switch)‡	242.0 ±42.5	271.3 ±64.1 (25–467)	361.9 ±69.7§ (176–730)
HIV-1 RNA• (log_{10} copies/ml)	Baseline (or before treatment)	5.21 ±0.16	5.33 ±0.3 (3.57–5.87)	5.73 ±0.11 (5.11–5.87)
	After 1–3 months treatment		4.33 ±0.44 (3.23–5.63)	3.72 ±0.55† (2.60–5.87)
	After 4–6 months treatment		4.37 ±0.53 (2.60–5.69)	3.05 ±0.6† (2.60–5.72)
	End-point (or before regimen switch)‡	5.42 ±0.24	4.46 ±0.46 (2.60–5.69)	4.08 ±0.38† (2.60–4.98)

*Values are expressed as mean ±SEM. Statistical significance was determined using one-way ANOVA test. †P<0.05. ‡t -test was used to compare arm A versus arm B switch from first line regimen. §P<0.01. •Roche Amplicor HIV-1 monitor test version 1.5 was used as viral load test. The lower and upper limits of detection for this test were 2.60 and 5.87 log_{10} copies/ml, respectively.

patients. Primary mutations related to NNRTI resistance (K103N, Y181C, V106M, M230L) were observed in nine of the 16 (56%) treated patients having NVP or EFV in their regimen. These mutations occurred within 5.8 ±1.5 months after initiating treatment (data not shown). The K103N mutation was predominant (7/16; 44%), followed by the Y181C mutation (5/16; 31%). Before initiating therapy, all of the 22 patients harboured GTG polymorphism at codon 106 and three patients receiving NNRTI harboured the V106M mutation (Table 3). Among these patients, the two receiving EFV developed a V106M mutation after 5 and 8 months of treatment, respectively, while one patient on NVP developed this mutation within 2 months of treatment, in addition to two other primary mutations associated with high-level resistance to NNRTIs (K103N and Y181C). Within arm B, the M184V mutation associated with 3TC resistance was the most prevalent (4/8, 50%), while one patient harboured the T215Y mutation associated with resistance to AZT. Other mutations associated with cross-resistance to NRTIs, mutations A62V and K70R, were found in one patient in arm B (patient 21). This patient also harboured the Y188L multi-NNRTI resistance mutation.

Although none of the 22 patients had taken any protease inhibitor (PI)-containing regimens, all of them harboured mutations associated with resistance to PIs. Mutations included K20R (4/22; 18%), M36I (16/22; 73%), V77I (6/22; 27%), L63P (11/22; 50%) and L10V (4/22; 18%).

Three out of four patients in arm B who developed primary mutations were able to maintain successive undetectable viral load (patients 17, 21 and 22) before viral rebound, while most patients on arm A with primary mutations (patients 7, 8, 9, 11, 12 and 14) experienced no major changes in VL.

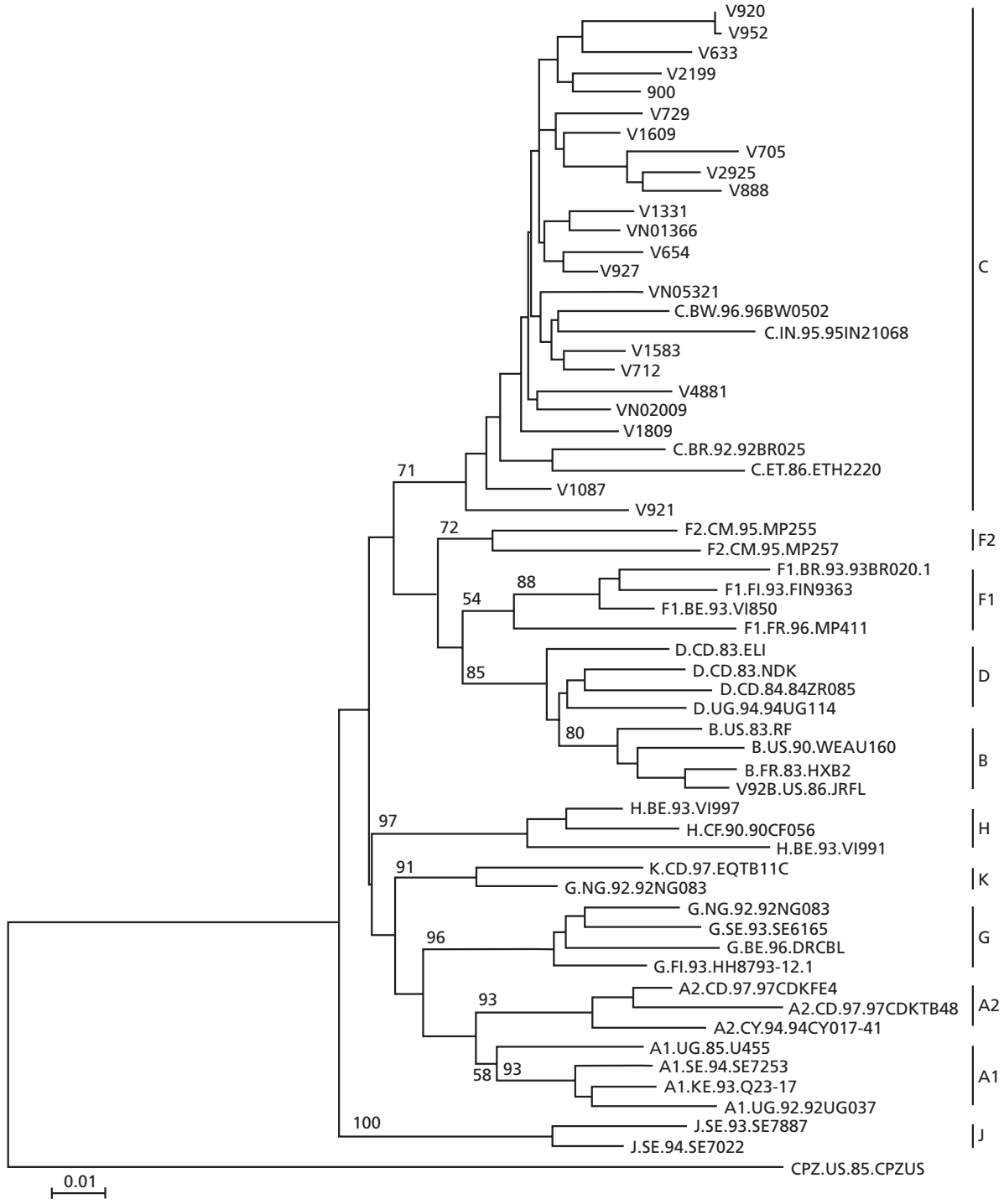
Dynamics of virological and immunological (V/I) parameters

Primary mutations accumulated faster in arm A (6.5 ±1.4 months) than in arm B (11.5 ±3.2 months) (Table 4). Despite an increased CD4 count by the time arm A-associated mutations developed, the virological parameter was not much different (ΔVL: −0.3 ±0.1 log_{10} RNA copies/ml). For four patients in arm B, the first mutations occurring after 6.3 ±2.6 months in HAART were NNRTI-related. They were followed by the emergence of M184V mutation associated with high level resistance to 3TC which arose at 11.5 ±3.2 months (n=4) after initiating therapy. Patients in arm B who were able to maintain successive undetectable VL (patients 17, 21 and 22) improved both virological parameters (mean VL decline of 1.3 ±0.1 log_{10}; P<0.05, n=3) and CD4 count (207 ±48 cells/μl; P<0.05, n=3) by the time M184V mutation developed. This beneficial outcome was time dependent (Figure 2).

RT polymorphism mutations

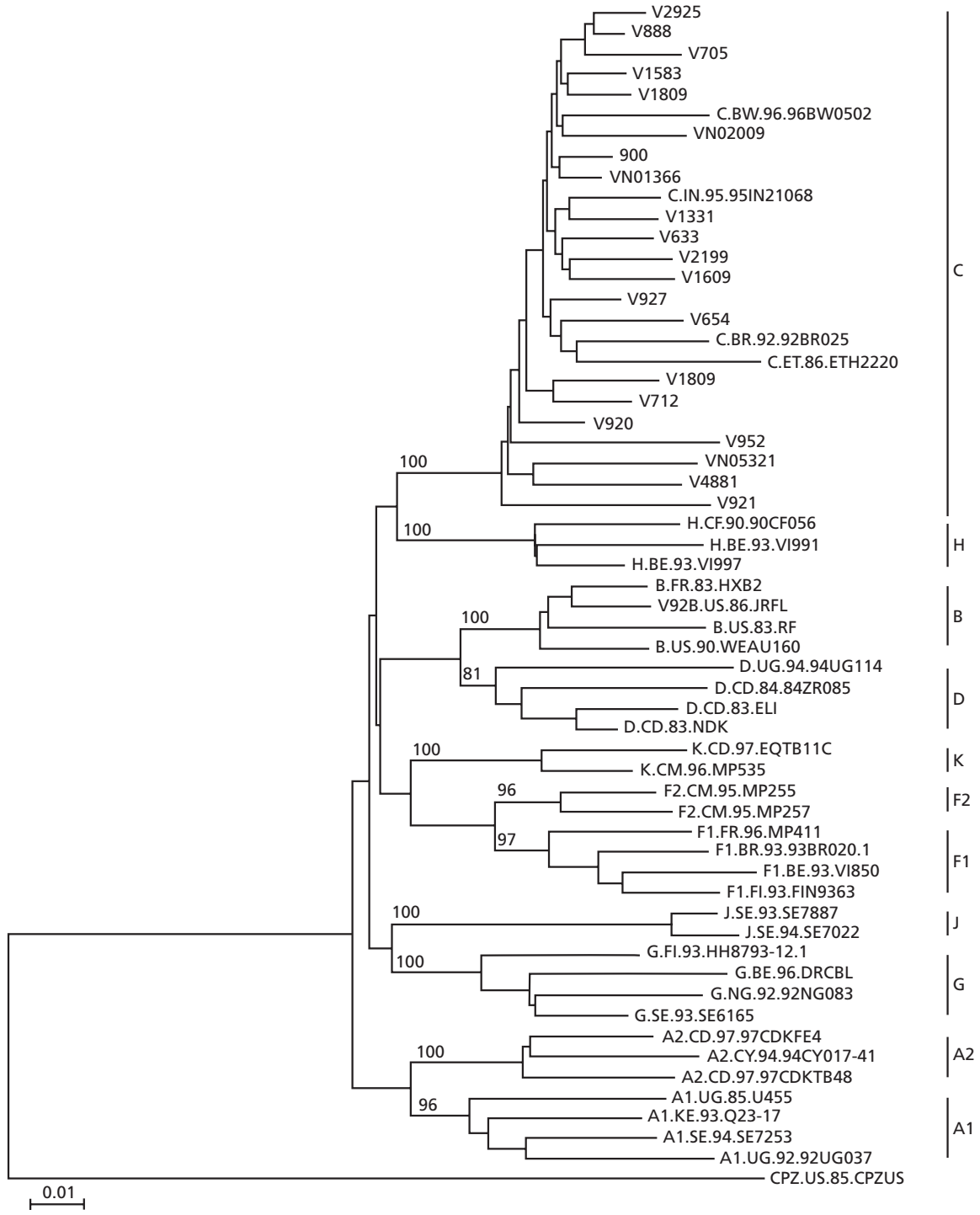
An analysis of RT polymorphisms for naive and treated patients who did or did not harbour primary mutations was performed (Figure 3). We compared these sequences with the consensus sequence (control) obtained from 51 naive Batswana infected with HIV-1 C (11). Few differences were observed between treatment-naive patients (baseline; n=22) and control (n=51) RT sequences. Depending on the regimen arm, we observed the preferential presence of

Figure 1A. Phylogenetic analysis of protease gene sequences of 22 HIV-1 isolates from Botswana



Comparison with reference sequences of HIV-1 *pol* genes from subtype A, B, C, D, F, G, H, J, K and O (outlier). Alignment was subjected to 100 bootstrap resamplings. Horizontal distance corresponds to genetic relatedness.

Figure 1B. Phylogenetic analysis of reverse transcriptase gene sequences of 22 HIV-1 isolates from Botswana



Comparison with reference sequences of HIV-1 *pol* genes from subtype A, B, C, D, F, G, H, J, K and O (outlier). Alignment was subjected to 100 bootstrap resamplings. Horizontal distance corresponds to genetic relatedness.

Table 2. HIV-1 *pol* mutation accumulation during the time of patient follow-up

Patient regimen	RT mutations	PR mutations
Naive		
1		K20R, M36I
2		V77I
3		K20R, M36I
4		M36I, L63P
5		L63P, V77I
6		M36I, L63P
Treated		
Arm A		
7 ddI/d4T/NVP	K103N, Y181C	L10V, M36I
8 ddI/d4T/NVP	K65R	L10V, L63P, V77I
9 ddI/d4T/NVP	K65R, K103N, Y181C, T69D, V106M	L10V, M36I, L63P
10 ddI/d4T/NVP	_	M36I
11 ddI/d4T/NVP	Y181C	K20R, M36I
12 ddI/d4T/NVP	K65R, K103N, Y181C	M36I
13 ddI/d4T/EFV	_	M36I
14 ddI/d4T/EFV	K103N, V106M	M36I
Arm B		
15 CBV/EFV	_	M36I, L63P
16 CBV/EFV	_	M36I
17 CBV/NVP	M184V, K103N	M66I, L63P, V77I
18 CBV/EFV	M184V, T215Y, M230L, V106M	M36I, L63P
19 CBV/EFV	_	M36I, L63P
20 CBV/EFV	_	L10V, K20R, M36I
21 CBV/NVP	M184V, A62V, G190A, K103N, Y181C, K70R, Y188L	L63P, V77I
22 CBV/EFV	M184V, K103N	L63P, V77I

RT, reverse transcriptase; PR, protease; CBV, combivir; ddI, didanosine; d4T, stavudine; NVP, nevirapine; EFV, efavirenz; CBV=3TC (lamivudine) plus AZT (zidovudine).

Table 3. Patients harbouring V106M mutation

Patient number	Genotype change	Drug regimens	Other mutations	Time of mutation emergence (months)
9	GTG → ATG	ddI/d4T/NVP	K103N, Y181C	2
14	GTG → ATG	ddI/d4T/EFV	K103N	8
18	GTG → ATG	CBV/EFV	M230L	5

polymorphisms such as K20R, K22N, E40D, V60I, I135T, I142V, K166R and K281R. In patients in arm B regimen, the frequency of I135T, I142V, D250E, Q174K and I178L polymorphisms were higher among patients harbouring the M184V primary mutation (50%, 75%, 75%, 50%, 75%, respectively) compared with patients without mutation (25%, 0, 25%, 25%, 0, respectively) (Figure 3D). While the frequency of Q174K in drug-naive individuals infected with HIV-1 C (control) was 24%, only 1% of drug-naive individuals exhibited this polymorphism in subtype B (http://hivdb.stanford.edu). Interestingly, three out of four patients harbouring the M184V mutation carried the Q174K polymorphism (patients 18, 21 and 22). While the I142V polymorphism occurred at the same frequency in naive patients infected with HIV-1 subtype B or C (4% and 5%, respectively), only patients under arm B regimen who exhibited the M184V primary mutation harboured this polymorphism at the increased frequency (3/4: patients 17, 21 and 22). Finally, the I135x polymorphisms (I135T and I135M) present at the frequency of 16% in drug-naive individuals infected with HIV-1 C, developed at the same time as the M184V primary mutation in three patients following arm B regimen (patients 17, 21 and 22).

Many other polymorphisms for mutations in RT nucleotide sequence, including E291D, V292I, I293V, R277K, T200A, T39E and Q334H, were detected in both naive and treated patients while two accessory mutations associated with NNRTI resistance, R211K and L214F, were found in most of the patients (95%). In addition, many polymorphisms were found in PR sequence from naive and treated patients (data not shown).

Co-localization of polymorphisms and primary mutation on RT T cell epitopes

We analysed the position of the above characterized primary mutations and polymorphisms on known RT CTL or T helper epitopes characterized using HIV-1 subtype B viruses (Table 5). We showed that mutations conferring drug resistance such as M184V or Y181C mutations, arose in highly polymorphic RT T Helper (171-191) and CTL (175–184) epitope regions. Patients developing these mutations also harboured Q174K (patients 18, 21 and 22) and/or I178L (patients 21 and 22) polymorphism mutations.

Discussion

We have described natural polymorphisms and drug-selected mutations in the RT and PR genes of 22 HIV-1 subtype C isolates from Botswana. In general, virological failure was associated with the emergence of major drug resistance mutations. In terms of regimen efficacy, our results indicate that initiating ARV therapy with the combination CBV/NNRTI delayed time to switch by 14 months, compared to 9 months for ddI/d4T/NNRTI-based regimens. In arm A, six of eight patients did not decrease their VL, suggesting that they were not adhering to their regimens.

This lack of adherence may be associated with pill burden and more complex treatment in the ddI/d4T-containing regimens compared to CBV-containing regimens. We observed that primary mutation Y181C, associated with NNRTI resistance, arises more frequently in patients having NVP in their regimen (5/8) than in patients taking EFV (0/8). Patients presenting mutations at both amino acid positions 103 and 181 are also more frequent in the NVP group than the EFV group (4/8 and 0/8, respectively). Interestingly, among the eight patients on EFV, five out of eight developed no mutations during the study. These results corroborated with previous data showing that certain mutations in the NNRTI binding pocket differentially affected susceptibility to these drugs. This is the case of mutation Y181C, commonly seen after exposure to NVP, which has little effect on EFV susceptibility and confers large reductions in susceptibility to NVP (Harris & Montaner, 2000).

By comparing time to treatment failure and change in VL, Keiser *et al.* (2002) showed that patients with NVP-containing regimens had a shorter time to treatment failure and less decrease in plasma HIV-1 RNA compared with EFV-based regimens. This study contradicted the recent 2NN clinical trial data showing that NVP and EFV, which exhibit distinct side effects and toxicities, presented an equivalent virological efficacy (van Leth *et al.*, Results of the 2NN study: A randomized comparative trial of first-line antiretroviral therapy with regimens containing either nevirapine alone, efavirenz alone, or both drugs combined, together with stavudine and lamivudine, Abstr. 176.000, *10th Conference on Retroviruses & Opportunistic Infections*. Boston, 2003). Of the patients having the CBV-based regimen, four of eight harboured mutation M184V. This primary mutation associated with high-level 3TC resistance in monotherapy (Schuurman *et al.*, 1995) has also been associated with diminished HIV fitness, polymerase processivity, pyrophosphorylysis, and nucleotide primer unblocking (Petrella & Wainberg, 2002), whereas NNRTI-associated mutations had been shown to have little impact on HIV fitness, behaving as wild-type virus (Dykes *et al.*, 2001). Interestingly, our data displayed a strong correlation with CD4/VL levels and resistance to 3TC, which correlated with the presence of M184V mutation in patients having the arm B regimen. The median CD4 increase of 207 cells/μl, correlated with a median VL decline from baseline of 1.3 $\log_{10}$ HIV-1 C RNA copies/ml (1.04–1.42), was strictly observed in patients maintaining undetectable viral load for at least 4 consecutive months after initiating HAART. Virological and clinical benefits of AZT/3TC

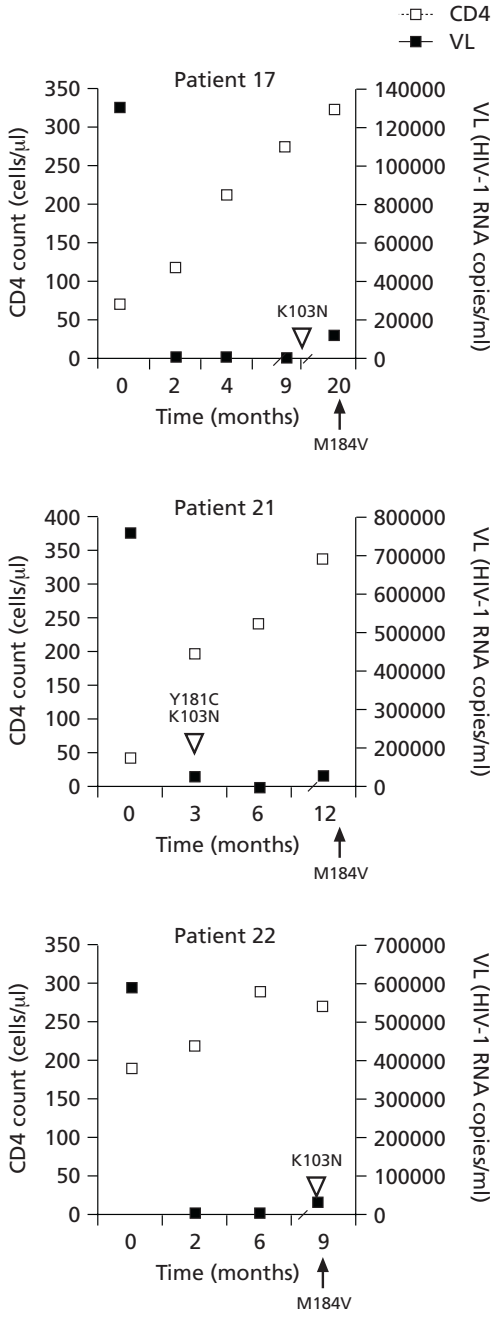
Table 4. Virological and immunological parameters in treated patients who (**A**) developed or (**B**) did not develop mutations

(A)	P	CD4 count† (cell/µl)	ΔCD4*	HIV-1 RNA† (log_{10} copies/ml)	ΔVL*	Mutation arising	Time (months)
Arm A							
	7	58		5.6			
		129	71	5.49	–0.12	K103N, Y181C	4
	8	0		5.87			
		25	25	5.02	–0.85	K65R	4
	9	7		5.87			
		434	427	5.17	–0.69	K103N, Y181C K65R, V106M	2
	11	169		3.57			
		245	76	3.48	–0.08	Y181C	1
	12	4		5.47			
		ND	ND	4.93	–0.53	K103N, Y181C K65R	12
	14	316		4.64			
		467	151	4.83	0.19	K103N, V106M V75T	8
			150 ±65‡		–0.3 ±0.1		
Arm B							
	17	69		5.11			
		316	247	4.07	1.04	M184V, K103N	20
	18	6		5.12			
		88	82	4.83	0.28	M184V, V106M	5
	21	45		5.87			
		339	294	4.55	1.32	M184V, A62V, K70R K103N, G190A, Y188C	12
	22	190		5.76			
		270	80	4.34	1.42	M184V, K103N	9
			207 ±48§		1.3 ±0.1§		

(B)	P	CD4 count (cell/µl)	ΔCD4	HIV-1 RNA (log_{10} copies/ml)	ΔVL	Mutation arising
	10	415		5.76		
		466	51	2.6	–3.15	None
	13	103		5.87		
		182	79	2.66	–3.21	None
			65 ±10		–3.2 ±0.0	
	15	155		5.72		
		456	301	2.6	–3.12	None
	16	232		5.87		
		730	498	2.6	–3.27	None
	19	199		5.69		
		103	96	5.87	0.17	None
	20	21		5.87		
		176	155	4.98	–0.89	None
			318 ±81		– 2.7 ±0.4§	

P, patient.
*, ΔCD4, difference in CD4 count; ΔCVL, difference in viral load.
†, Top value, baseline; bottom value, end–point of follow–up or time mutations arising for patients either (**A**) harbouring or not harbouring (**B**) primary mutations.
‡, Values are expressed as mean ±SEM. Statistical significance was determined by paired Student *t* test comparing each value to the baseline.
§, $P<0.05$.

Figure 2. CD4 and VL kinetics among arm B patients who exhibited the M184V mutation



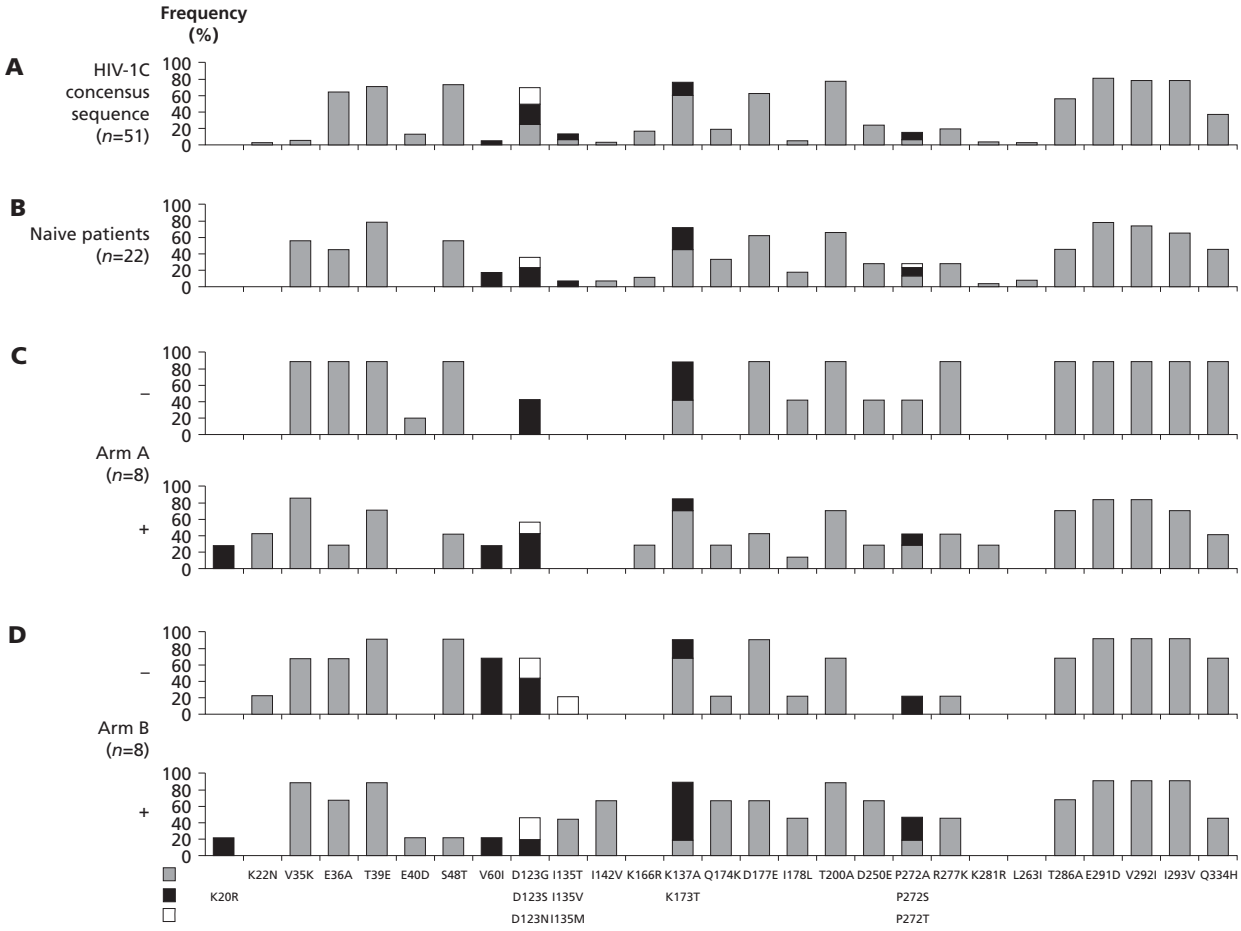
Longitudinal HIV-RNA load (■) and CD4 count (□) in arm B patients harbouring the M184V mutation and followed from baseline (0, pre-therapy) until time the M184V arose. ∇ is time of NNRTI mutation emergence.

combination therapy had been demonstrated by many clinical trials (Kuritzkes *et al.*, 1996; CAESAR Coordinating Committee, 1997; Maguire *et al.*, 2000; Ait-Khaled *et al.*, 1999; Pialoux *et al.*, 1998). The benefits observed in patients in the arm A regimen were less than those exhibited by patients in arm B, even among patients in arm A who did not fail their therapy.

The three-dimensional structure of HIV enzymes targeted by ARV drugs (reverse transcriptase and protease) has brought important insight to the understanding of the drugs' mechanism of action and the impact of specific mutations on their efficacy. The structure of the catalytic complex of RT described by Huang *et al.* (1998) clearly demonstrates that, inherent to this three-dimensional structure, any mutations occurring near the point mutations directly involved for specific drug resistance may reinforce the effects of these primary mutations. This is the case of mutation 44D/A and 118I which, in the context of AZT mutations (41L and 215Y), are both capable of generating resistance to 3TC (Hertogs *et al.*, 2000) as usually exerted by the M184V mutation.

In this study, we identified several polymorphism mutations unrelated to drug resistance (K20R, K22N, E40D, V60I, I135T, I142V, K166R, K281R). Recent studies have demonstrated that not only the virus but also the host immune system may influence HIV therapy outcome. It is interesting to note that primary mutations conferring a high level of drug resistance, such as M184V, arose in polymorphic RT T helper and CTL epitope regions. Furthermore, patients carrying these primary mutations preferentially harboured polymorphism Q174K and/or I178L. Such mutations and/or polymorphisms also affected host immune response (Samri *et al.*, 2000). Moore *et al.* (2002) identified HLA-specific polymorphisms within RT CTL epitopes. Indeed, in a cohort of Western Australian patients, they showed that the I135T polymorphisms located within the epitope RT known to bind HLA-B5101 may be associated with viral escape from immune recognition, in the presence of HAART, in acute HIV infection. Three of four patients with M184V mutation also displayed the I135x polymorphism by the time VL rebounded and M184V mutation arose. This polymorphism is located in a known CTL epitope. This may augur a complex interplay between development of resistance mutations and immunological control of the virus. This would not be surprising, as it has already been demonstrated that HLA molecules can predict the outcome of therapy in HIV-1 subtype B (Malhotra *et al.*, 2001). Interestingly, when HIV-1 C and HIV-1 B reference sequences were compared, it appeared that this RT CTL epitope (128–135) is highly conserved, suggesting that both subtypes may use the same mutational selection for viral escape. By contrast, K103N and V106M mutations occurred in a region of poor

Figure 3. RT polymorphisms in HIV-1C infected patients



Map of polymorphism frequencies at amino acid positions 20 to 334 of RT HIV-1C reference sequences used to establish the consensus (11) (A): sequences from the 22 patients at baseline (B): pre-therapy; sequences from patients presenting (+) or not presenting (–) primary mutations associated with drug resistance in arm A (C) or arm B (D) regimens.

immunogenicity (in reference to HIV-1 subtype B), which may explain the less beneficial effect of NNRTIs in terms of CD4 count recovery or viraemia control.

Finally, 73% of the patients exhibited more than two minor mutations associated with PI resistance. The presence of such mutations prior to initiation of PI-based regimens might lead to a faster emergence of viruses resistant to PIs. Although such accessory mutations had not demonstrated any decrease in drug sensitivity *in vitro*, we cannot rule out that such mutations may increase the risk of virological failure as shown by Perno *et al.* (2001) in an Italian cohort of 248 drug-naive patients or affect HIV-1 C fitness of resistant mutants.

The selection of appropriate first-line drug combinations may be the most important challenge, especially when there are limited options in drug regimens in case of failure. The presence of multiple secondary mutations associated with PI resistance, in addition to the many polymorphisms in the protease gene of HIV-1C isolated from Batswana patients, will have to be taken into consideration in the

Table 5. Polymorphisms and mutations within corresponding RT T cell epitopes among treated patients harbouring drug resistance mutations

Location	Amino acid sequences	Corresponding RT polymorphism mutations	NRTI mutations	NNRTI mutations
HIV-1 RT T-Helper epitopes (subtype B)				
171–191	*FRKQNPDIVIYQYMDDLYVGS*	Q174K (9, 18, 21, 22)*	M184V (17, 18, 21, 22)	Y181C (9, 21)
		I178L (21, 22)	M184V (21, 22)	Y181C (11, 21)
196–216	*GQHRTKIEELRQHLLRWGLT*	L214F (7, 8, 9, 11, 12, 14, 17, 18, 21, 22)	T215Y (18)	
HIV-1 RT CTL epitopes (subtype B)				
103–118	*AGLKKKKSVTVLDVGD*			K103N (7, 9, 12, 14, 17, 21, 22)
				V106M (9, 14, 18)
157–179	*PAIFQSSMTKILEPFRKQNPDIV*	Q174K (9, 18, 21, 22)		Y181C (9, 21)
175–184	*NPDIVIYQYM*	I178L (21, 22)	M184V (21, 22)	Y181C (11, 21)
209–220	*LLRWGLTTPDKK*	L214F (7, 8, 9, 11, 12, 14, 17, 18, 21, 22)	T215Y (18)	

*Patient's numbers are in parentheses.

indication for a second-line regimen. Further studies are needed to find whether such specific polymorphisms in association with drug-resistant mutations result in significant changes in HIV-1 C-specific T cell recognition and subsequent activation.

Acknowledgements

We thank the Botswana Ministry of Health for their encouragement. This study was approved by the national health research development committee of the Botswana Ministry of Health N°:HRU-13/18/1 Vol 11(5). We also thank Mr Moyo for statistical analysis, Mr C Vanderwarker for database management and Randy Wanat for editorial assistance. This work was sponsored by Secure (the Future Program of Bristol-Myers Squibb, Inc.), the Oak Foundation, and the FRSQ (Fonds de la Recherche en Santé du Québec) AIDS and Infectious Disease Network. Simane Gaseitsiwe, Thumbi Ndung'u, Musetsanagepe Modukanele, Ava Avalos and Ibou Thior are formerly or currently supported by National Institutes of Health-Fogarty International Center grant 5D43TW00004.

References

Ait-Khaled M, Rakik A, Griffin P, Gould J, Cutrell A & Fischl M (1999) Viral rebound during 48 weeks of abacavir/lamivudine/zidovudine combination therapy in antiretroviral-naive adults is infrequent with emergent virus showing a limited number of NRTI resistance mutations. *Antiviral Therapy* **4** (Suppl. 1):52–53.

Brenner B, Turner D, Oliveira M, Moisi D, Detorio M, Carobene M, Marlink RG, Schapiro J, Roger M & Wainberg M (2003) A V106M mutation in HIV-1 clade C viruses exposed to efavirenz confers cross resistance to non-nucleoside reverse transcriptase inhibitors. *AIDS* **17:**1–5.

CAESAR Coordinating Committee (1997) Randomised trial of addition of lamivudine or lamivudine plus loviride to zidovudine-containing regimens for patients with HIV-1 infection: the CAESAR trial. *Lancet* **349:**1413–1421.

Dykes C, Fox K, Lloyd A, Chiulli M, Morse E & Demeter LM (2001) Impact of clinical reverse transcriptase sequences on the replication capacity of HIV-1 drug-resistant mutants. *Virology* **285:**193–203.

Eshleman SH, Becker-Pergola G, Deseyve M, Guay LA, Mracna M, Fleming T, Cunningham S, Musoke P, Mmiro F & Jackson JB (2001) Impact of HIV-1 subtype on women receiving single dose NVP prophylaxis to prevent HIV-1 vertical transmission (HIVNET 012). *Journal of Infectious Diseases* **184:**914–917.

Essex M & Mboup S (2002) Regional variations in the African epidemics. In *AIDS in Africa*, 2nd ed. Edited by M Essex, S Mboup, P Kanki, R Marlink and S Tlou. New York: Kluwer Academic/Plenum Publishers, pp. 631–640.

Frater AJ, Beardall A, Ariyoshi K, Churchill D, Galpin S, Clarke JR, Weber JN & McClure MO (2001) Impact of baseline polymorphisms in RT and protease on outcome of highly active antiretroviral therapy in HIV-1-infected African patients. *AIDS* **15:**1493–1502.

Grossman Z, Vardinon N, Chemtob D, Alkan ML, Bentwich Z, Burke M, Gottesman G, Istomin V, Levi I, Maayan S, Shahar E, Schapiro JM & the Israel Multi-Center Study Group (2001) Genotypic variation of HIV-1 reverse transcriptase and protease: comparative analysis of clade C and clade B. *AIDS* **15:**1453–1460.

Harris M & Montaner JSG (2000) Clinical uses of non-nucleoside reverse transcriptase inhibitors. *Reviews in Medical Virology* **10:**217–229.

Hertogs K, Bloor S, De Vroey V, van Den Eynde C, Dehertogh P, van Cauwenberge A, Sturmer M, Alcorn T, Wegner S, van Houtle M, Miller V & Larder BA (2000) A novel human immunodeficiency virus type 1 reverse transcriptase mutational pattern confers phenotypic lamivudine resistance in the absence of mutation 184V. *Antimicrobial Agents & Chemotherapy* **44:**568–573.

Huang H, Chopra R, Verdine GL & Harrison SC (1998) Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* **282:**1623–1625.

Kantor R & Katzenstein D (2004) Drug resistance in non-subtype B HIV-1. *Journal of Clinical Virology* **29:**152–159.

Keiser P, Nassar N, White C, Koen G & Moreno S (2002) Comparison of nevirapine- and efavirenz-containing antiretroviral regimens in antiretroviral-naive patients: a cohort study. *HIV Clinical Trials* **3:**296–303.

Kuritzkes DR, Quinn JB, Benoit SL, Shugarts DL, Griffin A, Bakhtiari M, Poticha D, Eron JJ, Fallon MA & Rubin M (1996) Drug resistance and virologic response in NUCA 3001, a randomized trial of lamivudine (3TC) versus zidovudine (ZDV) versus ZDV plus 3TC in previously untreated patients. *AIDS* **10:**975–981.

Loemba H, Brenner B, Parniak MA, Ma'ayan S, Spira B, Moisi D Oliveira M, Detorio M, Essex M & Wainberg M (2002) Polymorphisms of cytotoxic T-lymphocyte (CTL) and T-helper epitopes within reverse transcriptase (RT) of HIV-1 subtype C from Ethiopia and Botswana following selection of antiretroviral drug resistance. *Antiviral Research* **56:**129–142.

Maguire M, Gartland M, Moore S, Hill A, Tisdale M, Harrigan R & Klein JP (2000) Absence of zidovudine resistance in antiretroviral-naive patients following zidovudine/lamivudine/protease inhibitor combination therapy: virological evaluation of the AVANTI 2 and AVANTI 3 studies. *AIDS* **14:**1195–1201.

Malhotra U, Holte S, Dutta S, Berrey MM, Delpit E, Koelle DM, Sette A, Corey L & McElrath MJ (2001) Role for HLA class II molecules in HIV-1 suppression and cellular immunity following antiretroviral treatment. *Journal of Clinical Investigation* **107:**505–517.

Moore CB, John M, James IR, Christiansen FT, Witt CS & Mallal SA (2002) Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science* **296:**1439–1443.

Novitsky V, Smith UR, Gilbert P, McLane MF, Chigwedere P, Williamson C, Ndung'u T, Klein I, Chang SY, Peter T, Thior I, Foley BT, Gaolekwe S, Rybak N, Gaseitsiwe S, Vannberg F, Marlink R, Lee TH & Essex M (2002) HIV-1 subtype C molecular phylogeny: consensus sequence for an AIDS vaccine design? *Journal of Virology* **76:**5435–5451.

Perno CF, Cozzi-Lepri A, Balotta C, Forbici F, Violin M, Bertoli A, Facchi G, Pezzotti P, Cadeo G, Tositti G, Pasquinucci S, Pauluzzi S, Scalzini A, Salassa B, Vincenti A, Phillips AN, Dianzani F, Appice A, Angarano G, Monno L, Ippolito G, Moroni M, d'Arminio Monforte A & the Italian Cohort Naive Antiretroviral (I.CO.N.A.) Study Group (2001) Secondary mutations in the protease region of human immunodeficiency virus and virologic failure in drug-naive patients treated with protease inhibitor-based therapy. *Journal of Infectious Diseases* **184:**983–991.

Petrella M, Brenner B, Loemba H & Wainberg MA (2001) HIV drug resistance and implications for the introduction of antiretroviral therapy in resource-poor countries. *Drug Resistance Updates* **4:**339–346.

Petrella M & Wainberg MA (2002) Might the M184V substitution in HIV-1T RT confer clinical benefit? *AIDS Review* **4:**224–232.

Pialoux G, Raffi F, Brun-Vezinet F, Meiffredy V, Flandre P, Gastaut JA, Dellamonica P, Yeni P, Delfraissy JF & Aboulker JP (1998) A randomized trial of three maintenance regimens given after three months of induction therapy with zidovudine, lamivudine, and indinavir in previously untreated HIV-1–infected patients. *New England Journal of Medicine* **339:**1269–1276.

Samri A, Haas G, Duntze J, Bouley JM, Calvez V, Katlama C & Autran B (2000) Immunogenicity of mutations induced by nucleoside reverse transcriptase inhibitors for human immunodeficiency virus type 1-specific cytotoxic T cells. *Journal of Virology* **74:**9306–9312.

Schuurman R, Nijhuis M, van Leeuwen R, Schipper P, de Jong D, Collis P, Danner SA, Molder J, Loveday C, Christopherson C, Kwok S, Sninsky J & Boucher C (1995) Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC). *Journal of Infectious Diseases* **171:**1411–1419.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG (1997) The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25:**4876–4882.

UNAIDS (2002) Report on the global HIV/AIDS epidemic. *Joint United Nations Programme on HIV/AIDS and the World Health Organization.* Geneva: UNAIDS.

Vergne L, Peeters M, Mpoudi-Ngole E, Bourgeois A, Liegeois F, Toure-Kane C, Mboup S, Mulanga-Kabeya C, Saman E, Jourdan J, Reynes J & Delaporte E (2000) Genetic diversity of protease and reverse transcriptase sequences in non-subtype-B human immunodeficiency virus type 1 strains: evidence of many minor drug resistance mutations in treatment-naive patients. *Journal of Clinical Microbiology* **38:**3919–3925.

Received 9 February 2004; accepted 25 May 2004