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Drug Resistance Pathways and Impact of Protease Mutation L10I/V in HIV-1 Non-B Subtypes

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Abstract

Background: Molecular pathways to drug resistance have yet to be fully characterized in HIV-1 non-B subtypes. Furthermore, polymorphisms such as protease L10I/V are ubiquitous in non-B subtypes, but their biological implications are still unknown. We evaluated resistance pathways emerging at treatment failure in a cohort of HIV-infected individuals in Mali, and characterized *in vitro* the role of L10I/V.

Methods: Genotypic resistance testing was performed on plasma obtained from 132 HIV-infected individuals from Mali before and after 9 months of treatment using population sequencing. CRF02_AG chimeric viruses containing 10I/V mutants CRF02_AG were constructed using site directed mutagenesis and susceptibility to protease inhibitors (PI) as well as replicative capacity were determined in a PBMC culture assay.

Results: At treatment initiation, 11/132 (8.3%; 95% CI 3.6-13.0%) patients harboured resistance mutations to NRTI (D67N, T69N, L210W, K219E and T215A) or NNRTI (K103N, V108I and V179E). Among these 11 patients, 5 with NNRTI mutations were in virological failure after 9 months of treatment. Six others with one Thymidine Analog Mutations (TAM) did not show complete resistance. Overall, 18/132 (14.0%; 95% CI 8.1-19.9%) patients failed at 9 months and resistance mutations to NRTI or NNRTI could be identified in 8 (6.10%; 95% CI 2.0-10.2%). NRTI mutation M184V was the most commonly observed, followed by NNRTI mutations Y181C and K103N. Polymorphisms in protease such as L10I/V were observed frequently. Their role was evaluated in vitro. CRF02_AG$_{wt_10L}$ showed a slight increase in IC50 for darunavir, lopinavir and nelfinavir compared to subtype B$_{HXB2_10L}$ with 1.2, 1.3 and 1.5 Fold-Changes (FC) respectively. Mutant's viruses CRF02_AG$_{L10I}$ and CRF02_AG$_{L10V}$ showed a slight increase in IC50 for indinavir with 1.30 and 1.20 FC and a slight decrease in IC50 for lopinavir with 0.78 FC and 0.75 FC respectively compared to CRF02_AG$_{wt_10L}$. We did not observe any difference in replicative capacity between CRF02_AG$_{wt_10L}$ and HXB2. However, compared to CRF02_AG$_{wt_10L}$, mutants, viruses CRF02_AG$_{L10I,}$ and CRF02_AG$_{L10V}$ showed a significant reduction in replication capacity by 10% ($p<0.03$) and 12% ($p<0.02$) respectively.

Conclusion: Primary resistance to NRTI and NNRTI impacts response to treatment. The presence of a single TAM mutation may have limited impact on first line treatment in CRF02_AG. A common polymorphism in non-B subtypes, L10V, may affect susceptibility of certain PIs. In the context of large-scale use of antiretroviral, monitoring the emergence of resistance in non-B subtypes is important to preserve treatment options.

Introduction

The availability of Antiretroviral (ARV) therapy has brought great hope for people infected with HIV-1 in resource-limited countries. However, the emergence of drug-resistant virus may compromise the effectiveness of treatments in these settings. In our previous study on primary resistance in Mali [1], we observed a prevalence that exceeds twice the 5% threshold set by the WHO as part of the Resistance Surveillance. Early studies on secondary resistance to ARV in sub-Saharan Africa reported rates varying between 3.7 to 49% after 24-163 weeks of Highly Active Antiretroviral Treatment (HAART) [2]. ARVs have been developed, tested and validated using mostly HIV-1 subtype B while patients worldwide are mainly infected by non-B subtypes. Non-B subtypes harbour several natural polymorphisms in viral genes such as Reverse Transcriptase (RT) and Protease (PR) some of which are known to cause drug resistance in subtype B [3]. We wanted to evaluate whether CRF02_AG isolates, which comprises 71% of the viral isolates in Mali [1] and are highly prevalent in West Africa, showed a particular pattern of resistance mutations under selective pressure from ARV. Several studies have shown the impact of genetic diversity on the resistance pathways to ARVs [4-6]. Unlike subtype B, CRF02_AG seems less likely to harbour mutations M41L and L210W under selective pressure of Nucleoside Reverse Transcriptase Inhibitors (NRTI) [7], or to develop the mutation D30N under pressure of Protease Inhibitors (PI) [8]. RT connection domain mutations G335D, A371V have been implicated in resistance to NRTI in subtype B [9-13]. The mutation A371V, when associated with Thymidine-Analog Mutations (TAM), lead to a significant decrease in susceptibility to Zidovudine (ZDV) and cross-resistance to lamivudine (3TC) and abacavir (ABC), but not to stavudine (d4T) or didanosine (ddI) [9-12]. G335D, in association with TAMs, also decreases susceptibility

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to ZDV [10-13]. However, their role in non-B subtypes has not been evaluated. Furthermore, polymorphisms such as protease L10I/V are ubiquitous in non-B subtypes [14-16], but their biological implications are still unknown for these subtypes. However, several studies have shown that, in subtype B isolates, mutation 10I/V is involved in the loss of virologic response to most PI except for darunavir [14,17-22]. For example, a study of Sevin et al. [23] *in vitro* has shown that the mutation at position 10 can increase saquinavir IC50. Perno et al. [14] showed that the simultaneous presence of the mutation 10I/V and M36I is strongly associated with treatment failure in subtype B infected individuals. M36I is naturally present in CRF02_AG and the frequency of L10I/V is increasing in this subtype, therefore better understanding its impact on drug susceptibility is important [1]. PI-based second-line regimens are likely to be increasingly used in West Africa. Thus, we investigated the impact of 10I/V mutation *in vitro* on resistance to PI in the context of CRF02_AG variants.

Method

Study population

As part of a cohort of subjects enrolled to receive antiretroviral therapy in Mali, we conducted a study to evaluate the profile of resistance mutations occurring in HIV-1 non-B subtype isolates. A total of 132 patients HIV-infected individuals from Mali were prospectively enrolled in this study between July 2007 and March 2008. Before treatment (T0), we evaluated the presence of resistance mutations by sequencing the *pol* gene and measured the Viral Load (VL) of all our patients. After 9 months of treatment (T3), we assessed also the presence of resistance mutations in patients who failed. Treatment failure was defined as a viral load > 400 copies/ml.

Viral load, viral extraction, PCR and sequencing

Plasma HIV-1 RNA was measured using Cobas Amplicor HIV-1 Monitor Test version 1.5 (Roche Diagnostics, Branchburg, New Jersey, USA). The ultrasensitive method was used with a limit of detection of 50 copies/ml. Viral RNA from patients with virologic failure was tested for drug resistance. The RNA extraction and the sequencing were performed as described in our previous article [1]. Briefly, Extraction of viral RNA was done using QIAamp spin Mini Kit of QIAGEN (Qiagen, Mississauga, Ontario, Canada) according to the protocol provided by Virco. RNA was then amplified using super script III HIFI one step RT-PCR system with HF expand (Invitrogen, California, USA) according to the manufacturer's protocol and using primers provided by Virco. Sequencing was performed at Genome Quebec (McGill University and Genome Quebec Innovation Centre, Montreal, Quebec, Canada) using eight primers (provided by Virco) covering the PR-RT genes, a total length of approximately 1600 bp with 1-99 aa for PR and 1-440 aa for RT.

Site directed mutagenesis on plasmid CRF02_AG

CRF02_AG plasmid (p97GH-AG2) was kindly provided by Prof. Mark A. Wainberg (Lady Davis Institute for Medical Research, McGill University AIDS Centre). This plasmid already contained a valine ($CRF02\_AG_{L10V}$) at position 10 (V) instead of Leucine (L). We first mutated the Valine (V) with Leucine (L) to obtain the wild type isolate ($CRF02\_AG_{10L}$) and then we mutated L to Isoleucine (I) to obtain our second mutant ($CRF02\_AG_{L10I}$). We used the Quick Change XL 10 Gold from Stratagene. We followed the manufacturer's protocol for the construction of primers and PCR. The primers used for mutation 10I were: 5′-CTCTTTG GCAACGACCAATAGTCGCAGTAAGAG-3′ and antisense 3′-GAGAAACCGTTGCTGGTT ATCAGCGTCATTCTC-5′. For the 10L, the primers used were: 5′-CTCTTTGGCAACGAC CATTAGTCGCAGTAAGAG-3′ and antisense 3′-GAGAAACCGTT-GCTGGTAATCAGCGTC ATTCTC-5′. After mutagenesis, the protease gene was cut for each mutant (10L and 10I) with BSTB1 (20,000 units/ml, Biolab New England) and EcoRI (100,000 units/ml, Biolab New England). Finally, we used T4 DNA ligase (2,000,000 cohesive end units/ml, Biolab New England) to insert the mutants protease into the plasmid previously cleared of its original protease. Before insertion, all mutations were confirmed by sequencing the gene *pol* with primers covering this region and provided by Virco. Moreover, a subtype B reference HXB2 was introduced as a control.

Viral production

The viruses were produced by transfection of 293T cells with 10 µg of plasmid using FuGENE6 [24]. After 48 hours, supernatants were harvested and the p24 antigen was measured by ELISA (HIV-1 P24 Antigen ELISA test kit-192, Perkin Elmer, USA) following the manufacturer's instruction to determined viral replication. The infectivity of virus produced was determined on CD4+CCR5+CXCR4+ MAGI cells (kindly provided by Dr. Ancuta's lab, CRCHUM, Montreal, Canada). Viruses recovered from the supernatants were titrated in PBMCs using limiting dilution culture method. After 5 days, the titer was measured as the rate of 50% tissue culture infectious doses (TCID50/ml) and calculated using the method of Reed & Muench [25].

Protease inhibitors

All PI used in our experiments were kindly provided by the National Institutes of Health (NIH): amprenavir (APV), atazanavir (ATZ), darunavir (DRV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV) and saquinavir (SQV). The compounds were diluted in Dimethyl Sulfoxide (DMSO) at a concentration of 10 mM and stored at -20°C until use.

Determination of IC50 and replication capacity

IC50 determination and evaluation of replicative capacity were conducted as previously described [26]. Briefly, PBMC activated with Phytohaemoagglutinin (PHA) for 3 days were infected with various viruses (1000 TCID50/ml per 10^6 cells). Viruses were preincubated with the cells for 2 hours. The drugs were added simultaneously and cells were suspended in 24-well plates in a final volume of 1.0 ml of R-20 medium supplemented with 10% interleukin-2. The cells were then placed in an incubator with 5% CO_2 at 37°C. We conducted two experiments and each experiment was performed in duplicate. For each experiment, the culture medium was changed twice weekly. In addition, a toxicity control was maintained with uninfected cells treated with a maximum concentration for each drug. Viruses without cells or drugs were also maintained in culture throughout the experiments to take into account the viral carryover. Five different drug concentrations were used for each compound. Thus, for APV, concentrations ranges varied from 0.005 to 0.08 µM, ATZ 0.0017 to 0.028 µM, DRV 0.001 to 0.016 µM, IDV 0003 to 0052, LPV 0.007 to 0.12 µM, NFV 0.00075 to 0.012 µM and SQV 0.0025 to 0.04 µM. After 7 days, the culture supernatant was harvested for determination of virus replication as measured by p24 antigen ELISA (HIV-1 P24 Antigen ELISA Test Kit-192 Perkin Elmer USA). Cell proliferation and viability were assessed by Trypan blue exclusion method. The inhibitory concentrations 50% and 90% (IC50, IC90) of each PI were determined by the dose-response analysis

using the program CalcuSyn for Windows software by T C. Chou & M. Hayball (Biosoft, Cambridge, United Kingdom) [27]. Replicative capacity was determined by measuring the production of p24 by ELISA (HIV-1 P24 Antigen ELISA Test Kit-192 Perkin Elmer USA).

Data analysis

Viral sequences were analyzed using Gene Code Software Corporation, Ann Arbor, MI, USA. Determination of subtypes and the analysis of resistance mutations were performed using the Virco algorithm (Virco BVBA, VircoNET2, Belgium). Statistical analysis was performed using the statistical package Statistical Analysis System (SAS) version 9.1. Treatment failure or not to antiretroviral (ARV), resistance and 50% inhibitory concentration (IC50) were considered variables of interest. Treatment failure was defined as a viral load (VL) greater than 400 copies/ml. Resistance was determined by the presence of at least one resistance mutation to any class of drugs: Nucleoside of Reverse Transcriptase Inhibitor (NRTI); Non Nucleoside of Reverse Transcriptase Inhibitor (NNRTI) and protease inhibitors (PI) according to the Virco's algorithm and verified by the list of mutations established by the World Health Organization (WHO) and the Internationl AIDS Society-USA and Stanford HIV drug resistance database. The IC50 was determined by the dose-response analysis using the program CalcuSyn for Windows software by T C. Chou & M. Hayball (Biosoft, Cambridge, United Kingdom). Mutant viruses were compared to the wild type CRF02_AG to assess the impact of these mutations on resistance to PIs tested. A subtype B reference HXB2 was introduced in order to compare the sensitivity of PI between subtype B and non-B. Fold change (FC) was determined for each PI in mutant viruses compared to wild-type. The student t-test was used to compare means of IC50. P value <0.05 was considered statistically significant.

Results

Study population and treatment regimen

132 patients were enrolled in this study between July 2007 and March 2008. The characteristics of patients at baseline are shown in table 1. After 9 months of treatment, 78 patients (59.0%; 95% CI 50.6-67.4%) had viral loads < 25 copies/ml, 36 (27.0%; 95% CI 19.4-34.6%) had 25-400 copies/ml and 18 (14.0% ; 95% CI 8.1-19.9%) had a VL> 400 copies/ml. 87.0% (95% CI 81.3-92.7%) of patients were on a stavudine (d4T) + lamivudine (3TC) + nevirapine (NVP) regimen (Table 1).

Evolution of drug resistance mutation and treatment failure

Before treatment initiation, 11/132 patients (8.3%; 95% CI 3.6-13.0) had primary resistance mutations to either NRTI or NNRTI. Among these 11 patients, 5 patients failed treatment at T3: sample from one patient with initial K103N could not be sequenced at failure, one other with initial T69N had no resistance mutation at failure, and the other 3 with initial V108I, V179E and D67N+K219E respectively failed with the same mutations. Only one patient acquired additional mutations (V108I at baseline + M184V and K101E at failure). The others 6/11 patients were virologically suppressed at T3 despite the presence of mutations at list one TAM mutations: L210W, K219E and T215A. Other 13 patients without mutation at baseline were failed at T3. Among these patients, samples from 4 patients could not be sequenced because of the poor sample quality. 4 had no mutation and others 5 were multi-resistant with NRTI an NNRTI mutation (Table 2).

Overall, 18/132 patients (14.0%; 95% CI 8.1-19.9%) failed at 9 months and resistance mutations to NRTI or NNRTI were identified in 8 (6.10%; 95% CI 2.0-10.2%): the 5 multi-resistant patients and the 3 patients who failed with their baseline mutation. M184V was the most commonly observed NRTI resistance mutations in 6/8 (75%) patients. For NNRTI, Y181C was observed in 3/8 (37%) patients followed by K103N observed in 2/8 (25%). No TAM was selected by the treatment (Table 2).

Characteristics	Patients (n=132)
Demographics	
Women	67%
Men	33%
Age	35 (20-65) years
CD4	188.5 (2-585) cell/mm^3
Viral load (VL)	217,000 (45-3,200,000) copies/ml
Antiretroviral Regimen	
D4T +3TC + NVP	87%
AZT+3TC+EFV	4%
AZT+3TC+IDV	4%
D4T+3TC+EFV	3%
D4T+3TC+IDV/rt	1%
AZT+3TC+NVP	1%

Age, CD4 count and viral load (VL) are expressed in median (inter quartile ranges). Frequency of men versus women is expressed in percentage.

Table 1: Characteristics of study patients at baseline (T0).

Currently, several studies done with subtype B isolates have shown the importance of polymorphisms in the connection domain of RT on drug sensitivity. It was recently found that these mutations are associated to NRTI and NNRTI resistance [9-13]. Notably, it has been shown that the combination of TAM or M184V with some mutations in the connection domain such as G333D/E, G335D, A371V and Q509L contributed significantly to dual ZDV-3TC resistance as well as other NRTI [10,13]. We observed some of these connection domain mutations present at T0: G335D was present in 83.3% (95% CI 76.9-89.7%) and A371V in 53.8% (95% CI 45.3-62.3%). Among patients who failed, 6 had mutations G335D or G335D+A371V in the connection domain at baseline. None of these patients had showed an association of these mutations with M184V or TAM at failure. Moreover, 3/6 patients failed with only M184V (Table 2).

No major PI mutations were observed. However, the polymorphisms 10I/V were observed in 22/132 patients (16.7%; 95% CI 10.3-23.1%) at T0.

In vitro susceptibility of CRF02_ AG_{L10I} and CRF02_AG_{L10V}

First, the susceptibility of wild type CRF02_AG_{10L} was compared to subtype B $HXB2_{10L}$ against several PI. No changes were observed for AMP and ATZ. CRF02_AG_{10L} IC50 for DRV, LPV, and NFV were slightly increased compared to subtype B $HXB2_{10L}$ with 1.2, 1.3 and 1.5 fold change respectively, and slightly decreased for IDV 0.71 FC (Table 3). Mutant viruses, CRF02_AG_{L10I} and CRF02_AG_{L10V} were constructed using site-directed mutagenesis, and IC50s to PI were compared to wild type CRF02_$AG_{10L.}$ Very little difference was observed in overall IC50 of the mutant CRF02_AG_{L10I} vs. wild-type CRF02_AG_{wt_10L}, except for a slight increase of IC50 for indinavir 0.0175 µM ± 0.0092 versus 0.0134 µM ± 0.0035 (FC 1.3) and a slight decrease in IC50 for LPV of 0.0230 µM ± 0.0050 versus 0.0292 µM ± 0.0030 (FC 0.78). Similar results were observed for mutant virus CRF02_AGL10V, with an increase in IC50

	Baseline (T0)				After 9 months of treatment (T3)		
ID	**Subtypes**	NRTI	NNRTI	PI	NRTI	NNRTI	PI
Patients with baseline drug resistance mutations who did not fail							
GT2026	CRF02_AG	K219E	0	0	-	-	-
GT2035	CRF13_CPX	L210W	0	0	-	-	-
GT2042	CRF06_CPX	T215A	0	0	-	-	-
GT2044	C	K219E	0	0	-	-	-
GT2045	CRF06_CPX	L210W	0	L10I	-	-	-
PG1005	CRF02_AG	T215A	0	0	-	-	-
Patients with baseline drug resistance mutations who failed							
GT2052	CRF02_AG	0	V108I	0	G335D, M184V	K101E, V108I, G190A	0
CE3008	CRF02_AG	0	K103N	0	ND	ND	ND
CE3012	CRF02_AG	D67N, K219E	0	0	D67N, K219E	0	0
CE3027	CRF06_CPX	G335D	V179E	0	0	V179E	0
CE 129	CRF02_AG	G335D, A371V, T69N	0	0	G335D		L10V
Patients without baseline drug resistance mutations who failed							
GT2014	CRF02_AG	0	0	0	0	0	0
GT2018	CRF02_AG	0	0	0	0	0	0
GT2038	A1	G335D, A371V	0	0	0	0	0
CE3049	CRF09_CPX	0	0	L10I	G335D, A371V	0	L10I
CE3080	CRF02_AG	G335D	0	0	M184V	K103N	0
CE3094	CRF02_AG	0	0	L90I, L10V	G335D, A371V, M184V	Y181C	L90I
CE3128	CRF02_AG	G335DA371V	0	L10V	M184V	K101Q, K103N	L10V
CE3131	CRF02_AG	G335D	0	L10V	M184V	Y181C	L10V
GT2043	CRF02_AG	0	0	0	M184V	Y181C	L10V
GT2001	CRF02_AG	0	0	0	ND	ND	ND
GT2009	CRF02_AG	0	0	0	ND	ND	ND
CE3003	CRF02_AG	0	0	L10I	ND	ND	ND
PG1019	CRF02_AG	0	0	L10V	ND	ND	ND

Genotypes and mutations were determined with Virco's algorithm.

ND: not determined.

Table 2: Patients with drug resistance mutations at baseline (T0) and after 9 months of treatment (T3.).

Drugs **Clones**	**Amprenavir (2.2)***		**Atazanavir (2.1)***		**Darunavir (2)***		**Indinavir (2.3)***		**Lopinavir (1.6)***		**Nelfinavir (2.2)***		**Saquinavir (1.8)***	
	IC50	FC	IC50	FC	IC50	**FC**	IC50	FC	IC50	**FC**	IC50	**FC**	IC50	FC
CRF02_AG$_{WT_10L}$	0.0199 ± 0.0014	0.95	0.0072 ± 0.0009	0.96	0.0039 ± 0.0005	**1.2**	0.0134 ± 0.0035	**0.71**	0.0292 ± 0.0030	**1.3**	0.0147 ± 0.0060	**1.5**	0.011 ± 0.0007	1.1
HXB2	0.02100 ± 0.0008	-	0.0075 ± 0.0005	-	0.0031 ± 0.0001	-	0.0188 ± 0.0015	-	0.0223 ± 0.0032	-	0.0097 ± 0.0022	-	0.0098 ± 0.0004	-

*Fold-change (FC) biological cut-off determined for each PI by Antivirogram-Virco. IC50 values represent the average of two experiments in duplicate. For each drugs, five different concentrations were used. Susceptibility of drugs has been determined by the fold change (FC) and calculated as the IC50 ratio of CRF02_AGWT$_{_10L}$/ subtype B reference virus HXB2.

Table 3: Comparison of IC50 (μM) between CRF02_AGwt$_{_L10}$ vs subtype B$_{HXB2_10L}$.

for IDV of 0.0156 μM ± 0.0074 versus 0.0134 μM ± 0.0035 compared to wild type CRF02AG$_{wt_10L}$ with 1.2 FC and a decrease in IC50 for LPV of 0.0220 μM ± 0.0024 (FC 0.75) (Table 4).

Replicative capacity

There was no difference in replicative capacity between our reference strain HXB2 and wild-type CRF02_AG$_{10L}$. On the other hand, compared to wild type CRF02_AG$_{10L}$, the two mutants CRF02_AG$_{L10L}$ and CRF02_AG$_{L10V}$ showed a significant reduction in replicative capacity by 10% ($p<0.03$) and 12% ($p<0.02$) respectively (Figure 1).

Discussion

In our study, we observed 11 patients with resistance mutations before treatment initiation, and 5 of these experienced treatment failure after 9 months of treatment. This highlights the impact of transmitted drug resistance on treatment outcomes. Of the other 6 patients with one TAM each: L210W, T215A and K219E, we did not observe any failure at T3 (CV < 400) (Table 2). Recently, Germanaud et al. [28] have made the same observation with other TAM such as D67N and K70R in patients under treatment for 6 months. This result could indicate that a single TAM is not sufficient to lead to resistance in

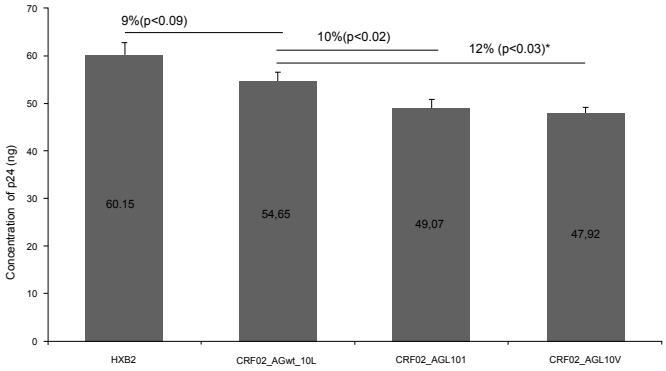
Drugs / Clones	Amprenavir (2.2)*		Atazanavir (2.1)*		Darunavir (2)*		Indinavir (2.3)*		Lopinavir (1.6)*		Nelfinavir (2.2)*		Saquinavir (1.8)*	
	IC50	FC	IC50	FC	IC50	FC	IC50	**FC**	IC50	**FC**	IC50	FC	IC50	FC
CRF02_AG$_{L10I}$	0.0184 ± 0.0017	0.92	0.0068 ± 0.0004	0.94	0.0043 ± 0.0002	1.10	0.0175 ± 0.0092	**1.30**	0.0230 ± 0.0050	**0.78**	0.0123 ± 0.0050	0.83	0.0106 ± 0.0006	0.96
CRF02_AG$_{L10V}$	0.0177 ± 0.0004	0.89	0.0069 ± 0.0004	0.96	0.0036 ± 0.0003	0.92	0.0156 ± 0.0074	**1.20**	0.0220 ± 0.0024	**0.75**	0.0131 ± 0.0070	0.89	0.0102 ± 0.0007	0.93
CRF02_AG$_{WT_10L}$	0.0199 ± 0.0014	-	0.0072 ± 0.0009	-	0.0039 ± 0.0005	-	0.0134 ± 0.0035	-	0.0292 ± 0.0030	-	0.0147 ± 0.0060	-	0.011 ± 0.0007	-

* Fold-change (FC) biological cut-off determined for each PI by Antivirogram-Virco. IC50 values represent the average of two experiments in duplicate. For each drugs, five different concentrations were used. Susceptibility of drugs has been determined by the fold change of IC50 (FC) and calculated as the IC50 ratio CRF02_AG$_{L10I}$/ CRF02_AG$_{WT_10L}$ and CRF02_AG$_{L10V}$ / CRF02_AG$_{WT_10L}$.

Table 4: Comparison of IC50 (µM) of mutants CRF02_AG$_{L10I}$ and CRF02_AG$_{L10V}$ vs wild type CRF02_AG$_{wt_10L}$.

this context. However, 3 of these patients had not fully suppressed their viral load (range 124-324 copies/ml) and one had a VL of 68 copies/ml. Thus, longer follow-up will tell whether this partial suppression is sustained, as these individuals are at risk for long-term treatment failure. This evaluation was done using population sequencing which underestimates minority variants. A recent study has shown that variants present under the level of detection of population sequencing unfavorably impact treatment outcomes [29].

Among our 13 patients without mutations at baseline, 4 patients failed without any drug resistance mutation at T3. This could reflect issues related to treatment adherence. In Mali as in many countries in West Africa, shortage of supplies including ARV medications often becomes an added burden to treatment adherence which is already complicated by social instability, fear of disclosure and tolerability issues. However, the small number of failure indicates that these burdens can be overcome. The others 5 have selected NRTI and NNRTI mutations. But, these mutations were compatible with the treatments used.



Results from 2 experiments in duplicate. PBMCs were infected with 1000 TCID$_{50}$/ml per 10^6 cells. p24 antigen was measured by ELISA in PBMCs supernatant after 7 days of culture. CRF02_AGwt_10L is wild type. CRF02_AGL10I and CRF02_AGL10V are mutants. HXB2 is the subtype B reference. % values represent the difference in p24 between HXB2 and CRF02_AGwt_10L on the one hand, and CRF02_AGwt_10L and the 2 mutants CRF02_AGL10I and CRF02_AGL10V on the other hand. *The student t-test (bilateral) was used to compare the average of p24 concentration. p<0.05 was considered statistically significant.

Figure 1: Replicative capacity in a cell culture assay.

At time T3, we observed 8 patients or 6.10% (95% CI 2.0-10.2%) with at least one resistance mutation to one class of ARVs used in this country. This result is encouraging for Mali compared to 14.2% (95%CI: 11.7-16.9%) observed in Ivory Coast after one year of ARV treatment [30]. However, our result is similar to 3.9% (95% CI: 08-11%) observed in Tanzania [31] and 12.5% (95% CI: 8-18.3%) in Senegal [32] after 1 year of treatment. These results show variability in the prevalence of resistance mutations in Africa [31]. Efforts are still being made to reduce this rate, improving compliance and good resistance surveillance.

Among these 8 patients, M184V (75.0%) selected by 3TC was the most frequent for NRTI. Y181C (37.5%) was the most common NNRTI mutation observed followed by K103N (25%). All these mutations were consistent with the regimens used in this country and with the pattern of drug resistance mutations observed at treatment failure in Africa. For example, a study in Burkina Faso showed that M184V was present in patients failing therapy at a rate of 57.3% [33], 64% in Tanzania [31], up to 75% in Ivory Coast [30] and 86 % in Uganda [34]. This is related to the presence of lamivudine in the first line regimens of most of these countries. In our study, Y181C was more prevalent than K103N (23.08% vs. 15.38%) unlike other studies in Africa where K103N was observed more frequently. For example, a study in Burkina Faso shows 44.4% and 16% [33], Tchad 47% and 35% [35] and up to 95% and 2% in Ivory Coast [30], for the K103N and Y181C respectively. This could be related to the use of NVP in most of our patients as opposed to efavirenz which may have been more broadly used in these studies. In addition, TAMs were not selected in our study. This is consistent with some other studies where M41L and L210W are infrequently observed in CRF02_AG [7]. The absence of TAM selection could be due to rapid selection of the M184V by 3TC increasing the sensitivity of d4T, thereby delaying the emergence of TAMs [36]. Unlike what is observed in subtype C, K65R was not selected by our d4T-containing regimens.

We observed polymorphisms or mutations in the connection domain in of our patients who failed treatment. The presence of polymorphisms or mutations in the connection domain of the reverse transcriptase can affect treatment response in subtype B in combination with TAM or M184V [9-13]. It has been clearly demonstrated that an association of the mutation A371V with certain TAMs (D67N, K70R) result in high-level resistance to ZDV and low-level resistance to 3TC, ABC and tenofovir (TDF) in subtype B isolates [9-12]. In

addition, several studies have shown that a combination of the G335D with M184V or TAM significantly contributes to ZDV and 3TC dual resistance in subtype B [10-13]. However, few studies have examined the impact of mutations in the connection domain mutations (G335D, A371V) on drug resistance in non-B subtype isolates. Moreover, a recent study has shown that mutations G335D and A371V alone do not cause resistance irrespective of subtypes, and act as simple polymorphisms in patients naïve to ARVs. But when these mutations are associated with M184V, they lead to high-level resistance to ZDV [11]. In our study, we observed that none of the 6 failing patients harbouring the G335D mutation at baseline had developed an association with M184V at failure. This observation speaks against an important clinical role of G335D in emergence of drug resistance in CRFAG_02 subtype. Moreover, 3 of these 6 patients failed at T3 with only M184V (Table 2). One hypothesis would be that the presence of the G335D mutation prior to treatment initiation would prevent its association with M184V in non-B subtypes.

No major PI resistance mutation has been observed in our study. This is consistent with the fact that very little PI-containing regimens were used, as treatment regimens were established using current guidelines. The L10I/V mutation has been associated to a loss of virologic response to most PI except darunavir in several studies involving subtype B isolates. First, Sevin et al. has shown that the mutation at position 10 increases 2.86-fold in SQV IC50, *in vitro* [23]. A study of Para et al. [21] showed that the presence of a substitution or a combination of substitutions at codons 10, 20, 48, 82, 84 or 90 was significantly associated with attenuated response to treatment with IDV and similarly at codons 10, 73, and 84 for SQV. Marcelin et al. [20] identified 12 positions in the protease gene including L10 involved in the reduction of the virological response to SQV in a study of 138 patients on protease inhibitors. In another study, the same group showed that L10I/V was among 14 protease mutations involved in the reduction of the virological response to FPV [19]. L10V was also associated with reduced phenotypic susceptibility and virologic response to TPV [17]. Vora et al. [22] identified 8 positions in the protease gene including L10I/V/F in the genotypic resistance to ATZ in a study of 62 patients on protease inhibitor. Furthermore, in 2007, study of Cesar et al of 191 patients treated with nelfinavir or lopinavir showed that the mutation L10I was strongly associated with treatment failure [37].

In non-B subtype, L10I/V polymorphisms are very prevalent. However their role in drug resistance in non-B subtypes has not been well studied. In our study, 16.7% (95% CI 10.3-23.1%) of our patients harboured these polymorphisms at baseline although they had never been exposed to PIs. However PI-based second-line regimens are likely to be increasingly used in West Africa. Therefore, it is important to have reliable data on the impact of these natural mutations on CRF02_AG susceptibility to PIs. In this study, wild-type CRF02_AG$_{wt_10L}$ seems to exhibit slightly higher IC50 to DRV, LPV and NFV (1.2, 1.3 and 1.5 FC) respectively compared to subtype B$_{HXB2_10L}$. However these changes do not seem to be clinically significant as they are within the biological cut-offs defined by most algorithms. Of note, LPV FC was close to its biological cut-off (1.6). Our mutant virus CRF02_AG$_{L10I}$ show a slight increase in IC50 against IDV compared to wild type CRF02_AG$_{wt_10L}$ with a 1.3 FC fold change. Although this is a minor increase, it could contribute to decreased susceptibility if associated with other PI mutations. This was also true for CRF02_AG$_{L10V}$, with 1.2 FC. Of note, our mutants displayed a slight hypersensitivity to lopinavir (10I 0.78 and 10V 0.75). This could indicate an increase in the sensitivity of lopinavir for CRF02_AG subtype. However, the clinical significance of this has yet to be determined.

M36I has been shown to decrease CRF02_AG isolates susceptibility to SQV [38]. This mutation is a natural polymorphism occurring in 99-100% of non-B subtype [1,39] and was present in our strain CRF02_AG. However, we did not observe this decreased susceptibility to SQV which might suggests that other mutations in the protease may compensate this effect of M36I.

Resistance interpretation algorithms are mostly based on subtype B [40]. A 2010 study of Yebra et al. [41] comparing 354 naïve patients with 128 non-B and 226 B subtypes has shown that genotypic drug resistance interpretation algorithms devised for viruses belonging to subtype B, displayed high level of discordance when applied to non-B strains from HIV-1. However, differential pathways of drug resistance have been described in non-B subtypes. Therefore it is important to continue to characterize the impact of natural polymorphisms as they are the main reason for discordant interpretations amongst algorithms [42].

Finally, we assessed the replicative capacity of our different strains. Wild type CRF02_AG$_{wt_10L}$ and subtype B$_{HXB2_10L}$ was similar. On the other hand, both mutants CFR02_AG$_{L10I}$, CRF02_AG$_{L10V}$ showed a significant reduction in replication capacity compared to wild type CRF02_AG$_{wt_10L}$. This reduction is of small magnitude. However it could explain why L10I/V is not as prevalent as M36I which is ubiquitous in non-B subtypes and has been shown to increase replication, mostly in G and A subtypes [43].

Conclusion

As with other cohort studies on antiretroviral treatment in this sub-region of West Africa such as Ivory Coast [30], Senegal [32] or Tchad [35], we observed a good response to treatment. This is encouraging in light of the current efforts to improve access to ARV in everyone who needs it. Moreover, recent studies have demonstrated a significant benefit of large scale use of ARVs on HIV transmission. As programs are implemented to provide this large scale access, it is important to monitor the emergence of resistance, as this could jeopardize these efforts towards HIV eradication. Our study provides some insights in pathways of resistance in CRF02_AG subtypes which seem to be similar to those observed with subtype B. Mutations in the connection domain are frequently observed in non-B subtype and did not seem to play a major role in treatment failure in our study. Whether the presence of the G335D mutation prior to initiation of treatment would prevent the association of this mutation with the M184V mutation among non-B subtypes remains to be evaluated. L10I/V polymorphisms on their own have limited impact on in vitro susceptibility to protease inhibitors in CRF02_AG isolates. Their impact in association with other PI mutations is still unknown. Overall, it will be important to continue to monitor the emergence of ARV resistance and quickly identify new resistance pathways to preserve treatment options.

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