

Short Communication: *In Vitro* Accumulation of Drug Resistance Mutations in Chimeric Infectious Clones Containing Subtype B or C Reverse Transcriptase and Selected with Tenofovir or Didanosine

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Abstract

Highly active antiretroviral therapy (HAART) contributed to the improvement in the life expectancy of HIV-infected patients. However, the emergence of drug-resistant mutations (DRM) is a major viral factor impacting therapeutic failure. Differences in DRM can occur among HIV-1 subtypes. We evaluate the kinetics of the selection of resistance mutations *in vitro* analyzing two chimeric clones that contain the reverse transcriptases of subtypes B or C (RTB' and RTC') in cells treated with increasing concentrations of tenofovir disoproxil fumarate (TDF) and didanosine (ddI). The mutation K65R is selected more quickly in RTC' than in RTB' viruses with TDF and ddI, and additional mutations (positions 45, 62, and 68) were selected after K65R fixation. Other primary mutations (M184V and Q151M) were selected with ddI treatment in conjunction with K65R only in RTC' viruses. Both patterns, M184V + K65R and Q151M + K65R, have a significant impact on NRTI resistance. Our data suggest that selection of TDF and ddI DRMs can occur earlier in subtype C HIV in patients when compared to subtype B.

ABOUT 35.3 MILLION PEOPLE were living with HIV-1 in 2012 worldwide with the majority of them living in sub-Saharan Africa. Highly active antiretroviral therapy (HAART) reduced the number of HIV-1-infected people and HIV-related deaths per year.¹ This regimen is composed of two nucleoside analogue reverse transcriptase (RT) inhibitors (NRTIs) and one nonnucleoside analogue RT inhibitor (NNRTI) or a protease inhibitor (PI) as the first line of treatment. PIs are not used as first-line treatment in low- to middle-income countries and are reserved for second-line regimens. Conversely, NRTIs are used worldwide, although the drugs of choice depend on the resources of the country.

Tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC) are widely used in high-resource countries. Zidovudine (AZT), lamivudine (3TC), and stavudine (d4T) are chosen in low-resource countries and are recommended by the World Health Organization.² Didanosine (ddI) is no longer used in most countries, although it is one of the few options available in some African and Asian countries. Moreover, ddI is still administered in children and is recommended as one of the second-line medications in South Africa.^{3–5} Integrase and entry inhibitors, when available, are

used in salvage regimens whenever second-line therapeutic failure is observed, confirmed by an increase in viral load and/or a decrease in CD4 measurements.² The large-scale implementation of HAART in countries with limited resources improved the quality of life and the life expectancy of HIV-1-infected patients,⁶ but drug resistance mutations (DRM) still represent an important factor involved in therapeutic failure.

HIV-1 is classified into many types, subtypes, and circulating recombinant forms. The epidemic in developed countries is dominated by subtype B, which is thus the target of most studies. Nevertheless, subtype C is responsible for the majority of HIV-1 infections worldwide due to its high prevalence in sub-Saharan Africa. Moreover, this variant is also found in India, China, and Brazil.⁷

Genetic differences among HIV-1 subtypes are around 10% in the *pol* gene. Therefore, it could be predicted that different resistance mutations or different mutation patterns could emerge in different HIV-1 subtypes under the same antiretroviral pressure.⁸ In fact, differences in DRM position, amino acid composition, and time of acquisition were observed during HAART among HIV-1 subtypes. Differences

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in the prevalence of resistance mutations at codon 106 that were selected by NNRTIs were observed between subtype B and C viruses. In this case, V106A accumulated in subtype B viruses treated with nevirapine while V106M emerged in subtype C viruses under efavirenz treatment. These events can be explained by differences in codon usage at that position.⁹ In phenotypic assays, V106M confers resistance to efavirenz (EFV) and nevirapine (NVP) while V106A impacts only NVP.¹⁰

Recently, our group has shown that differences in thymidine analogue mutation (TAM) pathways during AZT *in vitro* selection were observed between chimeric viruses generated with RTs from subtypes B and C, with the TAM-1 pathway (T215Y) being selected in subtype B and the TAM-2 (T215F, K70R) pathway being selected in subtype C viruses. In contrast, no differences at selected DRMs were observed with 3TC treatment between these two subtypes.¹¹ It is acknowledged, however, that TAM-1 has a higher impact in resistance to AZT and a large impact in cross-resistance.¹²

The K65R mutation, which confers resistance to all NRTIs except AZT, was reported to occur with low frequency in patients infected with subtype B. However, this DRM has been found to occur with higher frequency in subtype C viruses.¹³ In addition, K65R is selected more quickly in subtype C viruses when compared to subtype B viruses when subjected to the selective pressure of TDF *in vitro*.¹⁴ Nevertheless, preliminary data on clinical assays with TDF treatment somehow showed discordant results.¹⁵

Here we evaluated the acquisition kinetics of resistance mutations when chimeric infectious clones containing RTs from subtype B or C were subjected to *in vitro* selection with TDF or ddI, which is another NRTI known to select the K65R mutation in resistant viruses.

Different fragments of the wild-type (WT) subtype B and C RT sequence were cloned into molecular clones pHXB2 Δ RT and pHXB2 Δ NRT, respectively. The first plasmid pHXB2 Δ RT has the almost complete genome of HXB2 except for the RT gene, which was deleted in codons 25 to 554 and unique sites of restriction enzymes NgoMIV and MluNI were inserted through an adapter. We amplified NL4-3 RT, a subtype B clone, with primers RT2569 and RT22 in the first round of the polymerase chain reaction (PCR) (1,744 bp) followed by RTball and NgoMIV-INT1rev in the second round of the PCR (1,623 bp).¹⁶ Therefore, the PCR product will contain the same flanking restriction enzyme sites as the molecular clone and both of them were digested and ligated to generate HXB2 RTpNL 4.3 (RT_B).

The second plasmid, pHXB2 Δ NRT, also contains the HXB2 genome and the RT N-terminal region of the connection domain (codons 25 to 315) was deleted and two distinct restriction enzyme sites were inserted for cloning purposes (MluNI and Van91I). For subtype C RT cloning, a sample from a naive patient from the south of Brazil (C23) was amplified with primers RT2569 and 3'RTAA339 in the first round PCR (967 bp) and RTball and RT21 in the second round PCR (941 bp) as previously described.¹⁷ To clone the PCR fragment we used the same strategy explained above to generate CNRT23 (RT_C).

Several differences could be observed between RTB' and RTC' in amino acid sequences (V35T, E36A, T39D, K43R, S48T, Q102K, D121Y, K122E, C162S, K173N, Q174K, D177E, T200A, Q207E, and R211K) since each subtype has

a different polymorphic signature in the RT region. However, none has previously been associated with ddI or TDF resistance. All viral stocks were titrated through tissue culture infectious dose 50% (TCID₅₀) in MT4 cells before being used in selection experiments.

MT4 cells were infected with chimeric virus RTB' or RTC' in six independent infections (technical replicates) at a multiplicity of infection (MOI) of 0.002 to minimize stochastic effects at drug resistance mutation selection. These viruses were subjected to serial passage with increasing concentrations of TDF and ddI for 142 and 96 days, respectively. The start concentration was 10% of the half-maximal effective concentration (EC₅₀) (Fig. 1). If low levels of infection were detected by optical microscopy through cell death and/or characteristic cytopathic effect evaluation, the antiretroviral (ARV) concentration was maintained for the next passage. The ARV concentration was doubled if higher levels of infection were obtained in the previous passage. After each passage, viruses present in supernatants were subjected to genomic RNA extraction and RT gene amplification with specific primers.¹¹ Amplicons were sequenced by the Sanger method and manually analyzed (two different persons) for the presence of drug resistance mutations within the RT gene. When two peaks (WT nucleotide and mutate nucleotide) were observed, we concluded that a mixture existed and determined a partial selection. However, when only a peak of a nucleotide different from the WT was observed, we concluded that this mutation was fixed.

Replicative capacity analyses of viruses selected with TDF were performed in MT4 cells. These cells were infected with 5 ng of p24 for each virus in triplicate assays. RTB' WT and RTC' WT infections in the absence of TDF and RTB' carrying K65R, K65R + A62T, and K65R + G45R, as well RTC' carrying K65R, K65R + S68S/N, and K65R + S68S/G in the presence of 0.2 μ M TDF. Supernatants from infections were collected and analyzed for p24 levels at days 1, 3, and 5 after infection using Retro-Tek HIV-1 p24 antigen ELISA (Zep- tometrix) as described by the manufacturer.

Analysis of TDF and ddI selection revealed differences as drug concentrations were increased over time (Fig. 1A and B). The TDF concentration was frequently doubled every passage. In addition, a similar pattern was observed for six RTB' and RTC' independent infections (Fig. 1A). In contrast, the ddI concentration reached a plateau and concentration enhancement was very difficult due to the cytotoxic effects caused by the drug. Differences between RTB' and RTC' infections could be observed only at the last passages (Fig. 1B). The fact that RTB' and RTC' viruses have a similar pattern of drug concentration increase suggests that the replicative capacity of RTB' and RTC' viruses is similar.

To evaluate this MT4 cells were infected with RTB' and RTC' viruses in triplicate in the absence of drugs and were followed for 5 days. Supernatant was collected and analyzed at days 1, 3, and 5 for p24 levels. We can see that RTB' p24 levels were higher at day 3 while RTC' levels were higher only at day 5 (Fig. 1C) implying that RTB' viruses have a higher replicative capacity than RTC' viruses. These data are not surprising as subtype B background chimeras carrying subtype C RT usually have lower replicative capacity.¹⁸

The EC₅₀ value for ddI is 1,000 times higher than TDF. Thus, the final concentration of ddI at *in vitro* selection can reach only 0.1–1 mM, about 5–50 times the EC₅₀ for ddI.

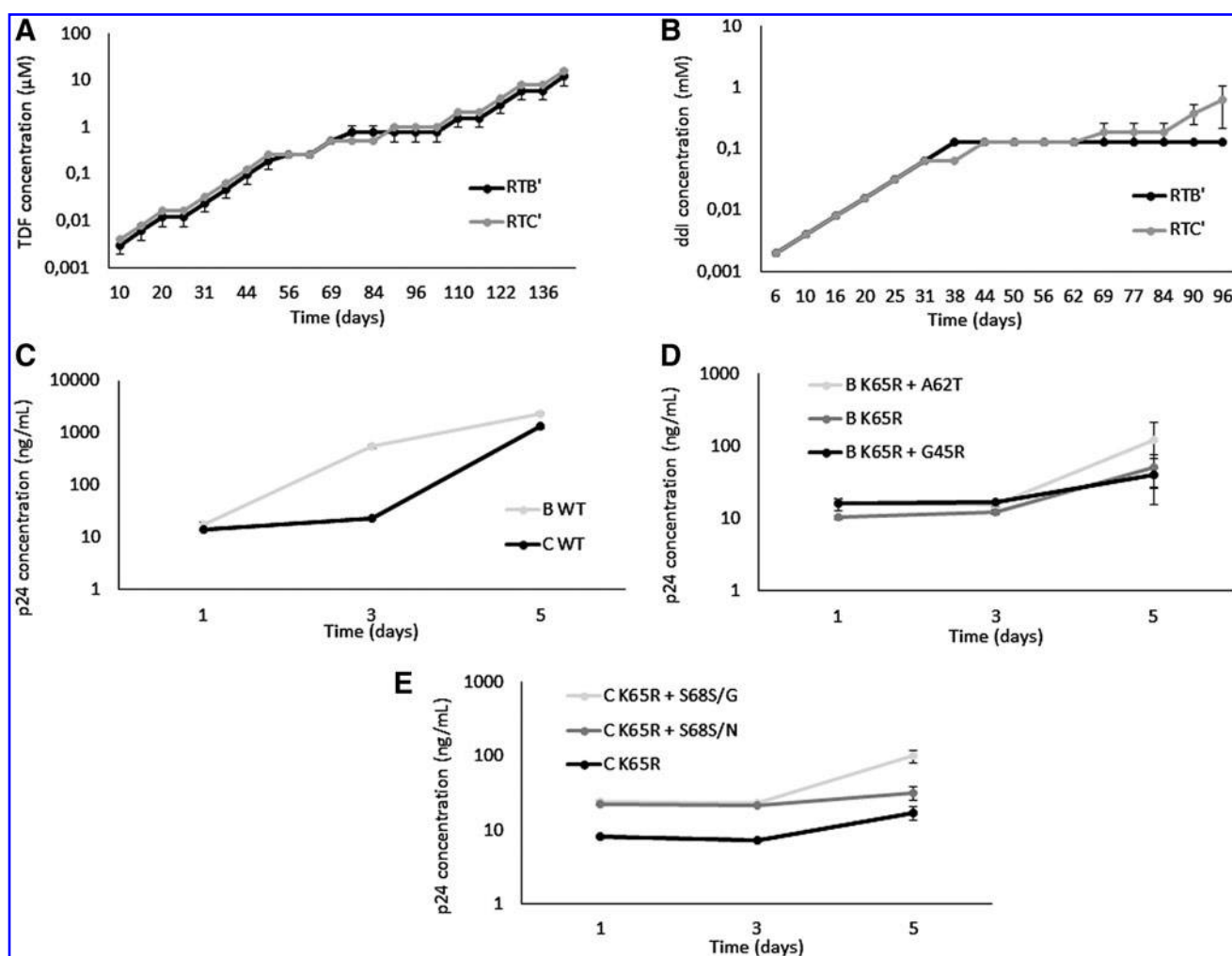


FIG. 1. Enhancement of drug concentrations at *in vitro* selections and replicative capacity analysis. Increase at tenofovir disoproxil fumarate (TDF) (A) and didanosine (ddI) (B) concentrations along *in vitro* selection. When HIV infection reaches higher levels of cell death and cytopathic effect at previous passage, the drug concentration was doubled. However, when low levels of each were observed, the drug concentration was maintained at the next passage. The average concentration of six independent infections for the reverse transcriptases of subtypes B and C (RTB' and RTC') and standard deviation values are shown over time. Viruses RTB' and RTC' WT (C) or viruses carrying resistance mutations (D, E) were used to infect MT-4 cells for 5 days in the absence or 0.2 μM TDF, respectively. Supernatant was collected and p24 levels were analyzed on days 1, 3, and 5.

Conversely, the final concentration of TDF can be increased to 8–16 μM , about 400- to 800-fold of the EC_{50} for TDF.

Our data demonstrate that K65R is the major mutation selected with TDF treatment, corroborating the literature.¹⁹ Interestingly, this mutation was both partially and completely selected faster in RTC' virus when compared to RTB' virus in all six replicates (Table 1). It was partially detected at 31–50 days (median: 47 days) of *in vitro* selection in RTC' viruses versus 62–84 days (median: 77 days) in RTB' viruses and completely emerged at 50–56 days (median: 53 days) in RTC' viruses versus 84–90 days (median: 90 days) in RTB' viruses. These results could not be explained by differences in replicative capacity since RTC' viruses have a replicative capacity lower than RTB' viruses (Fig. 1C).

Previous studies found results comparable to the results presented here. However, viruses used in these experiments were isolated from peripheral blood mononuclear cells (PBMCs) from HIV-infected patients. This methodological

approach has limitations since isolated viruses were not clonal and mutations could already be present in minority populations.¹⁴ The viruses used in our experiments were clonal and corroborate the results from Brenner *et al.*¹⁴ However, other studies have shown that there is no difference in required time to accumulate K65R in subtype B or C when samples from patients under a HAART-containing TDF regime were used.¹⁵

In fact, differences in treatment regimen were observed in those studies. When TDF monotherapy is used, as in our study, K65R is selected faster in subtype C when compared to subtype B recombinant viruses.¹⁴ Conversely, no differences in K65R accumulation time between RT subtypes B and C were observed when TDF was used as part of a triple therapy HAART.¹⁵ The impact of TDF could be lower as other ARVs also would affect the selection of resistance viruses when HAART was utilized. Nonetheless, Sunpath *et al.* demonstrated that K65R was selected at a higher frequency in

TABLE 1. DRUG RESISTANCE MUTATIONS FOUND DURING THE *IN VITRO* SELECTION OF RTB' AND RTC' WITH ESCALATING CONCENTRATION OF TENOFOVIR DISOPROXIL FUMARATE

TDF Time (days)	RTB' (1) Mutation (μM)	RTB' (2) Mutation (μM)	RTB' (3) Mutation (μM)	RTB' (4) Mutation (μM)	RTB' (5) Mutation (μM)	RTB' (6) Mutation (μM)	RTC' (1) Mutation (μM)	RTC' (2) Mutation (μM)	RTC' (3) Mutation (μM)	RTC' (4) Mutation (μM)	RTC' (5) Mutation (μM)	RTC' (6) Mutation (μM)
31	WT (0,016)	WT (0,016)	WT (0,016)	WT (0,032)	WT (0,032)	WT (0,032)	WT (0,032)	WT (0,032)	K65K/R (0,032)	WT (0,032)	WT (0,032)	WT (0,032)
44	WT (0,064)	NS	NS	WT (0,128)	NS	NS	WT (0,128)	K65K/R (0,128)	K65K/R (0,128)	K65K/R (0,128)	WT (0,128)	WT (0,128)
50	WT (0,128)	NS	NS	NS	NS	NS	K65K/R (0,256)	K65R (0,256)	K65R (0,256)	K65K/R (0,256)	K65K/R (0,256)	K65K/R (0,256)
56	WT (0,256)	WT (0,256)	WT (0,256)	WT (0,256)	NS	NS	K65R (0,256)	K65R (0,256)	K65R (0,256)	K65R (0,256)	K65R (0,256)	K65R (0,256)
62	K65K/R (0,256)	WT (0,256)	WT (0,256)	WT (0,256)	NS	NS	K65R (0,256)	NS	NS	K65R (0,256)	K65R (0,256)	K65R (0,256)
69	K65K/R (0,512)	NS	WT (0,512)	WT (0,512)	WT (0,512)	WT (0,512)	K65R (0,512)	K65R (0,512)	K65R (0,512)	K65R (0,512)	K65R (0,512)	K65R (0,512)
77	K65K/R (1,024)	WT (1,024)	K65K/R (1,024)	WT (0,512)	WT (0,512)	K65K/R (0,512)	K65R (0,512)	NS	NS	NS	NS	NS
84	K65R (1,024)	K65K/R (1,024)	K65K/R (1,024)	WT (0,512)	WT (0,512)	K65R (0,512)	K65R (0,512)	K65R (0,512)	K65R (0,512)	K65R (0,512)	K65R (0,512)	K65R (0,512)
90	K65R; A62A/T* (1,024)	K65R; A62A/T* (1,024)	K65R; A62A/T* (1,024)	K65R; G45G/R* (0,512)	K65R; G45G/R* (0,512)	NS	NS	NS	NS	NS	NS	NS
96	NS	K65R; A62A/T* (1,024)	K65R; A62A/T* (1,024)	K65R; G45G/R* (0,512)	K65R; G45G/R* (0,512)	K65R; G45R* (0,512)	K65R; S68K/N/R/S* (1,024)	K65R (1,024)	K65R (1,024)	K65R; S68S/N* (1,024)	K65R (1,024)	K65R; S68G/S* (1,024)
122	K65R; A62T* (4,096)	K65R; A62T* (4,096)	K65R; A62T* (4,096)	K65R; G45G/R* (2,048)	K65R; G45R* (2,048)	K65R; G45R* (2,048)	K65R; S68K/N/R/S* (4,096)	K65R; S68N/S* (4,096)	K65R (4,096)	NS	K65R (4,096)	K65R; S68G/S* (4,096)
142	K65R; A62T* (16,384)	K65R; A62T* (16,384)	K65R; S68S/N*; A62A/T* (16,384)	K65R (8,192)	K65R (8,192)	K65R; G45G/R*; S68S/N* (8,192)	K65R; S68S/R* (16,384)	NS	K65R (16,384)	K65R; S68S/N* (16,384)	K65R; S68G/S* (16,384)	K65R; S68G/R/S* (16,384)

*Mutation not associated with 3TC resistance. Numbers in parentheses are replicate numbers. NS, not sequenced; TDF, tenofovir disoproxil fumarate.

patients infected with subtype C viruses failing TDF-containing first-line HAART (69.7%) in a cohort from South Africa when compared to data from subtype B-infected patients (7–15%) in countries dominated by this variant.²⁰

TDF was used in several preexposure prophylaxis studies (PrEP) in mono or dual therapy, and it is highly efficacious in preventing infection in high-risk HIV-infected people.^{21–23} In some of them, when a patient enters the study already infected with HIV-1, there is a risk that K65R will emerge.^{22,23} All studies have been performed in countries in which HIV-1 subtype C is endemic. Our data suggest that K65R could emerge faster in subtype C viruses than in subtype B viruses in PREP studies when TDF is administered principally as monotherapy. This fact could be a limitation of this approach in countries in which the epidemic is dominated by subtype C viruses.

Interestingly, secondary mutations (A62T, S68G/N/R, and G45R) were partially or completely acquired for both subtypes after K65R fixation with TDF selection (Table 1). However, mutations in positions 45 or 62 were more common in RTB' viruses and substitutions at position 68 were found more frequently in RTC' viruses (Table 1). This could not be explained by differences in the genetic barrier since the same codon is shared by WT subtype B and C viruses at those positions.

Mutations at positions 62 and 68 have usually been detected in combination with K65R in patients treated with TDF, and these mutations accumulate after K65R selection.^{24–26} This suggests that mutations at positions 62 and 68 could work as secondary mutations, enhancing TDF resistance together with K65R. However, phenotypic studies did not find any impact of these accessory mutations in TDF resistance levels.^{24,25} Nevertheless, Ross *et al.*, analyzing viral populations in patients failing TDF-containing HAART, found that viral populations with K65R+S68N were more prevalent than populations in which only K65R was selected, suggesting an advantage for viruses carrying both mutations.²⁷

In contrast to mutations at amino acids 62 and 68, which were selected only at TDF higher concentrations, mutation G45R emerged at the same time as K65R at low drug concentrations. This mutation is located within a region that contains many RT mutations (M41L, K65R, D67N, insertion at 69, K70R) and could act as a secondary mutation. Surprisingly, little information about G45R exists in the literature. This mutation has also been found in association with K65R in other *in vitro* selection experiments with TDF.²⁸ However, this mutation has not been explored in TDF-treated patients in conjunction or not with K65R. The prevalence of G45R in the Stanford Database (<http://hivdb.stanford.edu/>) was 0.1% for naive or NRTI-only treated individuals.²⁹ Further studies to better evaluate this mutation in different subtypes are needed.

To assess whether those secondary mutations in conjunction with K65R would restore the replicative capacity of K65R viruses, MT4 cells were infected in the presence of 0.2 μ M TDF in triplicate. We observed similar levels of p24 between B K65R viruses and B K65R + A62T or G45R (Fig. 1D) indicating that these mutations were not improving the replicative capacity of subtype B viruses containing the K65R mutation. However, a slight improvement in replicative capacity of viruses carrying K65R was noted in conjunction with S68S/N and S68S/G principally (Fig. 1E).

The kinetics of K65R mutation selection was the same with ddI treatment in our experiments. Similar to what was found in TDF, the K65R mutation was selected faster in subtype C viruses than in the subtype B counterpart. The mechanism that explains the faster emergence of K65R in subtype C viruses when compared to subtype B isolates has already been elucidated. Although the genetic barrier for K65R in subtype B and C is similar, differences in codon usage in both subtypes occur. Codons 64 (AAA) and 65 (AAG) in subtype C favor pausing of DNA synthesis by RT yielding a higher mutation rate.^{30,31}

This result helps to elucidate the kinetics of K65R selection in HIV-1 subtypes B and C. We need to reiterate that ddI was also used in monotherapy and we do not know if the same DRM profile as well as the accumulation kinetics would occur if combination treatment was utilized *in vitro* or *in vivo*. As K65R confers cross-resistance to all NRTIs except AZT, this early emergence in subtype C viruses may impede NRTI ARV changes in second-line therapies since only AZT from NRTIs could be used successfully.

In addition, other known primary resistance mutations were coselected with K65R in subtype C viruses (Table 2). Mutations M184I/V and Q151M were selected in 50% of RTC' virus replicates in conjunction with K65R each. However, even if other primary mutations (M184I or L74V) were partially accumulated at the RTB' virus during *in vitro* selection with ddI, only K65R was fixed by the end of the experiment (Table 2).

According to the International Aids Society, M184V is not considered a resistance mutation for ddI because there was a reduction in viral load when patients were treated with ddI compared to placebo even when M184V was present.³² However, those mutations increased ddI resistance levels 4- to 8-fold in *in vitro* experiments.^{33,34} Moreover, this mutation confers cross-resistance to other NRTIs such as lamivudine and emtricitabine.

Interestingly, the NRTI multiresistant mutation, Q151M, was selected in three of six RTC' viruses with ddI in our *in vitro* selection experiments (Table 2). This mutation confers resistance to all NRTIs approved by the FDA except TDF.¹⁹ Q151M and additional secondary mutations, known as compensatory mutations, were initially selected under AZT and/or ddI treatment.³⁵ Moreover, Q151M is detected in low frequency in subtype B-infected patients.³⁶ However, Q151M was selected in a higher frequency with d4T/ddI treatment in subtype C-infected cohorts.³⁷ In addition, Q151M was detected in 11.3% of patients infected with HIV-1 subtype E with d4T treatment without ddI.³⁸ Our data suggest that ddI in addition to d4T plays an important role in the emergence of Q151M and viral subtype may influence the selection of this important DRM.

In our ddI treatment experiments we detected mutation F77I partially selected together with Q151M (Table 2). Although this mutation localizes at the same position as compensatory mutation F77L, studies should be performed to determine whether F77I could act as a compensatory mutation for Q151M and if there is a subtype C predominance.³⁵

One of six replicates of RTB' viruses partially selected L74V initially. However, this mutation could not be fixed and was not detected in further passages. Of note, this was the only replicate of the experiment that did not select K65R, which can be explained by the large impact in replicative

TABLE 2. DRUG RESISTANCE MUTATIONS FOUND DURING THE *In Vitro* SELECTION OF RTB' AND RTC' WITH ESCALATING CONCENTRATION OF DIDANOSINE

<i>ddI</i> <i>Time</i> <i>(days)</i>	<i>RTB' (1)</i> <i>Mutation</i> <i>(mM)</i>	<i>RTB' (2)</i> <i>Mutation</i> <i>(mM)</i>	<i>RTB' (3)</i> <i>Mutation</i> <i>(mM)</i>	<i>RTB' (4)</i> <i>Mutation</i> <i>(mM)</i>	<i>RTB' (5)</i> <i>Mutation</i> <i>(mM)</i>	<i>RTB' (6)</i> <i>Mutation</i> <i>(mM)</i>	<i>RTC' (1)</i> <i>Mutation</i> <i>(mM)</i>	<i>RTC' (2)</i> <i>Mutation</i> <i>(mM)</i>	<i>RTC' (3)</i> <i>Mutation</i> <i>(mM)</i>	<i>RTC' (4)</i> <i>Mutation</i> <i>(mM)</i>	<i>RTC' (5)</i> <i>Mutation</i> <i>(mM)</i>	<i>RTC' (6)</i> <i>Mutation</i> <i>(mM)</i>
31	WT (0,064)	WT (0,064)	WT (0,064)	WT (0,064)	WT (0,064)	WT (0,064)	WT (0,064)	WT (0,064)	WT (0,064)	WT (0,064)	WT (0,064)	WT (0,064)
38	NS	NS	NS	NS	NS	NS	K65K/R	K65K/R	K65K/R	K65K/R	K65K/R	K65K/R
44	WT (0,128)	WT (0,128)	M184M/I	WT (0,128)	WT (0,128)	WT (0,128)	K65K/R	K65K/R	K65K/R	K65K/R	K65K/R	K65K/R
50	K65K/R	M184M/I	M184I	L74L/V	WT (0,128)	K65K/R	K65K/R	K65K/R	K65K/R	K65K/R	K65K/R	NS
56	K65K/R	NS	NS	L74L/V;	K65K/R	K65K/R	K65R	K65R	K65R	K65R; Q151M	K65R;	K65K/R
	(0,128)			M184M/I;	(0,128)		(0,128)		NS	(0,128)	NS	(0,128)
				G45G/E*								
62	K65R (0,128)	WT (0,128)	M184I	L74L/V;	K65K/R	K65R	K65R;	K65R	K65R (0,128)	K65R; Q151M	K65R;	K65R;
			(0,128)	M184M/I;	(0,128)		M184M/I	(0,128)		(0,128)	Q151M	(0,128)
				G45G/E*			(0,128)					
69	NS	M16M/I;	K65K/R;	L74L/V;	NS	NS	K65R;	NS	NS	NS	NS	NS
		K65K/R;	M184M/I	M184M/I;			M184M/I					
		M184M/I;	(0,128)	G45G/E*			(0,128)					
		C162C/Y		(0,128)								
77	K65R (0,128)	M16M/I;	K65K/R;	NS	K65K/R;	K65R	K65R;	K65R;	K65R	K65R; Q151M	K65R;	K65R;
		K65K/R;	M184M/I		G45G/R*	(0,128)	M184M/I	M184M/I	M184M/I	(0,256)	Q151M	Q151M
		C162C/Y	(0,128)		(0,128)		(0,128)	(0,128)			(0,256)	(0,256)
84	K65R (0,128)	NS	K65K/R;	NS	NS	K65R	K65R;	NS	K65R;	K65R; Q151M	K65R;	K65R;
			M184M/I				M184I;		M184M/I/	(0,256)	Q151M	Q151M
			(0,128)				E138E/K*		V (0,128)		(0,256)	(0,256)
90	NS	K65R (0,128)	K65R	NS	NS	NS	K65R;	NS	NS	NS	NS	NS
			(0,128)				M184V					
							(0,256)					
96	K65R (0,128)	K65R	K65R	NS	NS	K65R;	K65R;	K65R;	K65R;	K65R; Q151M;	K65R;	K65R;
		(0,128)	(0,128)			GGG99GGA	M184I;	M184I;	F77I*	Q151M;	Q151M;	Q151M;
						(0,128)	M184V	V60V/I*	V; V60V/	F77I* (1,024)	V; F77F/I/	M184M/
							(0,256)	(0,256)	I* (0,256)	(1,024)	L* (1,024)	L* (1,024)

*Mutation not associated with 3TC resistance.
Numbers in parentheses are replicate numbers.
NS, not sequenced; ddI, didanosine.

capacity of HIV-1 that happens when L74V and K65R are selected. Perhaps, when it occurs, L74V reverts to WT during virus replication.^{39,40}

Some factors could influence the selection of resistance mutations and one of them is genetic background. Rath and colleagues found that genetic background is important for the selection of the nevirapine resistance pathway. However, Rath experiments with two isolates from the same clinical isolate with the same amino acid sequence, and probably the same genetic background, acquired different mutations. Other factors such as epistasis and stochastic effects also contribute to resistance mutations.⁴¹ Our experiments were performed comparing two genetic backgrounds and it would be interesting to determine whether the same results could be found in viruses B and C with different backgrounds, including other molecular clones, and with other cell culture model.

Our results reinforce the previous data on faster selection of K65R in subtype C viruses when compared to subtype B viruses. Independently of the ARV used in our experiments, TDF or ddI, K65R was selected more quickly in RTC' viruses. In addition, we observed different patterns of mutations associated with K65R in RTB' and RTC' viruses with TDF selection. The actual impact of these findings should be confirmed in patients under HAART regimens composed of TDF.

There was a more complex DRM profile when RTC' viruses were exposed to ddI leading to an accumulation of K65R and Q151M or K65R and M184I/V. However, only K65R was accumulated in RTB' viruses. The selection of Q151M + K65R and M184V + K65R mutation profiles confers resistance to all FDA approved NRTIs and can represent a great challenge in designing salvage therapies in second-line regimens. These data pose a major concern in using ddI where HIV-1 subtype C is the major subtype.

We demonstrated the earlier emergence of K65R in subtype C HIV than in subtype B HIV, suggesting that administration of TDF and ddI in subtype C viruses could result in faster therapeutic failure than in subtype B. Antiretroviral treatments need to be well monitored in countries dominated by subtype C viruses to avoid the accumulation of these complex DRMs.

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