Diminished Replicative Fitness of Primary Human Immunodeficiency Virus Type 1 Isolates Harboring the K65R Mutation

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The human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) resistance mutation K65R confers intermediate levels of resistance to several RT inhibitors, including a three- to fourfold reduction of tenofovir susceptibility. Here, we have used for the first time primary HIV-1 isolates from individuals who developed the K65R mutation while enrolled in a clinical trial of tenofovir to analyze the impact of this mutation on HIV-1 replicative fitness. A marked impairment in replicative fitness was observed in association with the selection of viruses carrying the K65R mutation in all patients. The mean replicative fitness among these viruses was 20% relative to the corresponding baseline wild-type virus, ranging from 10 to 32% depending on the accompanying RT mutations. These results support a reduction in in vivo replication for K65R mutant viruses.

Tenofovir disoproxil fumarate (TDF) is the oral prodrug of tenofovir, an acyclic nucleotide reverse transcriptase (RT) inhibitor with activity against human immunodeficiency virus type 1 (HIV-1), HIV-2, and most nucleoside-resistant HIV-1 strains (13, 20, 25), which has shown potent and durable efficacy in both drug-experienced subjects (9, 21) and patients naïve for antiretroviral therapy (6). To date, the K65R amino acid substitution in the HIV-1 RT is the only mutation selected, both in vitro and in vivo, by TDF, resulting in a three- to fourfold reduction in susceptibility to this drug (10). This mutation has also been selected, in vitro and in vivo, by zalcitabine, didanosine, abacavir, and stavudine (d4T) (7, 8, 19, 30, 31). Interestingly, despite the wider usage of abacavir and TDF, the occurrence of the K65R mutation remains infrequent in treatment-experienced patients (2 to 4%) (1, 5).

The low incidence of viruses harboring the K65R mutation among antiretroviral-treated patients supports the hypothesis of a defect in the replicative fitness of these viral strains (10). For example, several studies have reported a diminished processivity of HIV RTs containing the K65R mutation, compared to wild-type enzymes (4, 29). In addition, *pol* recombinant viruses carrying the K65R mutation had a reduction in replication capacity of up to 53% of the wild-type strain used as a control (4, 29). Finally, HIV-infected patients harboring viruses with the K65R amino acid substitution do not always experience rebound viremia and worsening of their clinical conditions (10, 21), perhaps as a consequence of the impaired replicative fitness of these mutant viruses. Here, we have analyzed primary HIV-1 isolates (i.e., baseline and posttherapy) from individuals who developed the K65R mutation while en-

rolled in clinical trial GS-99-903 to analyze the impact of this amino acid substitution on HIV-1 replicative fitness.

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The clinical trial GS-99-903 was a phase III, randomized, double-blind, multicenter study of the treatment of antiretroviral-naïve HIV-infected individuals, comparing (i) TDF administered in combination with lamivudine and efavirenz versus (ii) d4T, lamivudine, and efavirenz (6). Overall, the virological failure rate was similar between the two arms of the study: 36 of 299 (12%) and 38 of 301 (12.6%) for TDF and d4T, respectively (6). However, although emergence of the K65R mutation occurred infrequently in both treatment arms (2.7 versus 0.7% for TDF and d4T, respectively), the proportion of virological failures with K65R viruses was slightly higher for the tenofovir (8 of 36, 22%) than for the d4T (2 of 38, 5.3%) arm (6). For the present analysis, 4 out of 10 patients who developed the K65R mutation and had peripheral blood mononuclear cells (PBMC) stored for analysis were selected. Clinical and virological parameters are described in Table 1. Although all four patients showed HIV RNA rebound and discontinued therapy early, two patients maintained approximately 1-log plasma HIV RNA suppression through 32 weeks without changing treatment (patients 7728 and 7225) (Table 1). No significant changes in CD4 cell counts were observed in these individuals, with the exception of patient 7152, who showed a CD4 cell increase of 110 cells/ml from baseline after beginning a new antiretroviral therapy.

HIV-1 genotypic analyses on these subjects with the use of plasma samples have been reported elsewhere (6); however, in this study we used PBMC from patients for HIV-1 isolation. Thus, final *pol* sequencing analyses included samples from (i) uncultured PBMC and (ii) cell-free culture supernatant of each HIV-1 isolate in order to confirm the presence of these mutations in the viral isolates used to quantify replicative fit-

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TABLE 1. Clinical and virological parameters of four HIV-infected individuals who developed the K65R mutation

Patient	Wk	Antiviral treatment ^a	Viral load (log ₁₀ copies/ml)	CD4 count (cells/ μ l)	Viral replicative fitness (% relative to week 0) ^b
7101	0	TDF + 3TC + EFV	5.60	13	100
	12	TDF + 3TC + EFV	5.21	17	36
	24	TDF + 3TC + EFV	5.48	9	15
7728	0	TDF + 3TC + EFV	4.63	70	100
	32	TDF + 3TC + EFV	3.81	74	27
7225	0	d4T + 3TC + EFV	5.76	27	100
	36	d4T + 3TC + EFV	4.69	19	18
	56	Off therapy	5.33	26	10
7152	0	d4T + 3TC + EFV	5.80	19	100
	36	ZDV + ddI + IDV/r	2.76	129	32

^a 3TC, lamivudine; EFV, efavirenz; ZDV, zidovudine; IDV/r, ritonavir-boosted indinavir; ddI, didanosine.

^b To determine the replicative fitness of HIV-1 primary isolates and recombinant viruses obtained from patients, the final ratio of the two viruses produced from each growth competition experiment was determined by TaqMan real-time PCR and compared to production in the mono-infections as described previously (18, 28). Briefly, a relative fitness value for each virus in the competition was estimated by the production of each individual HIV-1 strain in the dual infection. A total relative fitness was calculated as the average of the two relative fitness values, corresponding to the competition between each subtype B HIV-1 isolate or recombinant virus and each one of the non-subtype B HIV-1 control strains. The viral replicative fitness was then compared and expressed as a percentage of the baseline virus for each patient.

ness. Viral RNA and proviral DNA were extracted from pelleted virus particles and uncultured PBMC, respectively, as described elsewhere (16). An HIV-1 genomic region encompassing the 3' end of the *gag* gene (including *gag* p7/p1 and *gag* p1/p6 cleavage sites), plus full-length protease (PR) and RT genes, was PCR amplified using the following sets of primers: external PCR, Apa1988 and 3R4226, and nested PCR, 5CAI1964B and 3CAI4155Lig (11). The full-length-protease-encoding region and the first 297 amino acids of the RT polymerase domain were sequenced (27), using the following primers: 5'SP6P66/OUT, 5'SP6P66, and HIV3ex (2, 26). As expected, we did not detect any primary mutation associated with resistance to protease inhibitors in any of the 10 HIV-1 isolates. However, the RT gene from each virus evolved differently and selected for a distinct combination of RT mutations (Fig. 1).

Primary HIV-1 isolates were obtained for all the paired specimens with the exception of the following samples: patient 7728, week 0 (baseline), and patient 7152, baseline and week 36 (Table 1). After multiple failed attempts to isolate HIV-1 from PBMC we decided to construct *pol* recombinant viruses using 3'Gag/PR/RT PCR products from these samples, as described elsewhere (28). We have previously demonstrated that, although this may not be the most accurate method to estimate overall HIV-1 replicative fitness, in many cases *pol* recombinant viruses show a significant reduction in viral fitness as a consequence of drug resistance mutations in the *pol* gene, similar to that observed with the corresponding HIV-1 isolate (28). Therefore, seven primary HIV-1 isolates and three *pol* recombinant viruses were used to evaluate their susceptibility to tenofovir and replicative fitness. PBMC were incubated with increasing concentrations of tenofovir (0 to 10 μ M, provided by Gilead Sciences, Inc., Foster City, Calif.) before being exposed to HIV-1 strains obtained from the four patients. The 50% inhibitory concentration of tenofovir for each virus was calculated after 6 days of culture, by an RT assay (23). Interestingly, we observed no difference in the susceptibility to tenofovir in viruses carrying the K65R mutation, from both the

TDF and d4T arms, compared with their own baseline wild-type strain (mean, 1.1-fold; range, 0.8- to 1.3-fold). This is in agreement with other reports of very low-level changes in tenofovir susceptibility with the K65R mutation that are further reduced with the M184V mutation (12, 29).

Recent studies using *pol* recombinant viruses (based on either patient-derived *pol* fragments or site-directed mutagenesis) have shown attenuated replication in viruses carrying the K65R mutation (4, 29). In this study; however, we have used primary HIV-1 isolates to evaluate the contribution of this mutation to viral replicative fitness. Seven HIV-1 isolates and three *pol* recombinant viruses were used to determine whether selection of K65R affected the replicative fitness of these mutant variants compared with their corresponding baseline wild-type viruses. HIV-1 replicative fitness can be evaluated by a variety of methods, of which the viral growth kinetic assay, although perhaps not the most sensitive, is one of the most widely used (15). In order to get a first glimpse of the replicative fitness, viral growth kinetics (in the presence of tenofovir, ranging from 0 to 10 μ M) was monitored by measuring RT activity in the cell-free supernatants of PBMC (10^5) infected with each virus (multiplicity of infection [MOI] of 0.02 IU/cell) (Fig. 1). Results showed that all four wild-type viruses corresponding to week 0 (baseline) samples replicated with similar kinetics in the absence of drug pressure, although a slight delay was observed for one of the *pol* recombinant viruses (patient 7728). As expected, replication of the wild-type viruses was compromised in the presence of tenofovir (data not shown). Conversely, all subsequent viruses that harbored the K65R mutation accompanied by other drug resistance mutations showed a marked decrease in replicative fitness relative to their baseline wild-type virus in the absence of tenofovir (Fig. 1). This impairment in replication was not recovered in the presence of drug; on the contrary, the replicative fitness of wild-type viruses was reduced, therefore narrowing the difference in replication between wild-type and drug-resistant mutants in the presence of tenofovir (data not shown).

To corroborate the viral growth kinetic data, we analyzed

FIG. 1. Replication kinetics of viruses obtained from four patients enrolled in the GS-99-903 study. PBMC were infected with each viral stock calibrated to infect cells at an MOI of 0.02 IU/cell. Virus production was monitored over time by measuring the RT activity in the cell-free supernatant. Values are representative of two independent infections. Drug resistance-associated mutations in each particular time point (week) are indicated. Mutations associated with resistance to protease inhibitors are depicted in *italics*, while primary mutations are indicated in **boldface**.

the replicative fitness of all 10 HIV-1 isolates and *pol* recombinant viruses using growth competition experiments as described elsewhere (17, 18, 28). Briefly, each HIV-1 primary isolate or recombinant virus obtained from the patients was competed against two different non-subtype B HIV-1 control strains (HIV-1_{A-92UG029} and HIV-1_{AE-CMU06}) in a 1:1 initial proportion with an MOI of 0.01 IU/cell, in the absence of tenofovir. One milliliter of these virus mixtures was incubated with 10^6 PBMC for 2 h at 37°C and 5% CO₂. Cells were washed three times with 1× phosphate-buffered saline and resus-

pended in culture medium (10^6 /ml). Cells were washed and fed with medium after 4 days. Supernatants and cells were harvested at day 8 and stored at -80°C for subsequent analysis. Viral production in each competition was quantified by TaqMan real-time PCR as described elsewhere (28). Figure 2 shows the replicative fitness of each drug-resistant mutant relative to its corresponding wild-type virus. As observed in the viral growth kinetics, each drug-resistant virus showed a decrease in replicative fitness. In patient 7101, the virus obtained at week 12, harboring the D67N+V106M+M184V mutations,

FIG. 2. Analysis of HIV-1 replicative fitness in four HIV-1-infected patients from the GS-99-903 study. A viral replicative fitness value was calculated for each HIV-1 isolate and *pol* recombinant virus with growth competition experiments and TaqMan real-time PCR as described in Table 1. Drug resistance-associated mutations are indicated (see Fig. 1 legend for details).

had a replicative fitness of 36% of the wild-type strain at baseline. Subsequent selection of the A62V and K65R mutations at week 24 decreased the replicative fitness to 15% of the wild-type value (Fig. 2). Similar results were observed in patients 7728 and 7152, where viruses with the A62V+K65R+M184V+Y188L or K65R+T69 Δ +K70R+V106M mutations had impaired replicative fitness, that is, 27 and 32% relative to their baseline wild-type strain, respectively. Interestingly, HIV-1 isolates obtained from patient 7225 at weeks 36 and 56 showed a similar profile of mutations in the *pol* gene: no primary mutations in the protease and a combination of K65R+L100I+K103N mutations in the RT. However, a slight difference in the replicative fitness of these two viruses was detected in both the viral growth kinetic assay (Fig. 1) and the growth competition experiment, with replicative fitness values of 18 and 10% of the wild-type baseline, respectively (Fig. 2). Although this small difference may not be statistically significant, changes in other genomic regions (e.g., the *env* gene [18]) could be playing a role in the overall fitness of drug-resistant viruses.

Multiple methods have been used to measure HIV-1 replicative fitness (14, 15), many times with contrasting results depending on the assay used to estimate viral replication. In the case of viruses carrying the K65R mutation, a previous study based on viral growth kinetics showed no difference in replication efficiency compared to the wild-type virus (8). However, more recent reports with single-cycle infection or competition assays have shown attenuated replication in viruses carrying this mutation (4, 29). In the case of K65R mutant viruses obtained from the GS-99-903 study, a reduction in the viral

replication capacity (mean, 45%; range 2 to 82%, of the wild type; ViroLogic Phenosense assay) has previously been reported (12). Here, we have used viral growth kinetics and growth competition experiments in PBMC to mimic the *in vivo* environment more closely than previous studies based on cell lines. We demonstrated that both primary HIV-1 isolates and *pol* recombinant viruses carrying the K65R mutation had reduced replicative fitness compared to their corresponding baseline wild-type viruses (mean, 20%; range, 10 to 32%). Moreover, no apparent difference was observed in the replicative fitness of viruses carrying the K65R mutation with and without the M184V mutation, supporting the idea of a major contribution of the K65R mutation in the impairment of HIV-1 replicative fitness. Previous studies with a single-cycle assay showed a reduction in the replication capacity of the K65R+M184V double mutant that was more notable than that for either single mutant (29). Differences between single-cycle and our multicycle assay system or the effect of additional mutations not present in these recombinant viruses may explain this discrepancy. Nonetheless, a recent study has shown how the decrease in replication is directly linked to the lower efficiency in the incorporation of natural deoxynucleoside triphosphates by the K65R mutant RT (4). In fact, while the K65R mutation seems to affect the ability of the RT to incorporate natural substrates, the M184V appears to impair their binding (4, 12). This effect was observed in patient 7101, where the double mutant (K65R+M184V) virus isolated at week 24 was less fit than the virus present at week 12 (D67N+V106M+M184V) (15 and 36% of the wild-type virus at baseline, respectively).

In the present study, we have described the first analysis of viral replicative fitness with the use of primary HIV-1 isolates harboring the K65R amino acid substitution. Among the patients with virologic failure who developed the K65R mutation, all had developed resistance to efavirenz and most had developed resistance to lamivudine as well (6), which appears to be associated with a reduction in replicative fitness of these mutant viruses. The idea of driving HIV-1 to reduced fitness is not completely new, and the role of several specific mutations in that purpose has already been described for RT (14). These replication-impaired viruses might be less pathogenic and better controlled than wild-type viruses (3, 14, 22). Therefore, patients who developed the K65R mutation during therapy may continue to benefit from TDF therapy because of maintenance of a less-fit mutant virus and/or partial drug activity. Similar results have already been shown in simian immunodeficiency virus-infected macaques treated with tenofovir (24). Thus, further studies are needed to evaluate the stable selection of certain drug-resistant HIV mutants with impaired replicative fitness which may contribute to improved clinical management of HIV disease.

Nucleotide sequence accession numbers. Nucleotide sequences were submitted to GenBank under the following accession numbers: AY736108 to AYAY736110 (patient 7101), AY736111 to AY736113 (patient 7225), AY736114 and AY736115 (patient 7728), and AY736116 and AY736117 (patient 7152).

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