Signature Nucleotide Polymorphisms at Positions 64 and 65 in Reverse Transcriptase Favor the Selection of the K65R Resistance Mutation in HIV-1 Subtype C

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Because of codon use, the existence of these nucleotide polymorphisms should not yield any variations in amino acids between the 2 subtypes.

Recently, our laboratory demonstrated the more rapid selection of K65R in tissue culture with tenofovir in subtype C than in subtype B viruses, using both clinical isolates and cloned viruses [1]. This was the first report to suggest that there might be significant differences between subtypes in regard to drug resistance that are not due to codon use. These findings stand in contrast to those of a large number of clinical studies that have failed to document significant differences between subtypes in regard to drug resistance [2–4].

However, clinical studies involving non-B subtypes may have been biased by small sample sizes of instances of virological failure, incomplete treatment regimens, mixtures of different subtypes, and nonproportional pooling of subtypes in the non-B category [3, 5]. Recent studies have reported high rates of K65R in subtype C–infected patients who experienced treatment failure and concluded that this increased risk is related to viral subtype [6–9].

Biochemical studies in our laboratory did not reveal significant differences between the reverse-transcriptase enzymes of subtypes B and C that could account for these subtype-specific differences in regard to K65R [10]. Recently, however, we reported that subtype C nucleotide sequences that encode amino acids at reverse-transcriptase positions 64 and 65 are responsible for the increased pausing at position 65, which had been shown earlier to be associated with facilitated misincorporation [11]. This pausing effect was attributed to 2 nucleotides at positions 64 and 65 that change only the genotype but not the phenotype of the virus in regard to drug resistance. These 2 silent polymorphisms appear to be responsible for the increased pausing and may help to explain the higher rates of K65R in subtype C that have now been observed both in tissue culture and in recent clinical studies [1, 6–9].

Here, we report the more frequent selection of K65R in a subtype B virus that contains the 2 subtype C nucleotide polymorphisms at reverse-transcriptase sites 64 and 65 in cell culture drug-selection experiments with nucleoside and nucleotide reverse-transcriptase inhibitors (NRTIs). As expected from our biochemical studies, this novel selection pattern is dependent on the presence of both polymorphisms in tandem, thereby confirming the template-specific and enzyme-independent nature of the mechanism. We believe that these 2 codons should be considered K65R-associated signature polymorphisms for subtype C viruses.

Methods. Mutant NL4-3 plasmids were generated using the
Figure 1. A, Location of the K65R mutation in the sequences of subtypes B and C of human immunodeficiency virus type 1. Differences in the nucleotide sequences between subtypes B and C are shown in boldface. Arrows and shaded letters indicate the nucleotide change leading to the K65R mutation. The polyadenosine sequence that is different in subtype C is underlined. B, Sequences of wild-type (wt) and mutated NL4-3 viruses. Single-nucleotide changes introduced into the 3 mutated viruses are shown in boldface.

QuikChange II XL Site-Directed Mutagenesis kit (Stratagene). The following primers were used (Integrated DNA Technologies): for NL4-3 (64), 5′-CTCCAGTATTTGCCATAAAAAAAAGACAGTACTAAATGGAG-3′ (forward) and 5′-CTCCAT-TTAGTACGTCTTTTTTTTTATGGCAAATCTGGAG-3′ (reverse); for NL4-3 (65), 5′-CTCCAGTATTTGCCATAAAAAGAAAGACAGTACTAAATGGAG-3′ (forward) and 5′-CTCCATTATGACTCTTTTTTTTTATGGCAAATCTGGAG-3′ (reverse); and for NL4-3 (64/65), 5′-CTCCAGTATTTGCCATAAAAAGAAAGACAGTACTAAATGGAG-3′ (forward) and 5′-CTCCATTATGACTCTTTTTTTTTATGGCAAATCTGGAG-3′ (reverse). Mutated residues are shown in boldface.

Seeded 293T cells (ATCC) were incubated in 2.5% fetal bovine serum (FBS)–Dulbecco Modified Eagle medium (Invitrogen) overnight at 37°C. Supernatants were removed and replaced with 2.5% FBS–Opti-MEM (Invitrogen) followed by a 30-min incubation at 37°C. NL4-3 plasmids were prepared for transfection with Lipofectamine 2000 (Invitrogen) in 2.5% FBS–Opti-MEM. 293T cells were transfected by replacing the 2.5% FBS–Opti-MEM with Lipofectamine-plasmid mixes. After a 10-h incubation at 37°C, supernatants were removed and replaced with fresh 2.5% FBS–Opti-MEM. Supernatants were collected after 24 h at 37°C, centrifuged at 1500 g for 5 min, filtered and aliquoted.

MT2 cells (National Institutes of Health AIDS Research and Reference Reagent Program) were infected with collected viruses (multiplicity of infection, 0.01) for 2 h at 37°C and subsequently washed with 10% FBS–Rosewell Park Memorial Institute 1640 medium (Invitrogen) to eliminate any unbound virus. Infected MT2 cells were seeded in 24-well plates (density, 2.5 × 10³ cells/well). The following NRTIs were used: tenofovir (Gilead Sciences), didanosine (Sigma), stavudine (Sigma), apicabine (Aventis Pharmaceuticals), and lamivudine (GlaxoSmithKline). These drugs were used either alone or in various combinations at starting concentrations below the median inhibitory concentrations of the compounds. Culture supernatants were collected at the peaks of infection and subsequently used for new rounds of infection with increasing drug pressure. Collected culture supernatants were sequenced using the Trugene HIV-1 Genotyping kit (Siemens Laboratories). All selections for resistance were performed at least 3 times, with similar results being obtained on each occasion.

Results. A comparison of the consensus sequences in reverse transcriptase of subtypes B and C revealed 3 polymorphisms in the region of codon 65 (Figure 1A). Previous biochemical investigations have excluded any significant involvement of the third polymorphism at position 66 in regard to any subtype-specific differences [11]. Examination of subtype C sequences in the Los Alamos database (http://www.hiv.lanl.gov/) revealed a few rare cases of an AAA codon at position 65. This was, however, always linked to the presence of an AAG codon at position 64, indicating that these 2 silent polymorphisms are tightly associated. This may be a result of folding of the RNA structure, since a single mutation may require adjacent compensatory mutations to restore viral replication capacity [12]. The observed subtype-specific differences were attributed to the shift of the polyadenosine homopolymeric nucleotide sequence in subtype C, which ends at exactly the site responsible for the A-to-G change resulting in the K65R mutation [11].

To investigate the impact of these subtype C nucleotide polymorphisms, we engineered HIV-1 NL4-3, a subtype B virus, to contain single-nucleotide polymorphisms associated with subtype C at positions 64 and/or 65 (Figure 1B). These viruses were then used in cell culture drug-selection experiments with NRTIs under single or combination drug pressure.

A first series of drug-selection experiments included teno-
Figure 2. Mutational preferences of wild-type (wt) and mutated NL4-3 viruses in MT2 cells under single (A) or combination (B) drug pressure with nucleoside and nucleotide reverse-transcriptase inhibitors. The duration of the study was 20 weeks, by which time the mutations listed were fully and uniquely expressed. The graphs show the time to expression of K65R (arrow) with the NL4-3 (64/65) virus under single (A) or combination (B) drug pressure. The concentration of the second drug of a combination is illustrated above or below the respective symbol when different from that of the first drug. 3TC, lamivudine; ABC, abacavir; ATC, apricitabine; d4T, stavudine; ddl, didanosine; FTC, emtricitabine; TFV, tenofovir.
fovir, didanosine, stavudine, apricitabine, abacavir, emtricitabine, and lamivudine as single drugs over 20 weeks or until a first resistance-associated mutation was detected (Figure 2A). Selections were done in 3 independent experiments, of which 1 is presented. As expected, both lamivudine and emtricitabine selected for M184I with all 4 viruses. Selections with tenofovir resulted in the acquisition of K65R with 3 of the 4 viral isolates, the exception being NL4-3 (65). Most important, didanosine, stavudine, apricitabine, and abacavir all selected for K65R with NL4-3 (64/65), whereas these drugs selected other mutations with the other 3 viruses. These single-drug selections demonstrate the favored emergence of the K65R resistance mutation for viruses containing the subtype C template sequence at positions 64 and 65 in tandem but not for the viruses NL4-3 (64) and NL4-3 (65) that contained only single substitutions.

The times required for K65R to be selected in MT2 cells in culture with the NL4-3 (64/65) virus did not vary significantly based on the drug that was used (Figure 2A). For didanosine, abacavir, and tenofovir, K65R was detectable after 9, 10, and 11 weeks, respectively. For stavudine and apricitabine, K65R became manifest after 13 and 16 weeks, respectively. The observed delays with stavudine and apricitabine were common to all viruses.

We also subjected these same viruses to drug combinations of tenofovir-emtricitabine, tenofovir-lamivudine, stavudine-didanosine, abacavir-emtricitabine, and abacavir-lamivudine for 20 weeks or until a first mutation was detectable. One of 3 independent experiments (Figure 2B) showed that abacavir-lamivudine never selected K65R, whereas tenofovir-emtricitabine selected K65R in all cases with the exception of NL4-3 (65). In contrast, the combinations tenofovir-lamivudine, stavudine-didanosine, and abacavir-emtricitabine only selected K65R with NL4-3 (64/65), which was not the case with the other 3 recombinant viruses. These results demonstrate that the propensity for selection of K65R in viruses containing subtype C sequences also applies when combinations of different NRTIs are studied.

We also investigated the amount of time required for K65R to appear when the NL4-3 (64/65) virus was used (Figure 2B). The results show that selection times ranged between 7 and 13 weeks for all combinations that were used.

**Discussion.** The presence of 2 subtype C–specific polymorphisms in tandem in a subtype B virus can drive selection toward the K65R resistance-mutation pathway. Of note, the 2 AAG codons at positions 64 and 65 in the NL4-3 (65) virus seem to be the reason for the reduced propensity to select K65R with tenofovir or tenofovir-emtricitabine, which may mechanistically be due to the interruption of the polyadenosine homopolymeric sequence [11].

Clinical data demonstrate an increased tendency to select K65R in subtype C viruses [6–9]. Although K65R confers moderate resistance against most approved NRTIs, such resistance may be accompanied by a loss of viral replicative fitness [13]. Another possible factor is the antagonism between K65R and thymidine analogue mutations [14]. The silent polymorphism (AAA to AAG) at position 70, commonly found in subtype C of HIV-1, could help to slow K65R from arising. That zidovudine selects for thymidine analogue mutations and is widely used as part of first-line regimens in developing countries may help to explain why K65R has not been commonly observed in certain settings where subtype C infections predominate [6].

In this context, it seems relevant that 2 areas in which subtype C is endemic that recently reported the frequent emergence of K65R both used stavudine rather than zidovudine as a part of treatment regimens [8, 9]. There now seems little doubt that stavudine should be considered a drug that strongly selects the K65R mutation in subtype C viruses. The present study, as well as a variety of clinical reports on the emergence of K65R, makes clear that the propensity of subtype C viruses to select for this mutation is not related to tenofovir to a greater extent than is true for other NRTIs, including abacavir, didanosine, stavudine, and apricitabine. Further investigations are required to understand the role of other polymorphisms that might potentially antagonize the development of K65R in subtype C.

Our results provide evidence that significant differences exist between HIV-1 subtypes in regard to the propensity to develop the K65R mutation. We propose that the K64 and K65 sequences in subtype C should be considered signature polymorphisms for the development of K65R. This is the first time that polymorphisms have been shown to have this type of impact on the development of resistance. This situation differs from those in which differences in codon use in regard to amino acid incorporation have been shown to be responsible for differences between subtypes in regard to drug resistance, for instance, mutations V106M and V106A [15].

Finally, it should be recognized that the greater propensity of subtype C viruses to select for the K65R mutation may have relevance to prevention studies involving NRTIs as well as to treatment protocols. Care must be taken to ensure that individuals enrolled in preexposure prophylaxis or microbicide trials that use tenofovir and/or other NRTIs as active agents are not in the acute phase of HIV-1 infection before seroconversion, for fear that the K65R mutation might potentially be selected by the equivalent of mono- or bitherapy in this circumstance.

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References