Identification of a rare mutation at reverse transcriptase Lys65 (K65E)
in HIV-1-infected patients failing on nucleos(t)ide reverse transcriptase inhibitors

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Objectives: The HIV reverse transcriptase (RT) mutation K65R confers resistance to nucleos(t)ide reverse transcriptase inhibitors (NRTIs). Here, analysing a large database, we report the selection of another rare K65E mutation in patients failing on NRTI-containing regimens.

Methods: Clinical and virological characteristics of patients harbouring the K65E mutation were analysed using a large RT sequence database from treatment-experienced individuals. Structural analysis of the K65E RT mutant complex was performed by means of docking simulations. The replication capacity was assessed using viruses harbouring the K65E mutation introduced by site-directed mutagenesis (SDM) in pNL4-3.

Results: Overall, in 23,530 sequences from patients failing on antiretroviral therapy, the prevalence of substitutions at position K65 in RT was 2.4%. In addition to K65R (n = 395) and K65N (n = 9), another mutation, K65E, was found in 15 patients. In 11 out of 15 cases, tenofovir, abacavir, didanosine or stavudine were present at the time of K65E selection. The molecular recognition of RT containing K65E supports evidence for the role of this mutation in resistance to tenofovir. The SDM pNL4-3 K65E variant harboured a very low replicative capacity (5% versus wild-type).

Conclusions: We investigated the role of a novel rare NRTI mutation located at position Lys65 of RT (K65E), found in drug-experienced patients failing on NRTIs. The low frequency of this mutation is probably related to the high impairment of replicative capacity induced by this mutation. This study should have significant clinical implications, as these findings warn clinicians that other minor substitutions at Lys65 (such as K65E) play a role in NRTI resistance.

Keywords: HIV, nucleoside analogues, drug resistance

Introduction

Combination antiretroviral therapy (ART) has led to marked decreases in mortality and morbidity worldwide. The failure to suppress viral replication during therapy leads to the selection and expansion of drug-resistant viruses. Control of the emergence of drug resistance has become an integral part of the successful management of HIV infection.

Nucleos(t)ide reverse transcriptase inhibitors (NRTIs) remain the most commonly utilized components of HIV antiretroviral combinations. Several pathways to resistance and cross-resistance between NRTIs have been described. For example, discriminatory mutations enable the reverse transcriptase (RT) to discriminate between dideoxy-NRTI chain terminators and the cell’s naturally produced dNTPs. In this context, NRTIs are not incorporated into a growing viral DNA chain. One of the key RT discriminatory mutations is K65R. This mutation is selected primarily by tenofovir1,2 and to a lesser extent by stavudine, abacavir and didanosine.3,4 The Lys65 residue, located in the fingers domain of HIV RT, interacts with the incoming dNTP.5 Thus, this residue is
critical for functional HIV-1 RT. Mutational analysis of Lys65 has been shown to influence the nucleotide-binding specificity of the enzyme and mutations at residue 65 also significantly contribute to resistance to NRTIs. Probably because Lys65 is critical for the function of RT, the K65R mutation is rarely selected in vivo and has an overall incidence of <5% in treatment-experienced patients. In addition, clinical studies and large genotypic databases have revealed that a less common mutation at Lys65 (K65N) may emerge during virological failure.5–8 Phenotypic resistance assays further demonstrated that K65N shows a resistance profile similar to that of K65R, but causes less resistance than K65R to abacavir, didanosine, tenofovir and lamivudine.9 Here, analysing a large clinical survey from four centres, we report the in vivo selection of another rare mutation at position 65 (K65E).

**Methods**

**Data collection**

The data in this study were obtained through the examination of four large independent panels of clinically derived RT sequence isolates from treatment-experienced individuals and from therapy-naive individuals. The data originated from routine antiretroviral resistance testing retrospectively collected from the period 2000–12. The prevalence of polymorphisms at HIV RT codon 65 was assessed using a large RT sequence database. Specifically, the prevalence of K65E in clinical isolates and its association with specific drug treatment and known drug resistance mutations were analysed from genotypes and treatment histories.

Additionally, the prevalence of substitutions at HIV RT codon 65 was assessed using a large RT sequence database from a panel of 516,863 recombinant HIV-1 isolates submitted to the Janssen Diagnostics BVBA database for routine clinical resistance testing between January 1999 and January 2012.

**Plasmids and site-directed mutagenesis (SDM)**

The pNL4-3 clone containing mutation K65E in the RT coding region was constructed by SDM using the QuickChange II Site Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. The HIV-1 construct was verified by nucleotide sequencing. The primers used for the introduction of K65E were K65E-S (5′-atactcagatgtttgattaagaaag cagtaataaggaa-3′) and K65E-AS (5′-cttcacttacgtcttgggctctgc tctgctgttt-3′). For the introduction of K65R, we used 1-K65R-S (5′-taacctcagatgtttgattaagaaag cagtaataaggaa-3′) and 2-K65R-AS (5′-cttcacttacgtcttgggctctgc tctgctgttt-3′). Additionally, the prevalence of substitutions at HIV RT codon 65 was assessed using a large RT sequence database from a panel of 516,863 recombinant HIV-1 isolates submitted to the Janssen Diagnostics BVBA database for routine clinical resistance testing between January 1999 and January 2012.

**Viruses stocks**

HEK 293T were maintained in complete medium, which consisted of Dulbecco’s modified Eagle’s medium supplemented with heat-inactivated fetal bovine serum (10%), 2 mmol/L L-glutamine, 170 mmol/L penicillin and 40 mmol/L streptomycin, at 37°C and 5% CO2. Viral stocks were generated by transfecting 5×10⁶ 293T cells with plasmids using Lipofectamine Plus reagent (Invitrogen). HIV-1 p24 gag antigen concentration in viral supernatants was determined after 48 h using p24 gag antigen (bioMérieux).

**Spreading viral infections in T cell lines**

MT2 T cells were used for infection experiments. Cells were cultured in complete RPMI-1640 culture medium at 37°C and 5% CO2. Viral stocks containing 25 ng of p24 gag antigen were added to 1×10⁶ MT2 cells. Culture supernatants were collected every 1–3 days over 5 days and p24 gag antigen was quantified. Each experiment was performed in duplicate. Two independent experiments were performed.

**Structural and docking analysis**

The X-ray crystallographic coordinates of RT complexed to DNA deposited in the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) with code 1RTD were used for the structural analysis, as reported in our previous work.10 The heterocyclic base was appropriately modified before docking into the enzyme. Also, the heterocyclic moiety of the n+1th nucleotide in the template overhang was modified according to the base complementarity with respect to the incoming tenofovir. Thus, the adenine moiety, which was in the original X-ray structure, was changed into thymine. Finally, the NRTI triphosphate was manually located in such an orientation paired with its complementary base in the template strand.

From this starting model, the K65R, K65E and K65N mutants were generated by single-residue replacement in both chains and energy minimized using the united atom AMBER force field11 and the GB/SA water implicit solvation model12 as implemented in MacroModel version 9.2 (Schrödinger).13 The fully optimized receptor coordinates were used for the docking simulations, carried out by AutoDock version 4.4.14 The map box was fixed equal to 70.190 Å.14 AMBER was used as the force field, the receptor was kept rigid and the drug flexible. This method applied a Lamarckian model of genetics, generating 50 allowed configurations per ligand. We carried out the maps for the A, C, HD, N, OA, P, SA and F probes; the dielectric constant was set equal to 80.

To select the AutoDock-generated poses able to receive the nucleophilic attack by the free 3’ OH of the DNA template (NRTI–template distance), a distance cut-off equal to 6.0 Å was adopted and the best pose, as ranked by the free-energy value, was identified.

All three-dimensional figures were created using the PyMOL graphics and modelling package version 1.3 (http://www.pymol.org).

**Results**

The prevalence of polymorphisms at HIV RT codon 65 was assessed using a large RT sequence database from treatment-experienced individuals (n = 23,530) recovered from four patient centres. Overall, the prevalence of HIV RT codon 65 substitutions was 2.4% (n = 419). As expected, the most commonly occurring mutation was K65R, observed in 395 patients (94%). In addition, mutations less commonly observed at codon 65 included K65N (2%; n = 9) and K65E (4%; n = 15). No other amino acid change was observed in this database from treatment-experienced patients. In contrast, such mutations were extremely rare when analysing 8,698 sequences from drug-naive patients (K65R, n = 2; K65N, n = 1; K65E, n = 1). In another panel of 516,863 HIV-1 clinical isolates from the Janssen Diagnostics BVBA database, the prevalence of HIV RT codon 65 substitutions was quite similar (2.1%; n = 11,018). A similar profile and rate of substitutions was observed: K65R (96%; n = 10,569), K65N (2%; n = 235) and K65E (2%; n = 183). In this database compiled since 1999, the frequency of occurrence of K65R, K65N or K65E evolves over time, with a peak of detection during the period 2003–05, when tenofovir started to be widely used in clinical practice (Figure 1).

Because there is no previously published report describing K65E, we specifically focused on the characteristics of viruses harbouring this novel mutation. The clinical and virological characteristics of the patients harbouring K65E in our dataset are presented in Table 1. At the time of detection of K65E, the median plasma viral load was 3.14 log₁₀ HIV-1 RNA copies/mL (range 1.6–4.7 log₁₀ copies/mL) and the median CD4 count was
325 cells/mm³ (range 114–3040 cells/mm³). In all cases but one, this mutation was observed under NRTI treatment. No specific NRTI was uniquely associated with the selection of K65E (Table 1), but the most frequent NRTIs combinations were emtricitabine/tenofovir (n = 4) and lamivudine/abacavir (n = 3).

When genotypic testing performed before ART was available (n = 8/15), the K65E mutation was not present, indicating that this mutation has been selected under treatment. When selected, the K65E mutation was observed in mixtures in population-based sequences (K/E) in 53% (n = 8). Regarding the association with other NRTI mutations, K65E was the unique NRTI mutation emerging during antiretroviral failure in six patients (40%); the lamivudine/emtricitabine-related mutation (M184V/I) was observed in seven patients (47%). Simultaneous detection of K65E and thymidine analogue mutations (TAMs) was observed in five patients (33%).

Regarding the subtypes of the viruses with K65E mutation, 53% (n = 8) were subtype B, 27% (n = 4) were subtype CRF02 AG, 7% (n = 1) subtype D, 7% (n = 1) subtype H and in 1 patient, the subtype could not be assigned. The frequency of these subtypes reflects the distribution of subtypes in the entirety of the databases (subtype B = 65%, subtype CRF02 = 16%, subtype C = 3% and others = 18%).

We examined the impact of the K65E mutation compared with the K65R mutation on virus fitness by introducing this mutation.
into the molecular HIV-1 clone pNL4-3. Wild-type (WT) NL4-3 and K65R and K65E mutants were generated in 293T cells by transfection and were used to infect MT2 cells, resulting in the production of WT, K65R and K65E viruses. As shown in Figure 2, K65E replication was significantly reduced compared with that of K65R after the first days of infection. At day 10, the fitness differences between WT and the K65R mutant ranged between 1.3- and 2-fold, while the fitness differences between WT and the K65E mutant showed that the K65E virus was up to 17-fold less fit than WT.

Attempts to verify the impact of K65E on NRTI resistance by phenotyping unfortunately failed due to the severe impairment of viral fitness. Therefore, we performed molecular modelling using docking analysis to investigate the role of the K65E mutation. According to our previously reported approach, we applied a distance-filtering criterion to analyse the docking results, assuming a major 'reagent-like' control in the NRTI mechanism of action. A distance >6 Å was considered to be not compatible with the binding of the drug with RT. After docking simulations, we found that the distance between tenofovir and the oxysriliic group of the nascent DNA chain was increased (d = 10.3 Å) in the presence of K65R, probably hampering the affinity between the RT and the nucleotide analogue (Figure 3). In particular, the arginine at position 65 is able to strongly interact with the two terminal phosphate groups of tenofovir, thus keeping the drug too far from the pre-reactive 3' OH of the DNA template. Similarly, the presence of K65E caused a larger distance between them (d = 11.4 Å) (Figure 3), due to a strong electrostatic repulsive effect exerted by the glutamate carboxylate group against the charged oxygen atoms of tenofovir phosphates. In contrast, in the presence of the K65N substitution, we found a distance compatible with the binding of the drug with RT (d = 5.7 Å) (Figure 3); however, such a geometric observation was not supported by the thermodynamic results. In the best geometrical pose, the asparagine side-chain oxygen of this mutant is oriented in front of the tenofovir terminal phosphate moieties, thus reducing its energy ranking position among all generated docking solutions (47th of 50) with respect to the corresponding best WT NRTI–template distance configuration (2nd of 50). The intermolecular energy terms, detailed in

Figure 2. Replication kinetics of full-length HIV-1 WT, K65R and K65E in MT2 T cell lines. Supernatants were collected every 2 days and assayed for viral p24 gag antigen. Each experiment was performed in duplicate. Two independent experiments were performed.

Figure 3. (a) WT, (b) K65R, (c) K65E and (d) K65N tenofovir RT–template complexes showing the distance between the drug and the free 3' OH of the DNA template. The RT and the DNA are shown in grey and orange, respectively. Tenofovir, the nucleotide at 3' and the residue at position 65 are shown in cyan, green and yellow, respectively. The distances, expressed in Å, are indicated as black broken lines. A distance >6 Å was considered to be not compatible with the binding of the drug with the RT. The energy ranking positions of each filtered pose are (a) 2nd, (b) 2nd, (c) 34th and (d) 47th out of the 50 generated docking solutions. TDF, tenofovir.
K65E HIV-1 resistance

Table 2. AutoDock evaluation of tenofovir free energy of interaction calculated for WT and mutated RT complexes

<table>
<thead>
<tr>
<th>Model</th>
<th>ΔGbind (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>−0.88</td>
</tr>
<tr>
<td>K65R</td>
<td>−0.37</td>
</tr>
<tr>
<td>K65E</td>
<td>+1.11</td>
</tr>
<tr>
<td>K65N</td>
<td>+0.62</td>
</tr>
</tbody>
</table>

Table S1 (available as Supplementary data at JAC Online), are consistent with this observation. Consequently, the energetic analysis of the best poses always correlates with an unproductive energetic profile (ΔG: −0.88 kcal/mol for WT, −0.37 kcal/mol for K65R, +1.11 kcal/mol for K65E and +0.62 kcal/mol for K65N) (Table 2).

Discussion

In this study, we describe a novel mutation at RT Lys65 (K65E) in HIV-1-infected patients failing on NRTIs, based on four large datasets of clinically derived isolates. Four independent lines of evidence support the relevance of this mutation in NRTI resistance, particularly to tenofovir: (i) epidemiological observations of increasing K65E during the period 2003–05, when tenofovir started to be widely used in clinical practice; (ii) this substitution was most exclusively observed in treatment-experienced individuals failing on NRTI-containing therapies in our database; (iii) a search of HIV-1 genotypes in the Stanford database (http://hivdb.stanford.edu/cgi-bin/RTPbsMutSummary.cgi?Gene=RT&Position=65) identified 30 isolates with K65E mutation being significantly associated with NRTI treatment compared with drug-naive patients; and (iv) docking analysis (geometric and the thermodynamic modelling) supported evidence for the role of K65E mutation in resistance to tenofovir.

Longitudinal data on the appearance of K65E suggested that the K65E substitution arose directly from WT, consistent with the single nucleotide substitution required to change WT K65 to K65E (AAA to GAA or AAG to GAG). The appearance of the K65E mutation is not likely to be related to variations in virus subtypes, as it was evidenced in different subtypes, including subtype B and CRF02_AG, which are the most prevalent subtypes in our dataset. This mutation appears at a similar rate to the K65N mutation in treatment-experienced patients. In this regard, K65E seems to have similar characteristics to K65N: both mutations seem to coexist with TAMs, in contrast to K65R. However, these results should be interpreted with caution, because the sample size of the K65E sequences is small and obtained from population genotyping. Therefore, it is not known whether the K65E mutation can coexist on the same genome as TAMs. In addition, similar to K65N, K65E is often detected as a mixture population, which in conjunction with its rareness suggests its deleterious impact on viral fitness might be important. This is supported by SDM experiments showing that NL4-3 K65E virus fitness was significantly reduced compared with NL4-3 K65R in MT2 cells. In this context, attempts to verify the impact of K65E on NRTI resistance by phenotyping unfortunately failed due to the severe impairment of viral fitness. Consistently with these findings, previous experiments showed that K65E impairs RT activity. These findings may have clinical implications: because K65E might alter viral fitness in patients failing on NRTIs, it is tempting to speculate that maintaining NRTI pressure (i.e. tenofovir or abacavir) could be beneficial for the patient. However, this hypothesis is difficult to address in prospective studies, given its very low prevalence in patients failing on highly active ART.

Similarly to K65R and K65N, the prevalence of K65E decreased overtime after 2005 in the Janssen Diagnostics BVBA database. This observation is consistent with previously published data: assessment of >1000 HIV resistance genotypes at a Spanish HIV/AIDS clinic showed a decrease in the incidence of the K65E mutation, from 15.2% of isolates during the period 2002–04 to 2.7% of isolates during the period 2005–06, despite elevated and stable rates of tenofovir use. In a large dataset of resistance test results by Monogram Biosciences (n = 107231), the prevalence in 2010 decreased compared with 2003 for K65R (4.3%–2.1%). This observation is probably the consequence of changes in strategies to administrate NRTI combinations: in early 2000, clinical studies have demonstrated an increased frequency of K65E in association with suboptimal stavudine and didanosine regimens or coadministration of tenofovir with abacavir or didanosine in first-line treatment regimens. After 2005, careful avoidance of such combinations and genotypic resistance testing has led to decreasing trends in K65R appearance. These considerations are of great importance, because they provide clinicians with reasonable clinical approaches in preventing HIV drug resistance. Highly potent emtricitabine/tenofovir and lamivudine/abacavir coformulated regimens with non-nucleoside reverse transcriptase inhibitors such as efavirenz or protease inhibitors help to prevent the advent of substitutions at position RT Lys65.

In this study, we investigated the role of a novel rare NRTI mutation located at position Lys65 of RT (K65E), found in drug-experienced patients failing on NRTIs. The low frequency of this mutation is probably related to the high impairment of replicative capacity induced by this mutation. However, this finding should have significant clinical implications, as it warns clinicians that other minor substitutions at Lys65 (such as K65E) play a role in NRTI resistance. In addition, because HIV-1 subtype C was rare in our database, more extensive studies, particularly with subtype C, should be performed to measure the frequency of its selection.

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Transparency declarations
P. V. D. E. is an employee of Janssen Diagnostics BVBA. All other authors: none to declare.

Supplementary data
Table S1 is available as Supplementary data at JAC Online (http://jac.oxford-journals.org/).

References