K65R in Subtype C HIV-1 Isolates from Patients Failing on a First-Line Regimen Including d4T or AZT: Comparison of Sanger and UDP Sequencing Data

Patricia Recordon-Pinson1,2, Jennifer Papuchon1,2, Sandrine Reigadas1,2, Alaka Deshpande3, Hervé Fleury1,2*

1 CNRS, UMR 5234, Bordeaux, France, 2 Laboratoire de Virologie (WHO accredited), CHU de Bordeaux, Bordeaux, France, 3 JJ Hospital, Byculla, Mumbai, India

Abstract

Background: We and others have shown that subtype C HIV-1 isolates from patients failing on a regimen containing stavudine (d4T) or zidovudine (AZT) exhibit thymidine-associated mutations (TAMs) and K65R which can impair the efficacy of Tenofovir (TDF) at second line. Depending on the various studies, the prevalence of K65R substitution as determined by the Sanger method ranges from 4 to 30%. Our aim was to determine whether ultra-deep pyrosequencing (UDPS) could provide more information than the Sanger method about selection of K65R in this population of patients.

Methods: 27 subtype C HIV-1 isolates from treated patients failing on a regimen with d4T or AZT plus lamivudine (3TC) plus nevirapine (NVP) or efavirenz (EFV) and who had been sequenced by Sanger were investigated by UDPS at codon 65 of the reverse transcriptase (RT). 18 isolates from naive patients and dilutions of a control K65R plasmid were analysed by Sanger plus UDPS.

Results: Analysis of Sanger sequences of subtype C HIV-1 isolates from naive patients exhibited expected polymorphic substitutions compared to subtype B but no drug resistance mutations (DRMs). Quantitation of K65R variants by UDPS ranged from <0.4% to 3.08%. Sanger sequences of viral isolates from patients at failure of d4T or AZT plus 3TC plus NVP or EFV showed numerous DRMs to nucleoside reverse transcriptase inhibitors (NRTIs) including M184V, thymidine-associated mutations (TAMs) plus DRMs to non-nucleoside reverse transcriptase inhibitors (NNRTIs). Two K65R were observed by Sanger in this series of 27 samples with UDPS percentages of 27 and 87%. Other samples without K65R by Sanger exhibited quantities of K65R variants ranging from <0.4% to 0.80%, which were below the values observed in isolates from naive patients.

Conclusions: While Sanger sequencing of subtype C isolates from treated patients at failure of d4T or AZT plus 3TC plus NVP or EFV exhibited numerous mutations including TAMs and 8% K65R, UDPS quantitation of K65R variants in the same series did not provide any more information than Sanger.

Introduction

We and others [1–2] have shown that subtype C HIV-1 isolates from Indian patients who fail on first-line HAART composed of stavudine (d4T) or zidovudine (AZT) plus lamivudine (3TC) plus nevirapine (NVP) or efavirenz (EFV) and according to WHO clinical and/or immunological criteria exhibit numerous drug resistance mutations (DRMs) to nucleoside reverse transcriptase inhibitors (NRTIs) and to non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the reverse transcriptase (RT) part of the viral genome, including thymidine-associated mutations (TAMs) and K65R (the prevalence of which was around 8% in our series). When the RT sequences were introduced into the ANRS and Stanford algorithms, both algorithms showed that the DRMs of the first line induce a decreased susceptibility to tenofovir (TDF), an NRTI drug that is still used as second line in some southern countries. This has implications for public health because patients who fail with a first-line regimen including d4T or AZT plus 3TC plus NVP or EFV and who switch to 3TC plus TDF plus ritonavir-boosted lopinavir (LPV/RTV) will in fact not be fully susceptible to TDF and therefore to the second-line regimen. TAMs and K65R are known to induce partial or full resistance to TDF [3]. Regarding K65R, similar studies carried out in the same context of failure on a first-line regimen including d4T, AZT or didoxoyninosine (ddI) showed a prevalence of 4% in a South African population where subtype C was predominant [4], 10.9%
isolate from South Africa [5], 14% in subtype C isolates from South Africa [6], 24% in Malawi with subtype C viruses [7] and up to 30% in Botswana [8].

From a molecular point of view, it has been demonstrated that the RT KKK nucleotide motif at codons 64, 65, 66 in reverse transcriptase of subtype C HIV-1 appears to lead to template pausing that facilitates the selection of K65R, even in isolates from untreated patients [9–12]. Moreover, it has also been shown that the KKK motif in this subtype can lead to PCR-induced K65R [13].

The aim of the present study was to clarify the prevalence of K65R in these subtype C isolates from patients failing on a first line including d4T or AZT. Since we had the prevalence of K65R [13].

Two subtype C MJ4 plasmids, one with K65K (wild type) and one with K65R were used to amplify RT region before sequencing by UDPS.

### Table 1. Primers used for GS Junior ultradeep sequencing of RT.

| sequence 5′-3′ | HXB2 position |
|---------------|---------------|
| RT PCR GS Junior |
| primer 5′ | AGTAGGACCTACACCTGTCGA | 2480 to 2499 |
| primer 3′ | CTGTTAGGTGTTGTTCTCT | 3399 to 3420 |
| Nested GS Junior |
| primer 5′ | GGGATGTTGATTTCCAATGAGACTTC | 2620 to 2647 |
| primer 3′ | GGGATGTTGATTTCCAATGAGACTTC | 2813 to 2840 |

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### Table 2. RT polymorphic substitutions (Sanger) compared to reference HIV-1 B in isolates from naive patients and amounts of K65R and K70R mutations in the same isolates plus control plasmids.

| naive patients | RT sequence by Sanger method | K65R | K70R |
|----------------|-----------------------------|------|------|
| 1031           | 35Q, 39D, 48T, 48S, 60L, 73K, 73I, 122K, 135R, 162N, 173A, 174K, 177E, 178L, 200A, 207E, 211K, 214F, 245Q | <0.4% | <0.4% |
| 1059           | 13R, 16N, 21G, 35T, 39D, 43R, 48T, 60L, 90V, 90H, 106M, 121Y, 135T, 135I, 158A, 158S, 162A, 162Q, 170P, 170L, 173A, 177E, 179D, 200A, 207E, 211K, 214F, 221H, 221Y, 245Q | 0.44% | 0.44% |
| 1067           | 13K, 13R, 35T, 36A, 39E, 48T, 60L, 77I, 77F, 123E, 142V, 146R, 173A, 174K, 177E, 178L, 195L, 200A, 202V, 207E, 211K, 214F, 245Q | 0.42% | 0.42% |
| 1113           | 28K, 32E, 35T, 36A, 39D, 48T, 60L, 121H, 173A, 174Q, 174R, 177E, 178L, 190R, 194H, 200A, 207E, 214C, 225L | <0.4% | <0.4% |
| 1116           | 20R, 35T, 39E, 48T, 60L, 102K, 102Q, 121Y, 135T, 138A, 162C, 173A, 200A, 207E, 207A, 211K, 214L, 214F, 245Q | 1.18% | 1.18% |
| 1120           | 35T, 39E, 39D, 40T, 48T, 60L, 64R, 121Y, 121C, 166R, 173T, 173A, 175N, 175H, 177E, 177E, 178L, 178L, 200A, 207E, 214F, 245Q, 250H | 0.35% | 0.35% |
| 1121           | 35T, 39E, 48T, 60L, 110H, 110D, 121Y, 135R, 173T, 177E, 177E, 200A, 207E, 214F, 217P, 217S, 245Q, 248R, 249R, 251V, 252G, 254F, 255K | 1.33% | 1.33% |
| 1122           | 101J, 35T, 36A, 39D, 48T, 60L, 121H, 139T, 139S, 173S, 174K, 177E, 178L, 180A, 200A, 207E, 211K, 214F, 245Q, 252C | <0.4% | <0.4% |
| 1123           | 13N, 35T, 39D, 43R, 48T, 60L, 121H, 121Y, 135T, 135I, 162A, 173A, 174R, 177E, 178L, 200A, 207E, 211K, 214F, 245Q | 0.85% | 0.85% |
| 1129           | 35T, 36A, 39D, 48T, 60L, 122K, 139A, 173T, 177E, 178M, 200A, 207E, 211K, 214F, 245Q | 0.75% | 0.75% |
| 1131           | 36A, 39E, 48T, 55T, 55P, 73K, 73I, 123S, 138E, 138V, 173A, 173E, 200A, 207E, 211K, 214F, 245Q | 3.08% | 3.08% |
| 1132           | 35T, 36A, 39D, 48T, 60L, 122P, 126C, 166R, 173T, 173A, 177E, 200A, 202L, 202V, 207E, 211K, 214F, 245Q | 1.02% | 1.02% |
| 1133           | 35T, 36A, 39D, 48T, 60L, 122P, 123E, 161Q, 161L, 166R, 173A, 177E, 200A, 207E, 211K, 214F, 220K, 220L, 245E, 248K | <0.4% | <0.4% |
| 1134           | 35T, 36A, 39D, 48T, 49K, 49R, 60L, 121C, 159I, 159V, 160C, 160F, 162S, 162C, 165I, 173T, 173A, 173E, 178M, 178L, 200A, 207E, 214F, 245Q | <0.4% | <0.4% |
| 1139           | 35T, 39N, 48T, 60L, 102R, 104R, 121Y, 162A, 173A, 200A, 207K, 211K, 214F, 245Q | <0.4% | <0.4% |

Footnote to Table 2. Two subtype C MJ4 plasmids, one with K65K (wild type) and one with K65R were used to amplify RT region before sequencing by UDPS. Amplicon pools were not prepared in equimolar concentrations but with different percentages of K65R mutation. The theoretical and observed values of K65R plasmid dilutions were 100%:94%, 5%:2.30% and 1%:0.90%.

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Methods

Subtype C HIV-1 isolates from Indian patients failing (according to WHO clinical and/or immunological criteria) on a first-line treatment including d4T or AZT plus 3TC plus NVP or EFV were sequenced on RT by the Sanger method, and the sequences were recorded in the Los Alamos database (GenBank JF895621–JF895675). Among these samples, 27 were randomly selected for investigation by UDPS using the Roche GS Junior equipment. RNA extracted previously from the samples was used to amplify a short region of RT with primers including specific sequences for the GS Junior system (Table 1). The reverse transcription used SuperScriptIII RTPCR enzyme (Invitrogen, Carlsbad, CA) with 10 ul RNA, one cDNA synthesis cycle at 50°C for 30 min and 40 cycles of PCR amplification. The nested PCR used FastStart HiFi (Roche) with 2 ul of RTPCR product, 40 cycles of PCR amplification. Amplicons were purified by AMPure kit (Agencourt Biosciences), quantified using QuantiT PicoGreen (Invitrogen) and pooled at equimolar concentrations. Clonal amplification on beads (EmPCR) was performed using the 454 Life Science reagents that enable bidirectional sequencing, composed of a 30 cycle PCR amplification. DNA containing beads were recovered and UDPS was performed on the GS Junior sequencer (454 Life Sciences). Most of the samples had HIV viral loads >100,000 copies/mL (mean: 379,753 copies/mL; IQR: 11209–5,817,977 copies/mL) and at least 1,000 clonal sequencing isolates from patients at failure were compared to those of subtype C isolates from 18 naive patients. Some of their bulk sequences have been previously published by our group [15]. All codons were analysed by Sanger while potential polymorphism at codons 65 and 70 was investigated by both Sanger and UDPS. As a control, we used two subtype C MJH plasmids, one wild type and one bearing K65R (both provided by Mark Wainberg’s group in Montreal). The UDPS results of the study are available in GenBank under accession number SRA 030640.

Results

Table 2 shows the Sanger results of viral isolates from naive patients and UDPS results of codons 65 plus 70 in these isolates, together with UDPS results of control plasmids for codons 65 and 70. Analysis of Sanger results for naive patients showed an extensive polymorphism compared to subtype B without involvement of substitutions 65R and 70R. K70R was <0.4% by UDPS in all isolates from naive patients. K65R ranged from <0.4% to 3.08% (mean 0.66±0.76 standard deviation, SD).

Regarding the 27 isolates from treated patients at failure, the drug resistance mutations (DRMs) according to the French ANRS algorithm and following bulk DNA sequencing (Sanger) are listed in Table 3. Most of them exhibited the M184V mutations to 3TC of the regimen plus TAMs to d4T and/or AZT and DRMs to NNRTIs. Only two samples (455 and 493) bore a K65R mutation, one (455) cumulating K65R and the Q151M nucleoside analog mutation (NAM). Table 4 compares the Sanger and UDPS data of these isolates at codons 65 and 70. Quantitation of K65R by UDPS ranged from <0.4% to 87%. In the two isolates with K65R by Sanger, percentages of K65R by UDPS were 27% and 87%. If we only consider positions 65 found not to have K65R with Sanger (25 samples), the quantities of K65R ranged from 0.4% to 0.80% (0.16±0.22 SD). K70R with UDPS ranged from <0.4% to 100%. Eight samples exhibited K70R with Sanger and the corresponding UDPS values ranged from 34.50 to 100%. Regarding positions 70 found not to have the K70R mutation with Sanger (19), all of them were <0.4% for 70R by UDPS except two samples (470 and 489 with values of 1.80 and 4.40% respectively).

Table 3. RT bulk sequences of 27 subtype C HIV-1 isolates failing on first line.

| patient | RT sequence by Sanger method | patient | RT sequence by Sanger method |
|---------|-------------------------------|---------|-------------------------------|
| 454     | 115F, 151M, 184V, 219Q, 90I, 181C, 221Y | 479     | 41L, 44D, 67N, 75M, 184V, 215Y, 98G, 101E, 179I, 181C, 190A, 221Y |
| 455     | 41L, 65R, 151M, 184V, 181V, 190A | 480     | 41L, 67N, 69D, 70R, 184V, 210W, 215Y, 98G, 106M, 179I, 181C, 190A |
| 456     | 67N, 70R, 184V, 215F, 219E, 98S, 181C | 481     | 41L, 67N, 69insert, 75M, 184I, 210W, 215Y, 90I, 103N |
| 461     | 41L, 44D, 69D, 184V, 90I, 179I, 181C | 482     | 41L, 67N, 69D, 184V, 215Y, 101E, 179I, 181C, 190A |
| 463     | 41L, 67N, 69D, 70R, 184V, 215Y, 188L, 221Y | 485     | no resistance mutation |
| 464     | 184V, 98G, 101E, 181C, 190A | 486     | 41L, 67N, 74V, 184V, 215Y, 101E, 138Q, 190S |
| 465     | 41L,44D, 67N, 74V, 184V, 210W, 215Y, 101E, 179I, 190A | 487     | 67N, 69D, 70R, 184V, 219Q, 98G, 179I, 190A |
| 466     | 41L, 67N, 69D, 75M, 184V, 210W, 215Y, 101E, 179I, 190A | 488     | 41L, 184V, 215Y, 103N, 225H |
| 469     | 41L, 67N, 69N, 70R, 184V, 215F, 219E, 103N, 190A | 489     | 74V, 184V, 215Y, 101E, 179I, 190C |
| 470     | no resistance mutation | 493     | 65R, 75A, 219E, 179I, 181C, 190A, 221Y |
| 471     | 41L, 184V, 215Y, 98G, 101E, 190A | 495     | 41L, 184V, 210W, 215F, 90I, 103N |
| 472     | 41L, 67N, 70R, 184V, 215Y, 219E, 181C | 496     | 41L, 67N, 70R, 75M, 184V, 215F, 219Q, 106M, 190A |
| 473     | 41L, 44D, 67N, 70R, 184V, 215Y, 103N, 190A | 501     | 41L, 67N, 75M, 184V, 210W, 215Y, 101E, 190S |
| 475     | 41L, 67N, 69D, 75M, 184V, 215Y, 101E, 179I, 188L, 190A | | |

DRMs are noted according to ANRS.
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Table 4. Comparison of Sanger and UDPS sequencing results for RT codons 65 and 70; same isolates as in Table 3.

| Patient | K65R Sanger | K70R Sanger | K65R UDPS | K70R UDPS |
|---------|-------------|-------------|-----------|-----------|
| 454     | N           | <0.4%       | N         | <0.4%     |
| 455     | O           | 87%         | N         | <0.4%     |
| 456     | N           | <0.4%       | O         | 100%      |
| 461     | N           | <0.4%       | N         | <0.4%     |
| 463     | N           | <0.4%       | O         | 34.6%     |
| 464     | N           | 0.14%       | N         | <0.4%     |
| 465     | N           | <0.4%       | N         | <0.4%     |
| 466     | N           | 0.5%        | N         | <0.4%     |
| 469     | N           | <0.4%       | O         | 94%       |
| 470     | N           | <0.4%       | N         | 1.8%      |
| 471     | N           | <0.4%       | N         | <0.4%     |
| 472     | N           | 0.46%       | O         | 81.6%     |
| 473     | N           | <0.4%       | O         | 97.1%     |
| 475     | N           | <0.4%       | N         | <0.4%     |
| 476     | N           | 0.24%       | N         | <0.4%     |
| 480     | N           | 0.25%       | O         | 84.4%     |
| 481     | N           | 0.21%       | N         | <0.4%     |
| 482     | N           | <0.4%       | N         | <0.4%     |
| 485     | N           | <0.4%       | N         | <0.4%     |
| 486     | N           | 0.3%        | N         | <0.4%     |
| 487     | N           | 0.25%       | O         | 90.1%     |
| 488     | N           | 0.5%        | N         | <0.4%     |
| 489     | N           | <0.4%       | N         | 4.4%      |
| 493     | O           | 27%         | N         | <0.4%     |
| 495     | N           | 0.4%        | N         | <0.4%     |
| 496     | N           | 0.8%        | O         | 98.15%    |
| 501     | N           | <0.4%       | N         | <0.4%     |

Sanger Y: presence of mutation; N: absence of mutation. UDPS: Frequency of mutations observed in samples.
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Discussion

Sanger and UDPS results in isolates from naïve patients

Although UDPS has limitations particularly with regard to polymerization and pyrosequencing errors [13,16], recent studies with different methods (UDPS, allele specific PCR) have shown that K65R is identified more frequently in subtype C HIV-1 from naïve patients [14,17]. In our naïve patients, there was a clear difference between K70R (mean 0%) and K65R (mean 0.66%) (Table 2). Our data on position 65 are in agreement with those of Kozal et al [14], ranging from 0.4% to 1.33% apart from one sample (1131) at 3.08%. We were not expecting selection of K65R mutations observed in samples.

Sanger and UDPS results of isolates from treated patients at failure

The Sanger results of isolates from treated patients were as expected with a predominance of M184V, numerous TAMs of pathway1 (M41L, D67N, K70R, L210W, T215Y/F) and DRMs to NNRTIs (mainly K101E, K103N, V106M, Y181C, G190A). As mentioned above, 2 isolates of the series exhibited a K65R substitution.

With regard to UDPS results at codon 70, the quantitative data were different from those recorded in naïve patients: 8 isolates exhibiting K70R with Sanger had UDPS K70R values above 34.60%, while 2 samples without K70R with Sanger (470 and 489) had K70R variants at quantities above the <0.4% background observed in naïve patients. We hypothesize that these isolates are undergoing a process of selecting K70R mutations. Regarding the K65R values apart the two samples with K65R by Sanger, the UDPS quantities of K65R variants were low and below those of isolates from naïve patients. Our results are not in accordance with those obtained by another group [18] using an allele- specific PCR which exhibited minority variants of K65R in four subtype C HIV-1 isolates out of 30 patients having received NRTIs at first line; it must be pointed out that this technique uses an intercalating dye and high-melt resolution assay which can be difficult to interpret due to genomic variability in the flanking region of codon 65.

K65R substitutions are generated in subtype C isolates from naïve patients due to the 64–65–66 motif. There are some constraints in experienced patients failing on a suboptimal regimen with d4T or AZT plus 3TC plus NVP or EFV. We first hypothesize that 194V, which was the most prevalent mutation observed in our series of treated patients at failure, has dampened the emergence of 63R as noted by others [3]. Second, there is an antagonism between TAMs and K65R, while the latter can be found in association with NAMs (Q151M) and is considered to be increasingly selected in the presence of DRMs to NNRTIs and particularly Y181C and G190A. As noted above, the prevalence of K65R in this clinical context of failure ranges from 4 to 30%. In our series, we estimate this prevalence to be 8% and the UDPS data did not reveal any process of K65R selection that cannot be assessed by Sanger sequencing.

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Author Contributions

Conceived and designed the experiments: PP HF. Analyzed the data: PP SR HF. Contributed reagents/materials/analysis tools: PP JP AD. Wrote the paper: PP SR HF.

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K65R in Subtype C HIV-1 Isolates

Discussion

Sanger and UDPS results in isolates from naïve patients

Although UDPS has limitations particularly with regard to polymerization and pyrosequencing errors [13,16], recent studies with different methods (UDPS, allele specific PCR) have shown that K65R is identified more frequently in subtype C HIV-1 from naïve patients [14,17]. In our naïve patients, there was a clear difference between K70R (mean 0%) and K65R (mean 0.66%) (Table 2). Our data on position 65 are in agreement with those of Kozal et al [14], ranging from <0.4% to 1.33% apart from one sample (1131) at 3.08%. We were not expecting selection of K65R in this population of patients who were quite distant from primary
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