The preferential selection of K65R in HIV-1 subtype C is attenuated by nucleotide polymorphisms at thymidine analogue mutation sites

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Objectives: We recently reported the preferential selection of the K65R resistance mutation in subtype C HIV-1 compared with subtype B and showed the underlying mechanism to be dependent on subtype C-specific silent nucleotide polymorphisms, i.e. genomic mutations that change the genotype but not the phenotype. The number of clinical reports demonstrating elevated numbers of K65R nevertheless suggests the existence of factors limiting the increased incidence of K65R mutations. Thus, we investigated the contributions of subtype C-specific silent nucleotide polymorphisms at thymidine analogue mutation (TAM) sites 70, 210 and/or 219 that might reduce the previously described preferential selection of K65R in subtype C HIV-1 associated with subtype C-specific nucleotide polymorphisms at sites 64/65.

Methods: Cell culture drug selections were performed with various drugs in MT2 cells.

Results: The use of nucleoside/nucleotide reverse transcriptase inhibitors [N(t)RTIs] as single drugs or in combination confirmed the more frequent selection of K65R by multiple N(t)RTIs in a subtype B virus that contained the 64/65 nucleotide polymorphisms of subtype C than in a wild-type subtype B virus. This effect was attenuated in the presence of several silent TAM nucleotide polymorphisms, except when stavudine was employed in the selection protocol.

Conclusions: These results further demonstrate that stavudine can preferentially select for K65R in subtype C virus and also provide a basis for understanding the importance of silent nucleotide polymorphisms in regard to altered HIV drug resistance profiles.

Keywords: subtype differences B/C, nucleotide polymorphisms, K65R resistance mutation, cell culture drug selections, N(t)RTIs, TAMs

Introduction

We recently described the more rapid tissue culture selection of K65R mutations with tenofovir in clinical isolates of subtype C than subtype B HIV-1. Careful analysis of these drug resistance profiles concluded that there must be an underlying subtype-specific mechanism that accounted for this difference. The reverse transcriptase (RT) enzymes of subtypes B and C behave in similar fashion in biochemical assays, suggesting that differences in nucleotide sequences between subtypes might be involved. Further investigation revealed that two subtype C-specific silent nucleotide polymorphisms that change the genotype but not the phenotype at positions 64 and 65 of RT are involved and that both polymorphisms had to be present in tandem in order for a selection pattern distinct from that of subtype B virus to occur. An increasing number of reports now indicate that levels of K65R mutations are on the rise, especially in patients infected with subtype C HIV-1 who have failed treatment. These reports are in disagreement with multiple studies conducted on subjects infected with non-B subtypes of HIV-1. Small sample sizes of virological failures, incomplete treatment regimens, mixtures of different subtypes and non-proportional pooling of non-B subtypes may have biased the results of these studies. Despite the elevated numbers of patients infected harbouring K65R mutations, it is nonetheless likely that several factors might limit the proportion of K65R that might otherwise occur.
First, zidovudine is widely used as part of first-line regimens in developing countries with a high prevalence of subtype C and is known to favour the selection of thymidine analogue mutations (TAMs), which antagonize the development of K65R.\textsuperscript{7,16–19} Second, K65R is associated with a loss of viral replicative fitness,\textsuperscript{20} which might compromise the ability of this mutation to be generated. Indeed, phenotypic polymorphisms at TAM sites of HIV-2 have been shown to preclude the development of K65R with nucleoside/nucleotide reverse transcriptase inhibitors (N(t)RTIs) used as single drugs or in combination.\textsuperscript{21} Therefore, we wished to assess the involvement of known silent nucleotide polymorphisms at three different TAM sites in HIV-1 subtype C that, in contrast to those in HIV-2, do not result in a change in phenotype.

Materials and methods

Due to known antagonistic effects between TAMs and K65R,\textsuperscript{16–19} we compared the consensus sequences of subtypes B and C with a focus on sites known to develop TAMs, since nucleotide polymorphisms at such sites might reduce the probability of selection of K65R by increasing the likelihood of selection of other mutations. Three TAM sites harbour single nucleotide polymorphisms at the third position of the codon, i.e. K70K, L210L and K219K, representing differences between subtypes B and C (Figure 1a). At positions 70 and 219, the change is from A to G, whereas a G to A switch occurs at position 210.

To investigate the impact of these silent nucleotide polymorphisms on the preferred development of K65R in subtype C virus, we first generated HIV-1 NL4-3 clones containing single nucleotide mutations at positions 64 and 65 to create viruses behaving as if they were subtype C, i.e. NL4-3 (64/65), as previously described.\textsuperscript{6} Next, we introduced polymorphisms at TAM positions 70, 219 and/or 210 (Figure 1b). These three TAM polymorphism-containing viruses, i.e. NL4-3 (64/65/70), NL4-3 (64/65/70/219) and NL4-3 (64/65/70/219/210), were compared with wild-type NL4-3 and NL4-3 (64/65) in cell culture drug selections with N(t)RTIs under single or combination drug pressure.

Based on known TAM antagonisms toward K65R,\textsuperscript{16–19} all three viruses containing TAM polymorphisms were expected to show an attenuated frequency of K65R selection compared with NL4-3 (64/65) and to behave in an manner more similar to that of wild-type virus, i.e. NL4-3 (wt). Such a result would explain, at a nucleotide level, discrepancies between previous cell culture results with NL4-3 (64/65) in which K65R had been selected at high frequency,\textsuperscript{6} compared with clinical data available from patients infected with subtype C virus who often did not possess K65R at such a high incidence.\textsuperscript{7–9}

In a first round of selections, we included tenofovir, didanosine, stavudine, apricitabine and abacavir as single drugs for 20 weeks or until a first resistance mutation had appeared (Figure 2). We did not include lamivudine, emtricitabine and zidovudine, since no subtype-specific differences are expected with these drugs; i.e. lamivudine and emtricitabine preferentially select for M184I/V mutations, while zidovudine favours the development of TAMs, notably K70R.\textsuperscript{6} Selections with tenofovir resulted in the appearance of K65R in all viruses tested, whereas the four other drugs, i.e. didanosine, stavudine, apricitabine and abacavir, failed to select for K65R with NL4-3 (wt) but did select for K65R when NL4-3 (64/65) was employed, consistent with earlier results.\textsuperscript{6} In contrast, the preferential selection of K65R that was observed with NL4-3 (64/65) was attenuated in more than one-third of the TAM polymorphism-containing viruses that were exposed to didanosine, apricitabine or abacavir but not to stavudine. Interestingly, none of the acquired resistance mutations were TAMs. These single drug selections demonstrate that silent TAM nucleotide polymorphisms may be able to shift the resistance mutation selection pattern away from NL4-3 (64/65), which favours K65R.

In drug combination selections, we subjected the same viruses to tenofovir/emtricitabine, tenofovir/ lamivudine, stavudine/didanosine, abacavir/emtricitabine and abacavir/lamivudine for 20 weeks or until the first resistance mutation had appeared (Figure 2). As expected, abacavir/lamivudine selected for the M184I mutation in all cases, and no subtype differences could be noted.\textsuperscript{6} The selection of K65R in the case of NL4-3 (64/65) was attenuated in many of the TAM polymorphism-containing viruses that were exposed to tenofovir/emtricitabine, tenofovir/lamivudine or abacavir/emtricitabine. In contrast, the combination of stavudine/didanosine continued to select for K65R in all cases with the exception of subtype B virus, i.e. NL4-3 (wt).

These results demonstrate that silent TAM nucleotide polymorphisms may be able to diminish the likelihood of selection of K65R when various N(t)RTI combinations are studied, although not in the case of stavudine/didanosine.

Discussion

These results show that silent nucleotide polymorphisms found at TAM sites can attenuate the tendency of viruses containing the 64/65 subtype C sequence to favour the K65R resistance mutation pathway. The recombinant viruses that were tested developed K65R under drug pressure at high rates, but below the rates of viruses that did not contain subtype C-specific TAM polymorphisms, as previously reported.\textsuperscript{6} These data are consistent with those that might be associated with K65R development in patients infected with subtype C HIV previously treated with TAM-selecting drugs such as zidovudine.\textsuperscript{7–9}

In contrast, the results obtained with stavudine indicated that K65R selection occurred in all circumstances tested, consistent with the results of clinical studies in areas in which subtype C virus is pandemic that have pointed to high rates of K65R in stavudine-treated individuals.\textsuperscript{7–9} Other studies have shown that RNA template sequences that differ between subtype C viruses

\begin{table}[h]
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\begin{tabular}{|c|c|c|}
\hline
\textbf{Drug Combination} & \textbf{K65R Frequency} & \textbf{Conclusion} \\
\hline
Tenofovir/emtricitabine & 100% & \textbf{K65R selected} \\
Tenofovir/lamivudine & 0% & \textbf{K65R not selected} \\
Stavudine/didanosine & 100% & \textbf{K65R selected} \\
Abacavir/emtricitabine & 100% & \textbf{K65R selected} \\
Abacavir/lamivudine & 100% & \textbf{K65R selected} \\
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\end{tabular}
\caption{Results of drug combination selections.}
\end{table}
and those of other subtypes are responsible for these differences in propensity for selection of K65R. None of the TAM polymorphism-containing viruses developed TAMs in the absence of K65R but rather seemed to favour other mutations such as M184I. A possible explanation may lie in the known antagonism between K65R and TAMs, and ultrasensitive assays for the detection of individual mutations that are present at levels below limits of detection may help to resolve this issue.

In summary, the data presented here help to explain the fact that patients who have been treated with zidovudine as part of first-line regimens in developing countries may possess TAMs that antagonize the later development of K65R mutations following subsequent therapy. Other nucleotide polymorphisms may, of course, also militate against the development of K65R, and this should be investigated.

Figure 1. (a) Location of the K65R mutation in HIV-1 subtype B and C sequences. Representation of the sites relevant to this study and the surrounding nucleotide sequences, and comparison of the subtype B and C consensus sequences. All subtype C nucleotides that are different from subtype B sequences are shown in bold. Frequencies of subtype-specific silent nucleotide polymorphisms relevant to this study are shown in percentages that were calculated for each subtype from alignments of complete sequences available from the HIV Sequence Database of Los Alamos National Laboratory. The nucleotide change leading to the K65R mutation is shaded in black and indicated by an arrow. Of note, the underscored poly(A) sequence is shifted in subtype C. (b) Sequences of wild-type and mutated NL4-3 viruses. The sequence of NL4-3 (wt) corresponds to the subtype B consensus sequence in (a). Single nucleotide changes to the sequence in the four mutants are shown in bold. All the mutations introduced have no effect on the phenotype.
Figure 2. Mutational preferences of wild-type and mutated NL4-3 viruses in MT2 cells under single and combination drug pressure with NtRTIs. Viruses: wt denotes wild-type subtype B NL4-3 virus; 64/65, 64/65/70, 64/65/70/219 and 64/65/70/210/219 denote subtype B NL4-3 viruses with subtype C sequences at positions 64, 65, 70, 210 and/or 219. Drug abbreviations: TFV, tenofovir; ddI, didanosine; d4T, stavudine; ATC, apricitabine; ABC, abacavir; FTC, emtricitabine; 3TC, lamivudine. The duration of the study was 20 weeks. The mutations listed were fully developed and unique at 20 weeks or earlier. *Can vary between experiments (M184I or K65R). †The mutation developed later than week 20. All selections for resistance were performed at least three times. Selection of K65R is shown in bold.

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Transparency declarations
None to declare.

References
18. Martin-Carbonero L, Gil P, Garcia-Benayas T et al. Rate of virologic failure and selection of drug resistance mutations using different triple

