

Introduction of the 64/65 Nucleotide Polymorphisms of Subtype C into Subtype B HIV-1 Selects for the K65R Mutational Pathway in Cell Culture

Cédric F. Invernizzi*, Dimitrios Coutsinos, Daniela Moisi, Maureen Oliveira, Bonnie Spira, Bluma G. Brenner and Mark A. Wainberg

McGill University AIDS Centre, Lady Davis Institute, Jewish General Hospital, Montréal, Québec, Canada



✉ Cédric Invernizzi, Ph.D.
3755 Côte-Ste-Catherine, Rm 331
Montréal, QC H3T 1E2, Canada
☎ +1-514-340-8222 ext. 5283
✉ cedric.invernizzi@mail.mcgill.ca

Abstract

Background

Previously, our group showed that subtype C HIV-1 selects more rapidly for K65R than subtype B HIV-1 in cell culture. However, our biochemical studies that compared subtype B and C reverse transcriptase (RT) enzymes did not reveal any major differences that would explain these observations. Recently, we presented subtype-specific differences in fidelity of RT in (+) strand DNA synthesis at the site responsible for the K65R mutation. This novel nucleotide template-based mechanism may therefore be decisive in the selection of the K65R resistance mutation pathway in subtype C, as compared to subtype B. In this study, we analyze this mechanism in more detail and confirm the suggested mutational pathway outcome in cell culture.

Methods

Mechanistic *in vitro* assays: Recombinant subtype B and C HIV-1 RT enzymes were expressed and purified in *E. coli*. Gel-based nucleotide extension assays were used to study DNA synthesis from subtype B and C DNA and RNA templates that spanned the region of the *pol* gene where the mutation K65R occurs. **Selections in cell culture:** NL4-3 (64), NL4-3 (65) and NL4-3 (64/65) plasmids (subtype B with subtype C nucleotide sequence at residues 64 or/and 65) were generated from NL4-3 (wt) plasmid (subtype B) by site-directed mutagenesis. CBMCs and MT2 cells were infected with viruses harvested from 293T cells (MOI 0.01) over 2 h and subsequently washed. Infected cells were seeded and drugs (3TC, FTC, TFV, ABC, d4T and ddI) were added separately or in combination. Selection at a certain drug concentration was defined as complete when in repeated passages the culture consistently peaked at the same interval as the control well without any drugs. Collected culture supernatants were sequenced for genotypic analysis.

Results

***In vitro* assays:** Reverse transcription of subtype B and C RNA templates did not show any differences in (-) strand DNA synthesis when subjected to different RT enzymes. However, (+) strand DNA synthesis revealed different pausing patterns while copying subtype B or C DNA templates. When subtype C RT was employed to synthesize (+) strand DNA from a subtype C template, preferential pausing was observed at the nucleotide position just preceding the position responsible for the AAG to AGG mutation on codon 65, which gives rise to K65R. In contrast, the use of subtype B RT together with a subtype B template reveals a different pattern of pausing sites during (+) strand DNA synthesis. Interestingly, these observed pausing patterns are only template-dependent and are independent of the specific subtype of the RT enzyme used. **Cell culture:** Selections with TFV alone and in combination with 3TC in CBMCs revealed that NL4-3 (64/65) tested in both cases positive for K65R, whereas NL4-3 (wt) acquired M184V with the combination and never developed any mutation with TFV after 35 weeks. In MT2 cells, 3TC and FTC selected for M184V with all four viruses, and TFV selected for K65R in the case of three viral isolates. Interestingly, ABC, ddI and d4T all selected for K65R with NL4-3 (64/65), whereas with the three other viruses ABC selected for M184V, ddI mostly favored L74V/V75I and d4T did not select for any mutations. This trend of the double mutant NL4-3 (64/65) behaving differently from the three other viruses and favoring the development of K65R, was also confirmed with the d4T+ddI combination, as well as combinations containing either ABC or TFV.

Conclusions

These mechanistic biochemical data and resistance selections all demonstrate that nucleotide sequence changes at both positions 64 and 65 within HIV-1 subtype C, distinct from subtype B, redirect the selection pattern with multiple N(t)RTIs toward the K65R resistance mutation pathway, which is not the case for single changes at either of these positions. Hence, the 64/65 changes in subtype C are required in tandem and represent signature polymorphisms for the development of K65R in this subtype in regard to N(t)RTI selective pressure.

Acknowledgements

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The Mechanism:

Selection of K65R Is Facilitated in Subtype C

In Vitro (-) Strand DNA Synthesis from (+) Strand RNA of the 65 Region of *pol*

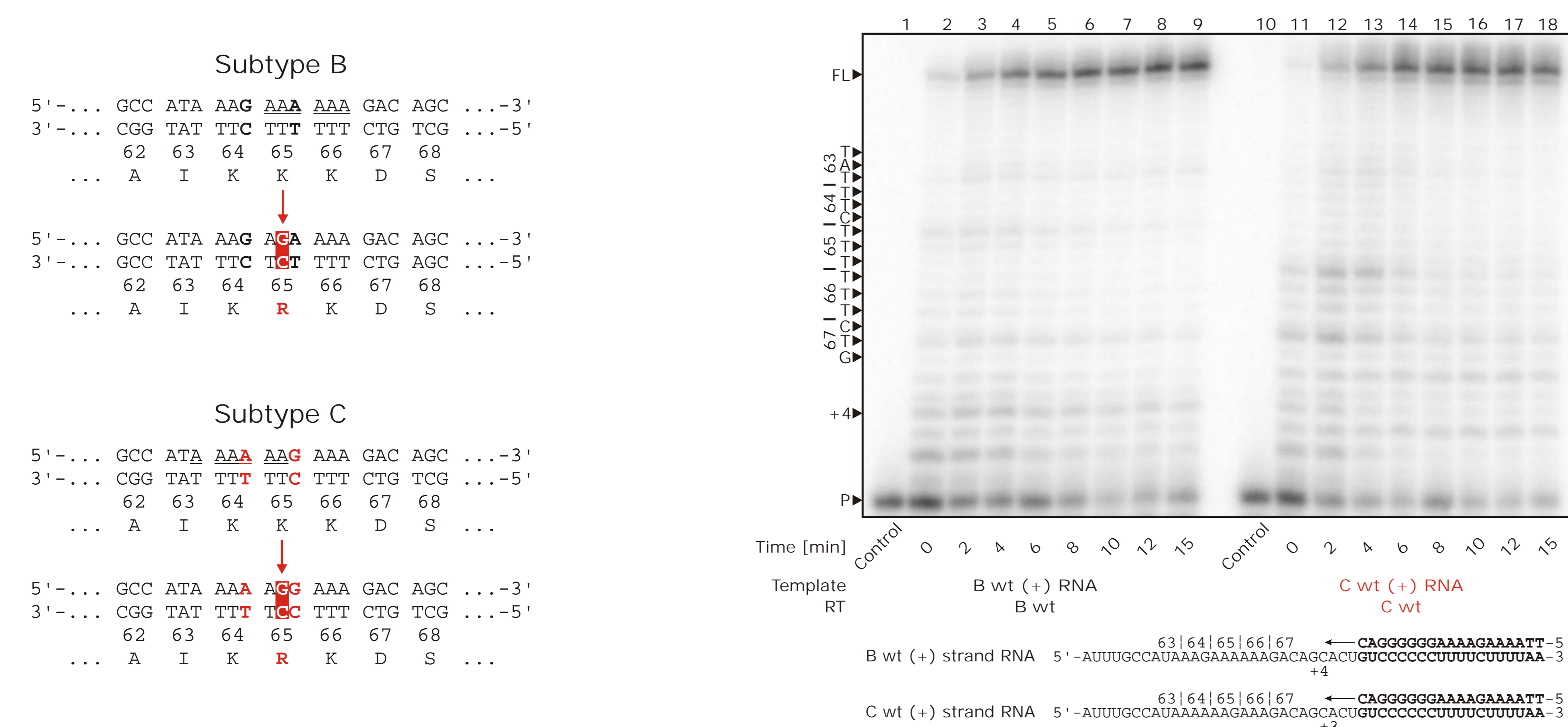


Figure 1. *Left:* Comparison of genomic sequences of subtype B and C HIV-1. Indicated is the double-stranded DNA of the *pol* gene that spans codons 62 to 68. In bold are the bases that differ between the two subtypes. Highlighted in red is the site of acquisition of the K65R mutation due to an A to G change. Underscored is the adenine stretch, which ends at the bolded G in subtype C, whereas the stretch is shifted towards codon 66 in subtype B. *Right:* Lanes 1 through 9 depict (-) strand DNA synthesis with subtype B wild-type RT on a subtype B (+) strand RNA template and lanes 10 through 18 depict (-) strand DNA synthesis with subtype C wild-type RT on a subtype C (+) strand RNA template. The data shows no pausing for either enzymes or templates in the 63 to 67 region. Minor pausing attributable to initiation is seen at the +3 and +4 positions. Templates and primers used in the reaction are shown at the bottom. In bold are the primers and primer annealing regions of the templates.

No differences between subtype B and subtype C are revealed in the (-) strand DNA synthesis.

In Vitro (+) Strand DNA Synthesis from (-) Strand DNA of the 65 Region of *pol*

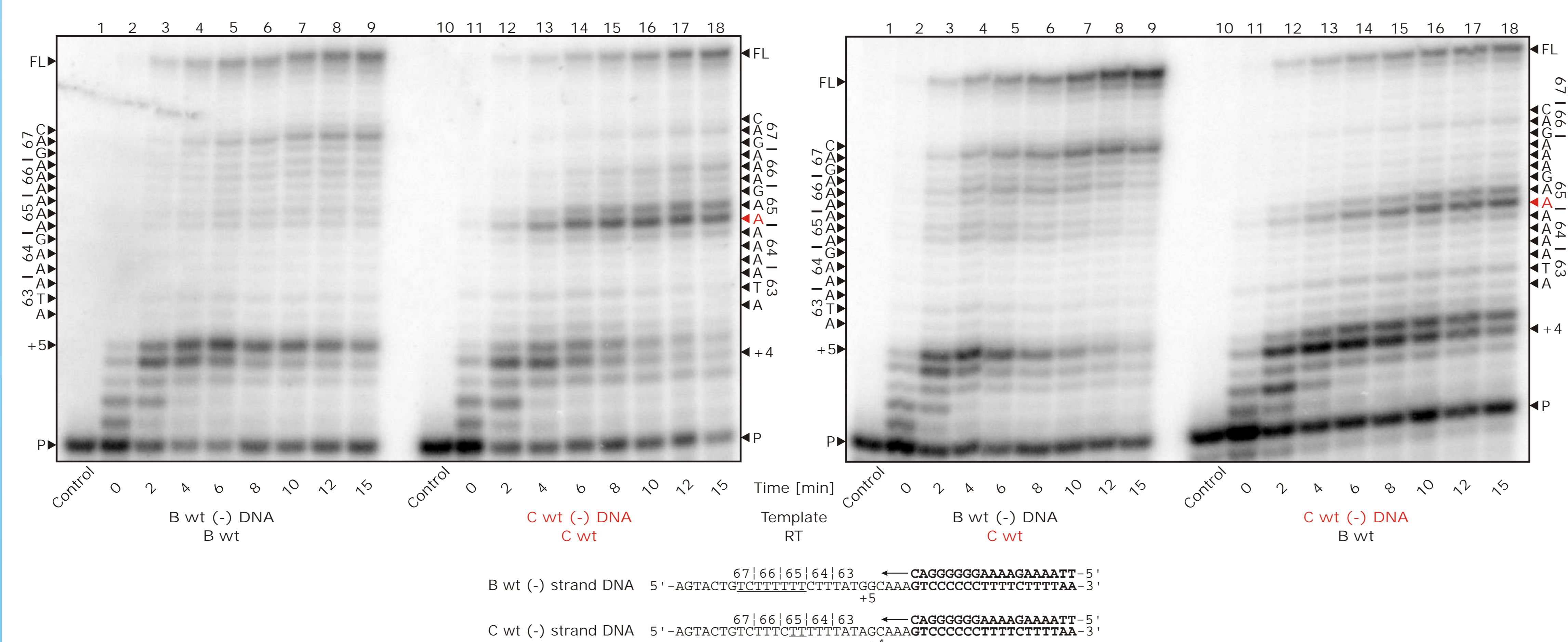


Figure 2. *Left:* Lanes 1 through 9 depict (+) strand DNA synthesis with subtype B wild-type RT on a subtype B template. A ladder of pausing is seen throughout the 65, 66 and 67 codons. Pausing at position +5 is attributable to early stage initiation events. Lanes 10 through 18 depict (+) strand DNA synthesis with subtype C wild-type RT on a subtype C template. Strong pausing is seen at residue 65 and is associated to the more rapid development of the K65R mutation in subtype C. Pausing at position +4 is attributable to early stage initiation events. *Right:* Lanes 1 through 9 depict (+) strand DNA synthesis with subtype C wild-type RT on a subtype B template and lanes 10 through 18 depict (+) strand DNA synthesis with subtype B wild-type RT on a subtype C template, both showing similar results as seen on the left. Therefore, the pausing events are independent of the RT enzymes and depend solely on the sequences used. Templates and primers used in the reaction are shown at the bottom. In bold are the primers and primer annealing regions of the templates and underlined are the regions where pausing is seen.

Only subtype C exhibits strong pausing associated to the more rapid development of K65R in the (+) strand DNA synthesis, which is independent of the subtype of the RT used.

The Effect:

K65R Is Selected Faster and More Frequent in Subtype C

Resistance Mutation Selection in Cord Blood Mononuclear Cells (CBMCs)

NL4-3 (wt)

5'... AAG AAA ...-3'
3'... TTC TTT ...-5'
64 65
... K K ...
Subtype B sequence

Drugs	Virus			
	NL4-3 (wt)		NL4-3 (64/65)	
	Mutation	Week	Mutation	Week
TFV	none	>35	K65R	25
TFV + 3TC	M184V	15	K65R	20

NL4-3 (64/65)

5'... AAA AAG ...-3'
3'... TTT TTC ...-5'
64 65
... K K ...
Subtype C sequence at positions 64/65 in a subtype B backbone

Figure 3. Differential time to emergence of resistance in wild-type and mutant NL4-3 viruses in cord blood mononuclear cells (CBMCs) treated with different N(t)RTIs. A significant temporal difference between the subtype B NL4-3 (wt) and the subtype C-like NL4-3 (64/65) viruses was apparent when treated with TFV. Furthermore, K65R was only selected in NL4-3 (64/65) when using the TFV + 3TC combination, whereas NL4-3 (wt) favored development of M184V. On the left, the sequence at positions 64 and 65 is shown for the subtype B virus NL4-3 (wt), the sequence matching NL4-3 (64/65) is shown on the right. Duration of study: 35 weeks.

Subtype C selects more rapidly for K65R than subtype B.

Resistance Mutation Selection in MT2 Cells

NL4-3 (wt)

5'... AAG AAA ...-3'
3'... TTC TTT ...-5'
64 65
... K K ...
Subtype B sequence

NL4-3 (64)

5'... AAA AAA ...-3'
3'... TTT TTT ...-5'
64 65
... K K ...
Subtype C sequence at position 64 in a subtype B backbone

NL4-3 (65)

5'... AAG AAG ...-3'
3'... TTC TTC ...-5'
64 65
... K K ...
Subtype C sequence at position 65 in a subtype B backbone

NL4-3 (64/65)

5'... AAA AAG ...-3'
3'... TTT TTC ...-5'
64 65
... K K ...
Subtype C sequence at positions 64/65 in a subtype B backbone

Drugs	Virus			
	NL4-3 (wt)	NL4-3 (64)	NL4-3 (65)	NL4-3 (64/65)
3TC	M184I	M184I	M184I	M184I
FTC	M184I	M184I	M184I	M184I
ABC	M184I	M184I	M184I	K65R
ddI	L74V	M184I	V75I	K65R
d4T	none	none	none	K65R
TFV	K65R	K65R	none	K65R
ABC + 3TC	M184I	M184I	M184I	M184I
ABC + FTC	M184I	M184I	M184I	K65R
d4T + ddI	V75I	V75I	none	K65R
TFV + 3TC	K65R / M184I / T215F	D67E / K70E / M184I	K65R / D67G / M184I	K65R
TFV + FTC	K65R	K65R	K65N	K65R

Figure 4. Mutational preference of wild-type and mutant NL4-3 viruses in MT2 cells treated with different N(t)RTIs. Only the subtype C-like virus NL4-3 (64/65) exhibited a preference for the development of K65R with many single drugs and combinations that included TFV, ABC, d4T and ddI. Subtype B virus NL4-3 (wt) and the two viruses containing only single mutations NL4-3 (64) and NL4-3 (65) selected mostly for other mutations than K65R. These results show that both changes at positions 64 and 65 are required to shift the original subtype B virus to a subtype C-like behavior in terms of K65R selection. At the top, the sequences at positions 64 and 65 are shown for the four viruses. NL4-3 (wt) denotes subtype B virus, whereas NL4-3 (64), NL4-3 (65) and NL4-3 (64/65) denote subtype B viruses with subtype C sequences at positions 64 or/and 65. Duration of study: 20 passages.

Subtype C favors selection of K65R, which is triggered by the two polymorphisms found at positions 64 and 65.

Conclusions

The decreased transcription fidelity of HIV-1 RT, when copying HIV-1 subtype C nucleic acid template at RT positions 64 through 66, results in the preferred selection of the K65R resistance mutation pathway. Hence, in subtype C, template factors, in addition to drug pressure, increase the probability of developing K65R. The specific K64K and K65K sequences in subtype C should be considered as signature polymorphisms for the development of K65R. These results urge for the analysis of resistance mechanisms to be studied in all HIV-1 subtypes.

The sequence drives subtype C into the K65R resistance mutation pathway