Infection with the frequently transmitted HIV-1 M41L variant has no influence on selection of tenofovir resistance

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Objectives: In ~10% of newly diagnosed HIV-1 patients, drug-resistant viral variants are detected. In such transmitted HIV-1 variants, the thymidine analogue mutation (TAM) M41L is frequently observed as a single resistance mutation and these viral variants often belong to phylogenetic transmission clusters. The presence of at least three TAMs, in particular patterns with M41L/L210W, impairs the efficacy of the extensively used drug tenofovir. We investigated whether the presence of a single M41L mutation at baseline influences the selection of resistance to tenofovir and emtricitabine in vitro and in vivo.

Methods: The impact of M41L on the development of drug resistance to tenofovir and emtricitabine was determined by extensive in vitro selection experiments and investigation of the virological outcome of patients on a first-line regimen.

Results: The presence of a single M41L mutation did not influence the selected mutational profile or the genetic barrier to resistance to tenofovir and/or emtricitabine during long-term in vitro selection experiments. In vivo, virological outcome of first-line regimens containing tenofovir and emtricitabine was comparable between patients diagnosed with HIV-1 harbouring M41L (n=17, 16 were part of one transmission cluster) and WT virus (n=248).

Conclusions: Detection of a single M41L reverse transcriptase mutation at baseline did not influence the development of resistance in vitro or virological outcome on tenofovir-containing regimens in patients belonging to a large transmission cluster. Our results indicate that a high genetic barrier regimen may not be required when patients are diagnosed with HIV variants containing a single M41L mutation in reverse transcriptase.

Keywords: transmission, drug resistance, emtricitabine, Truvada®, Atripla®

Introduction

Approximately 10% of all newly diagnosed HIV-1 patients in Europe and the USA present with drug-resistant HIV-1, often harbouring only one drug resistance mutation.1–3 Such viral variants can be derived from patients experiencing therapy failure, in whom extensive drug resistance profiles are regularly observed.4 After transmission of these drug-resistant HIV-1 variants, improvement of replication capacity can drive (partial) reversion of the resistance profile.5 Meanwhile, the originally transmitted, more extensively resistant variant is archived and may potentially re-emerge after treatment initiation. Alternatively, drug-resistant HIV-1 variants may be found in untreated patients who themselves are infected with these variants by onward transmission.6,7 In these patients, the risk of archived extensively drug-resistant HIV variants is limited.8 Therefore, insight into the source of transmitted drug-resistant HIV-1 variants may help to distinguish between patients with and without archived extensively drug-resistant HIV variants that are not detected at the moment of diagnosis but may endanger therapy efficacy. Phylogenetic analysis may provide relevant insights regarding transmission networks and such onward transmission of drug-resistant HIV-1.8,9

Among patients diagnosed with drug-resistant HIV-1, the reverse transcriptase (RT) M41L mutation is one of the most frequently observed transmitted resistance mutations with a prevalence of 20%–31%.1–3 M41L belongs to the group of thymidine
analogue mutations (TAMs) (M41L, D67N, K70R, L210W, T215Y/F and K219E/Q) that are selected by the NRTIs zidovudine and stavudine, which have been used extensively in the past.\textsuperscript{10,11} TAMs not only decrease susceptibility to zidovudine and stavudine, but also lead to cross-resistance to other NRTIs such as tenofovir. Clinical studies have shown that the presence of three or more TAMs, in particular patterns with M41L or L210W, impairs the virological efficacy of tenofovir.\textsuperscript{12–14} In patients infected with WT virus, TAMs are rarely observed upon virological failure of tenofovir-containing first-line regimens. If resistance to tenofovir is selected in these cases, mutations K65R and/or K70E are primarily detected.\textsuperscript{15–19}

Little is known about the biological and clinical consequences of transmitted HIV variants with a single M41L in relation to the use of tenofovir. The presence of a single M41L does not lead to an evidently decreased susceptibility to tenofovir in vitro.\textsuperscript{20} However, it remains unknown whether the presence of M41L decreases the genetic barrier to resistance or skews the resistance pathway towards accumulation of TAMs in case of virological failure. The tenofovir and emtricitabine coformulation Truvada\textsuperscript{21} is the most frequently used first-line NRTI backbone in clinical practice and is often combined with low genetic barrier drugs such as efavirenz, nevirapine or raltegravir. Concerns for rapid selection of TAMs or the presence of more extensively drug-resistant HIV-1 variants in the quasispecies may result in the choice of more complex initial regimens with a higher genetic barrier. However, complex regimens often lead to an increased pill burden, elevated costs and may also increase toxicity. Insight into the effect of M41L at baseline on the selection of resistance by tenofovir/emtricitabine may ease first-line treatment choices for patients. Furthermore, as tenofovir/emtricitabine is also used for pre-exposure prophylaxis (PrEP) to prevent HIV-1 transmission in high-risk individuals, it is essential to gain insight into whether the efficacy of tenofovir/emtricitabine PrEP could be affected by circulating viruses with the M41L mutation. We investigated the impact of a single M41L at baseline by studying the selection of drug resistance to tenofovir and/or emtricitabine in vitro and therapy response in vivo.

Methods

Virus panel

To compare in vitro development of drug resistance to tenofovir and emtricitabine, three subtype B M41L viruses were generated: two patient-derived M41L viruses (pM41L-1 and -2) and a site-directed mutant (SDM-M41L). The clinical isolate resulting in pM41L-1 was selected from a large transmission cluster. To ensure results were not specific for the genetic background within that particular cluster, a second isolate with a single M41L that did not cluster was chosen resulting in pM41L-2 (data not shown, for methods see below in the Phylogenetic analysis section). The N-terminus of RT (amino acids 25–314) of both patient-derived viruses was amplified, digested and ligated using the previously described NRT vector system.\textsuperscript{21} The M41L mutation was introduced by site-directed mutagenesis into WT HXB2 (primer M41L-RT: 5′-ATT TGT ACA GAG CTG GAA AAG GAA G-3′, nucleotides 2658–2682) using the aforementioned vector system. Clones were verified by sequence analysis. Virus stocks were obtained by transfection of HEK293T cells with plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The TCID\textsubscript{50} was determined by endpoint dilution in MT2 cells (baseline viruses) or SupT1 cells (in vitro selection viruses).

In vitro drug selection and evolution

A chequerboard approach was used to determine the drug concentration of tenofovir in combination with emtricitabine. SupT1 cells were inoculated with WT (HXB2) at an moi of 0.001 for 2 h before a range of emtricitabine (0–0.9 μM) and tenofovir (0–2.0 μM) concentrations were added. Cells were monitored regularly for CPE. Twice a week, half of the culture was replaced by culture medium with the same drug concentrations. The highest drug concentrations of the cultures in which CPE developed after 2 weeks were used in the first passage of the in vitro selection experiments. The initial emtricitabine concentration in the in vitro selection experiments of 0.011 μM emtricitabine was below the 50% effective concentration (EC\textsubscript{50}) in SupT1 cells, while the initial tenofovir concentration of 2 μM tenofovir was above the EC\textsubscript{50}.

In vitro selections were started in parallel 5-fold cultures in which 2×10\textsuperscript{5} SupT1 cells were infected with an moi of 0.001. The initial drug concentration was 0.011 μM emtricitabine, 4 μM tenofovir or 0.011 μM emtricitabine + 2 μM tenofovir. Cultures were regularly monitored for CPE and replenished twice a week with fresh culture medium supplemented with drug(s). When full-blown CPE was observed, cell-free supernatant was harvested and stored at −80 °C. Subsequent passages were started by infecting 2×10\textsuperscript{5} SupT1 cells with 10–50 μL of virus of the previous passage. Drug concentrations were gradually increased to 0.352 μM emtricitabine, 32 μM tenofovir or 0.132 μM emtricitabine + 24 μM tenofovir. After five (tenofovir and emtricitabine) or six passages (emtricitabine), viral RNA was isolated for genotypic analysis of the entire RT gene. Increased drug levels of tenofovir and/or emtricitabine repeatedly inhibited viral replication in several of the WT and M41L cultures, which were therefore discontinued.

Phenotypic drug susceptibility

Viruses resulting from the in vitro selection experiments were expanded by infecting 2×10\textsuperscript{6} SupT1 cells in the absence of drugs. The in vitro drug susceptibility of the virus panel was determined using a multiple cycle assay.\textsuperscript{22} Resistance was defined as the fold increase in EC\textsubscript{50} compared with the WT laboratory strain HXB2. At least two independent experiments were performed to calculate phenotypic drug resistance. The susceptibility of all viruses with a single M41L to tenofovir and emtricitabine was comparable to WT virus (tenofovir EC\textsubscript{50}: 1.9 μM, emtricitabine EC\textsubscript{50}: 1.6 μM in MT2 cells) (Table S1, available as Supplementary data at JAC Online).

Study population

We investigated individuals who were newly diagnosed with HIV-1 in two centres in the Netherlands: University Medical Center Utrecht and Rijnstate Hospital in Arnhem. All patients were participants of the Dutch ATHENA Cohort, which has been approved by the local and national institutional review boards. Inclusion criteria were as follows: (i) baseline genotypic resistance test generated between 2007 and 2010; (ii) initiation of first-line therapy with a tenofovir/emtricitabine-containing regimen; (iii) ≥1 year of clinical follow-up after therapy initiation; and (iv) detection of WT virus or a variant harbouring M41L as the only resistance mutation according to the IAS-USA list of resistance mutations.\textsuperscript{23} Virus subtypes were determined using REGAv3.\textsuperscript{24}

Clinical outcome

Low genetic barrier ART regimens were defined as tenofovir/emtricitabine with an NNRTI or raltegravir as a third drug. Regimens that contained either a boosted protease inhibitor or more than three drugs were classified as high genetic barrier regimens. Virological failure during the first year of treatment was defined as follows: (i) not achieving HIV RNA <50 copies/mL or (ii) HIV RNA >200 copies/mL after achieving HIV RNA <50 copies/mL. The
analyses were conducted on an intent-to-treat basis. Statistical analysis was conducted using Fisher’s exact test (categorical data) or the Mann–Whitney U-test (numerical data) in SPSS Statistics 19 (IBM).

**Phylogenetic analysis**

Baseline population sequences of the HIV pol gene from all patients included in the cohort analysis were aligned using ClustalW. To avoid bias caused by the presence of M41L, amino acid position 41 in RT was removed from the alignment. A maximum-likelihood phylogenetic tree was calculated with MEGA 5.2.1 using the general time-reversing model of nucleotide substitution with Γ-distributed rate heterogeneity with 1000 bootstrap replicates.

**Results**

**Impact of M41L on in vitro selection of resistance to tenofovir and emtricitabine**

To assess the impact of the M41L mutation on the acquisition of resistance to tenofovir and emtricitabine, extensive in vitro selection experiments were performed. We compared WT virus HXB2, a site-directed mutant with M41L in the genetic background of HXB2 (SDM-M41L) and two patient-derived viruses containing M41L as the only major resistance-related mutation (pM41L-1 and -2). One patient-derived virus, pM41L-1, was selected as a representative virus from a large transmission cluster (Figure 1). A second, genetically distinct patient-derived virus, pM41L-2, did not cluster with any known sequence (data not shown).

During these experiments, the drug pressure was gradually increased and comparable selection rates were observed for WT and SDM-M41L (data not shown), suggesting that M41L does not affect the rate of selection of resistance mutations against tenofovir and emtricitabine. After five or six passages, the entire RT gene was sequenced to investigate the selected resistance profile.

Under tenofovir pressure alone (Table 1, Table S1 and Table S2), the M41L mutation persisted in 5 out of 6 cultures, while no TAMs were selected. For WT virus as well as M41L-containing viruses, the dominantly selected resistance profile was K65R. The single presence of K65R in WT virus background resulted in a 21-fold increase in the EC50 of tenofovir compared with WT virus and a 12- to 15-fold increase when K65R appeared in the background of M41L. D67G was selected by two M41L-containing viruses: once solitary and once accompanied by K65R. The combination of M41L and D67G resulted in a 4-fold increase in the EC50 of tenofovir.

Under single emtricitabine pressure (Table 1, Table S1 and Table S3), M41L persisted in all cultures and no selection of TAMs was observed. Again, the resistance patterns of WT and M41L-containing viruses were very similar. In the majority of cultures (15/17), mutations at position 184 were selected. M184V conferred a very high increase in the EC50 of emtricitabine (>300-fold) independently of the presence of M41L. The average increase in the EC50 of emtricitabine for a virus harbouring M184I was 120-fold. In two cultures, K65R was selected: once as the only mutation in WT background and once in combination with M184V by pM41L-2. The effect of a single K65R on the fold change in the EC50 of emtricitabine was comparable in the absence and presence of M41L (19- to 21-fold for WT virus and SDM-M41L).

Although all viruses were able to replicate under tenofovir pressure in combination with emtricitabine during initial passages, viral replication was completely inhibited in the majority of cultures during later passages. Only four cultures could be successfully propagated under high drug pressure and in all four experiments K65R was selected (Table 1 and Table S4).

**In vitro evolution of M41L variants**

To ensure that the observed selection of drug resistance mutations was specifically caused by the selective drug pressure of tenofovir and/or emtricitabine, all viruses were propagated in 5-fold for 10 passages in the absence of drugs (Table S5). This revealed persistence of M41L in all cultures for 10 passages. No additional resistance-related mutations were selected in any of these cultures. Interestingly, in all cultures changes were observed at polymorphic positions. Selection of additional polymorphisms may be a result of adaptation to in vitro culture in a T cell line, the absence of immunological pressure or can be related to restoration of the viral replicative capacity. Indeed, position 162 in RT has been described to have a compensatory role increasing the replicative capacity of viruses harbouring M41L. Both patient-derived viruses had a polymorphism at position 162 and changes at this position were observed in 4/13 cultures (Table S5).

**Impact of M41L on first-line treatment with tenofovir and emtricitabine**

To investigate the possible influence of M41L on the efficacy of tenofovir/emtricitabine-based therapy, we compared the therapy outcome of patients diagnosed with an HIV-1 M41L variant with patients harbouring WT HIV-1. All patients from two centres in the Netherlands (University Medical Center Utrecht and Rijnstate Hospital in Arnhem) from whom a baseline genotypic analysis was performed between 2007 and 2010 were enrolled in this study. Of 530 patients, 279 initiated tenofovir/emtricitabine-containing first-line regimens and had > 1 year of clinical follow-up. Of these, 31 patients were diagnosed with drug-resistant HIV-1, of which 17 patients had a variant harbouring only M41L. The remaining 248 patients were diagnosed with WT virus. The latter two groups were included in the analysis (Figure 2).

**Baseline characteristics of included patients**

Of the 265 included patients, 90% were male. The main transmission route was MSM (72%). Most patients were infected with HIV-1 subtype B (80%). At baseline, the median CD4 count was 290 cells/μl and median log plasma HIV RNA was 5.1 copies/ml. Except for a slightly higher HIV RNA (5.5 versus 5.1, P=0.033), the baseline characteristics of patients diagnosed with an M41L variant were comparable to patients with a WT virus (Table 2).

Phylogenetic analysis of the viral sequences isolated from all included patients (n=265) revealed that 16/17 variants harbouring M41L belonged to a large transmission cluster (bootstrap value 98%). This indicates that these patients are part of a transmission network with circulating HIV variants harbouring M41L as a single drug resistance mutation. These 16 clustering viruses belonged to HIV-1 subtype B, whereas the non-clustering virus was subtype C. Although the two participating centres are located in distinct geographic regions of the Netherlands, viruses from...
both centres frequently clustered, indicating highly interlacing epi-
demics (Figure 1).

**Clinical impact of transmitted HIV-1 variants
harbouring M41L**

The majority of included patients who initiated a tenofovir/
emtricitabine-containing therapy continued the initial first-line
regimen during follow-up. Only 23/265 patients (8.7%) (tempor-
arily) stopped tenofovir/emtricitabine treatment or switched to
other NRTIs, mainly due to toxicity ($n=7$) or because the patient
decided to discontinue treatment ($n=6$). The presence of M41L, if
known at the time of therapy selection, regularly shifted therapy
choice towards a higher genetic barrier regimen. Indeed, a com-
parison of initial therapy regimens revealed that patients
diagnosed with an M41L variant received a high genetic barrier
regimen significantly more often than patients diagnosed with
WT virus [$11/17$ (64.7%) versus $98/248$ (39.5%), Fisher’s exact
test: $P=0.044$].

The rate of virological failure during the first year of treatment
was comparable between patients diagnosed with an M41L vari-
ant or WT virus [$1/17$ (5.9%) versus $12/236$ (5.1%), $P=0.586$].
Interestingly, none of the patients diagnosed with M41L variants
who initiated a low genetic barrier regimen experienced viro-
logical failure, which was not statistically different from patients
infected with WT virus on a low genetic barrier regimen [$0/6$
(0.0%) versus $7/150$ (4.7%), $P=1.000$]. In the single patient
infected with an M41L variant who experienced therapy failure
on a high genetic barrier regimen consisting of tenofovir, emtrici-
tabine and ritonavir-boosted atazanavir, resistance testing did not

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**Figure 1.** Maximum-likelihood phylogenetic tree of baseline sequences. The first available sequence of all patients included in the cohort analysis ($n=265$) was used to calculate a maximum-likelihood tree. Sequences from patients from Utrecht Medical Center are indicated with triangles and sequences from patients from Rijnstate Hospital are indicated with circles. WT sequences are indicated with open symbols and sequences harbouring M41L in RT are indicated with filled symbols. The arrow indicates the virus used for pM41L-1.
reveal selection of additional NRTI mutations. For 7 of the 12 patients diagnosed with WT virus, genotypic resistance analysis was successfully performed during virological failure, which revealed no selection of TAMs, K65R or K70E. In one patient who received a high genetic barrier regimen, an NRTI mutation (M184V) was selected. In another patient treated with a low

Table 1. Resistance patterns and drug susceptibility after in vitro selections

<table>
<thead>
<tr>
<th></th>
<th>WT (number of cultures)</th>
<th>Fold increase in EC_{50} compared with WT HIV</th>
<th>M41L (number of cultures)</th>
<th>Fold increase in EC_{50} compared with WT HIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFV</td>
<td>K65R (2/2)</td>
<td>21×</td>
<td>K65R (5/6)</td>
<td>12–15×</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D67G (2/6)</td>
<td>4×</td>
</tr>
<tr>
<td>FTC</td>
<td>M184I/V (4/5)</td>
<td>120 to &gt;300×</td>
<td>M184I/V/T (11/12)</td>
<td>65 to &gt;300×</td>
</tr>
<tr>
<td></td>
<td>K65R (1/5)</td>
<td>21×</td>
<td>K65R (1/12)</td>
<td>10–20×</td>
</tr>
<tr>
<td></td>
<td>D67G (1/5)</td>
<td>ND</td>
<td>D67G (1/2)</td>
<td>5×</td>
</tr>
<tr>
<td>TFV + FTC</td>
<td></td>
<td></td>
<td>K65R (4/4)</td>
<td>ND</td>
</tr>
</tbody>
</table>

TFV, tenofovir; FTC, emtricitabine; ND, not determined.
The fold change in EC_{50} compared with WT virus was determined in at least two independent experiments. More extensive susceptibility data can be found in Table S1.

As we were unable to propagate WT virus in the presence of a combination of TFV and FTC, no data available.

Figure 2. Inclusion of patients and clinical outcome of cohort analysis. TFV, tenofovir; M41L, variant harbouring M41L as only resistance mutation; LGB, low genetic barrier regimen (tenofovir and emtricitabine with efavirenz, nevirapine or raltegravir); HGB, high genetic barrier regimen (tenofovir and emtricitabine with a boosted protease inhibitor or regimens containing more than three drugs); VF, virological failure.

reveal selection of additional NRTI mutations. For 7 of the 12 patients diagnosed with WT virus, genotypic resistance analysis was successfully performed during virological failure, which revealed no selection of TAMs, K65R or K70E. In one patient who received a high genetic barrier regimen, an NRTI mutation (M184V) was selected. In another patient treated with a low
The P value was calculated for characteristics of patients diagnosed with WT versus M41L variant using Fisher’s exact test (categorical data) or Mann–Whitney U-test (numerical data).

The most frequently observed mutation in baseline genotypes of newly diagnosed patients and the influence of this frequently transmitted variant had not been elucidated.

It was previously observed that the presence of a complex TAM profile was negatively associated with the presence of K65R. Indeed, single-genome sequencing demonstrated that TAM profiles were rarely observed on the same viral genome as K65R, implying a detrimental impact of this combination of mutations. Interestingly, in our experiments the presence of a single M41L did not prevent the selection of K65R, as we observed similar rates of selection of K65R for HIV with and without M41L. It has been shown that the presence of multiple TAMs decreases the ability of K65R to discriminate between NRTIs and the natural nucleoside, resulting in a decreased level of resistance. In line with that study, our data indicate that the presence of a single M41L also decreases the level of resistance to tenofovir caused by K65R (Table 1 and Table S1).

Recent evidence indicates that resistance or compensatory mutations related to TAMs or M184 variants can also be selected in the connection domain and RNaseH. Therefore, a possible limitation of this study is that only the N-terminus of RT was used for the generation of patient-derived viruses. However, sequence analysis of the entire RT gene revealed that mutations were selected in the C-terminus in only a minority of the cultures and no mutations described to be associated with therapy were observed.

In addition to the extensive in vitro experiments, we investigated the impact of HIV M41L on therapy outcome. In our cohort of 265 patients, 17 patients were diagnosed with an HIV-1 variant harbouring a single M41L mutation, of whom 16 belonged to a large phylogenetic cluster. Phylogenetic clustering is indicative of circulation of HIV-1 within a transmission network and limits the risk of initial transmission of a more extensively resistant HIV that reverted in plasma.

Although these are relatively small numbers, this is, to our knowledge, the largest group of patients described with a similar transmitted mutation for whom outcome data are available. Of these 17 patients, 16 achieved and maintained a suppressed
Influence of M41L on tenofovir drug resistance

HIV RNA during the first year of therapy, which is similar to the virological outcome of patients infected with WT virus. A potential limitation is that the viruses of patients diagnosed with HIV M41L variant have a limited genetic diversity. However, this particular variant is often encountered in clinical practice in several countries. Moreover, none of the three genetically distinct M41L-containing HIV-1 variants in our extensive in vitro experiments selected for any TAMs. Combined, these observations suggest that our results are broadly applicable.

Our findings suggest that a tenofovir/emtricitabine-containing regimen can be efficacious for treatment of HIV variants harbouring a single M41L mutation, especially when phylogenetic analysis indicates that the virus belongs to a transmission cluster.

Our data have important clinical implications. Our in vitro and clinical data demonstrate that low genetic barrier regimens may be effective in treating patients diagnosed with a single M41L. This is especially true when the presence of additional TAMs in the quasispecies is unlikely based on clinical or phylogenetic evidence. Additionally, M41L did not skew the virus towards selection of multiple TAMs. This suggests that the presence of a single M41L does not affect the genetic barrier towards selection of resistance to tenofovir and/or emtricitabine. Furthermore, our data indicate that the presence of a single M41L does not affect the efficacy of tenofovir/emtricitabine as PrEP or impact the potential selection of additional resistance mutations under PrEP. Taken together, these results suggest that initiation of a high genetic barrier regimen may not be required when patients are infected with a drug-resistant strain containing M41L as the only drug resistance mutation.

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Transparency declarations

None to declare.

Author contributions

A. M. J. W. and M. N. designed the study and laboratory experiments. C. A. B. B. was involved in the study design. M. P. conducted the experiments and the majority of the analyses. A. v. L. was involved in the analysis and database management. T. M., N. L. and C. R. were responsible for the clinical data and the interpretation of these data in the study. A. M. J. W., M. N. and M. P. wrote the manuscript and the other authors contributed to the manuscript. All authors read and approved the final manuscript.

Supplementary data

Tables S1 to S5 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


