Maraviroc treatment in non-R5-HIV-1-infected patients results in the selection of extreme CXCR4-using variants with limited effect on the total viral setpoint

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Objectives: Using deep sequencing methods, we intensively investigated the selective pressure of maraviroc on the viral population in four patients with dual/mixed HIV-1 experiencing treatment failure.

Methods: Patients received maraviroc add-on therapy (n = 4). Tropism was determined by Monogram’s Trofile assay and/or ‘deep’ sequencing. Longitudinal ‘deep’ sequence analysis used triplicate HIV V3 RT-PCR on plasma samples. Sequences were interpreted using the geno2phenocoreceptor algorithm with a 3.5% false-positive rate (FPR) cut-off.

Results: Patients had a median viral load of 4.7 log10 HIV RNA copies/mL with a median of 24% chemokine (C-X-C motif) receptor 4 (CXCR4)-using virus at baseline. Following maraviroc exposure, the chemokine (C-C motif) receptor 5 (CCR5)-using virus (R5) plasma viral load decreased by at least 1 log10, and only non-R5 variants with extremely low FPR values predominated after 21 days. Virus with an FPR ≤1.8% accounted for more than 90% of the circulating virus, having expanded to occupy the ‘space’ left by the suppression of R5 variants. Population genetic estimates of viral fitness in the presence of maraviroc showed a steep rise around an FPR value of 2%.

Conclusions: Longitudinal analysis of independent R5 and non-R5 HIV populations shows that maraviroc selects viruses with an extremely low FPR, implying that the antiviral activity of maraviroc may extend to a broader range of HIV variants than previously suspected.

Keywords: V3 loop, genotyping, 454 deep sequencing, dual/mixed tropism

Introduction

HIV-1 infection is dependent on viral entry into a susceptible host cell. Entry is mediated by the binding of virus to the host CD4 receptor and one of two chemokine co-receptors present on the cell surface—either chemokine (C-C motif) receptor 5 (CCR5) or chemokine (C-X-C motif) receptor 4 (CXCR4).1-3 The majority of new infections appear to be due to CCR5-using ‘R5’ virus, which continues to predominate during the course of the infection.4,5 As time progresses however, ~50% of patients with HIV subtype B have a shift in their viral population to include a growing amount of CXCR4-using ‘X4’ virus, which is strongly correlated with rapid disease progression.5-7 Many patients with X4 virus have a viral population that contains both X4 and R5 virus (M; mixed infection). In some cases, the virus may also be able to use both co-receptors (D; dual infection).8,9 For the purposes of this paper, both X4 and dual/mixed (D/M) populations will be referred to as ‘non-R5’.

The co-receptor that HIV uses to enter the host cell (‘tropism’) is largely but not entirely dependent on the characteristics of the V3-loop region within the gp120 protein encoded by the env gene.10,11 The V3-loop region is usually 35 amino acid residues in size and bookended by cysteine residues between which a disulphide bridge forms, resulting in the loop structure.12 Particular mutations have been associated with CXCR4 co-receptor use, including the substitution of basic residues at V3-loop positions 11 and/or 25.13,14 This observation formed the basis of the simpler rule-based co-receptor prediction methods.13 Multiple
interpretive algorithms have since been designed to predict co-receptor use based on the sequence of relevant regions of the viral genome, in particular the envelope’s V3-loop region. The use of machine-learning techniques has allowed prediction methods to become more complex, resulting in the improved sensitivity of genotypic methods.15,16

Maraviroc is the first clinically available CCR5 antagonist.17 Patients screened as having R5 virus in the clinical trials of maraviroc had a significantly greater virological response to maraviroc than those with non-R5 virus populations, demonstrating the need to identify HIV tropism prior to therapeutic initiation of maraviroc.18–20 A number of both phenotypic and genotypic screening methods have been developed in order to determine HIV-1 tropism in patient samples. Initially, the most widely used method of identifying tropism was the phenotypic Original Trofile Assay (Monogram Biosciences).21 Since its introduction, adjustments have been made to increase its sensitivity and it is now referred to as the Enhanced Sensitivity Trofile Assay (ESTA).22 More recently, a population-based genotypic tropism assay has been developed in which the V3 loop is sequenced and interpreted using a bioinformatic algorithm such as geno2pheno,coreceptor (g2p).16,23,24 The same concept can be achieved with enhanced sensitivity using next-generation sequencing (NGS) technology.25,26 NGS can be used to quantify viral variants accounting for <2%–3% of the viral population, in comparison with the approximate 20% limit of standard sequencing technologies.25–27 This allows a more thorough evaluation of the non-R5 and R5 variants present within a sample.

Using deep sequencing methods to identify tropism, we longitudinally investigated and quantified the selective pressure of maraviroc on the dynamics and composition of the viral population in four patients with non-R5 virus who had experienced maraviroc treatment failure.

Methods

Study population
The four patients were participants in the AIDS Therapy Evaluation in the Netherlands (ATHENA) observational cohort, which has been approved by local and national institutional review boards. Three patients harboured non-R5 HIV populations by ESTA. The remaining patient harboured R5 virus, but was predicted to harbour non-R5 virus by ‘deep’ sequencing. Despite the presence of non-R5 virus, the potential value of maraviroc as an addition to an ongoing failing therapy regimen was investigated. No other drugs were added, to preserve future treatment options in these heavily pre-treated individuals.

Patient A started treatment in 1995 and was treated sequentially with two duo nucleoside reverse transcriptase inhibitor (NRTI)-based regimens, followed by four protease inhibitor (PI)-based regimens and one non-nucleoside reverse transcriptase inhibitor (NNRTI)-based regimen. Patient A discontinued all highly active antiretroviral therapy components (Combivir, tenofovir and ritonavir-boosted atazanavir) on his own initiative prior to starting maraviroc. Patient B started treatment in 1995 with mono and duo NRTI-based and PI-based regimens followed by one PI+NNRTI-based regimen. At the time of starting maraviroc, Patient B was being treated with Combivir, tenofovir and ritonavir-boosted tipranavir. Patient C had started triple drug therapy in 1997 with a PI-based regimen followed by two NNRTI-based regimens. Patient C was being treated with tenofovir, emtricitabine and nevirapine when maraviroc was introduced; however, during the course of maraviroc treatment, nevirapine was substituted with ritonavir-boosted lopinavir as part of the background regimen. Patient D started therapy in 1995 and was treated with mono and duo NRTI regimens, three PI-based regimens and one NNRTI-based regimen. Patient D was being treated with tenofovir and ritonavir-boosted tipranavir when maraviroc was added.

Four longitudinal samples were collected from each patient over a median of 53 days (IQR 27 days). The median length of maraviroc add-on therapy was 3 weeks (range 13–26 days). The period between the start of maraviroc and the first sample analysed ranged from 6 to 26 days (median 11 days). Three of the four patients had two post-maraviroc samples sequenced.

Laboratory methods
RNA was extracted from frozen plasma and the V3 loop of the gp120 protein encoded by the HIV-1 env gene was amplified in triplicate using nested RT–PCR.25,26 Second-round PCR was performed with primers incorporating unique sample-specific tags, ‘barcodes’, for sample identification. In preparation for clonal amplification using emulsion PCR, the resulting triplicate PCR amplicons were quantified, combined in equal proportions and purified.25,26 Amplicon libraries were diluted to 1×10^7 molecules/µL, such that five molecules of DNA were present for every DNA capture bead. Approximately 790000 beads were loaded into each of the four regions of the 454 pyrosequencing picotitre plate. Samples were sequenced in both the forward and reverse directions using 454 GS-FLX (Roche, 454 Life Sciences).

Sequence processing and tropism interpretation
A median of 14000 sequence reads per sample was obtained. After processing for sequence quality, a median of 12500 sequence reads per sample was included in the analyses. Viral tropism was interpreted using the g2p algorithm with a false-positive rate (FPR) cut-off of 3.5% for each variant sequence. As previously reported for ‘deep’ sequence analyses, sequences below this FPR value were inferred as being non-R5.25,26 The percentage of non-R5 variants within each viral population was determined from the total useable read counts for each sample. Having calculated this, the viral load of non-R5 and R5 populations could be determined separately. The non-R5 plasma viral load (pVL) was calculated by simply multiplying the percentage of non-R5 by the total pVL, with the R5 pVL as the remainder.

Fitness analysis
Following g2p scoring, sequences were stratified into five bins based on their g2p FPR (FPR < 2%, 2% ≤ FPR < 3.5%, 3.5% ≤ FPR < 10%, 10% ≤ FPR < 20%, 20% ≤ FPR). The observed frequency of the sequences within each bin was calculated and plotted over time for each timepoint and patient (allele frequencies). Each bin represents a different ‘level’ of tropism and level of response to maraviroc.

Relative viral fitness in the presence of maraviroc was calculated according to Wright’s recursive formula for allele frequency evolution under haploid selection, with the generation time assumed to be 1 day.28 We fitted a four-parameter fitness curve based on the flexible gamma cumulative distribution function to the longitudinal deep sequence data, given this model. The expected allele frequency of evolution was calculated from the initial observed allele frequencies at the earliest timepoint given the four model parameters of the fitness function. Here, a relative fitness of 1.0 is equal to the most-fit allele in the patient viral population.

Results
All four patients were confirmed as having non-R5 virus at baseline by 454 deep sequencing, the baseline being defined as the
Figure 1. Change in pVL (log_{10} copies/mL) and non-R5 percentage following exposure to maraviroc in four patients experiencing treatment failure and screened as having D/M HIV-1. Longitudinal samples were collected over a median of 53 days; patients were exposed to maraviroc for a median of 21 days. Viral tropism was determined from the V3 sequences generated using deep sequencing (454 GS-FLX titanium) and interpreted using the g2p algorithm with a 3.5% FPR cut-off point. The vertical broken line indicates the date at which the patient started maraviroc as an additive therapy. When the patient was exposed to maraviroc the overall pVL remained relatively constant, despite the decrease of at least 1 log_{10} in the R5 population, due to the expansion of the non-R5 population.
median of 11 days of maraviroc exposure (Figure 1). We note
that the proportion of non-R5 virus subsequently increased to 99% after
21 days of maraviroc exposure. In all four patients, the predominant non-R5 variant contained a basic residue (Lys or Arg) at
V3-loop positions 11 or 25 (Figure 2).

**Unusually, the majority of sequences in the forward direction from the first two timepoints for Patients A and C contain a frameshift mutation
**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Days on MVC</th>
<th>Pre-therapy (weeks 1–4)</th>
<th>Post-therapy (weeks 1–4)</th>
<th>V3 loop amino acid sequence</th>
<th>g2p FPR</th>
<th>Pre-therapy prevalence (%)</th>
<th>Post-therapy prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A**</td>
<td>37</td>
<td>1.7</td>
<td>22.5</td>
<td>C–14B TRPNNNT RKG IHI GPG G A L Y A T G Q I I I G N I R E A H C</td>
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<td>51.2</td>
<td>2.5</td>
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<td></td>
<td>26</td>
<td>10.2</td>
<td>84.1</td>
<td>C–16D TRPNNNT RK S I T LG P G R A F Y T T E R I I G D I R Q A H C</td>
<td>1.7</td>
<td>22.5</td>
<td>0.0</td>
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<tr>
<td>B</td>
<td>14</td>
<td>80</td>
<td>72.2</td>
<td>C–2C** TRPNNNT R K S I H M G P G A L Y A T G E I V G D I R Q A H C</td>
<td>1.8</td>
<td>0.36</td>
<td>80.1</td>
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<tr>
<td></td>
<td>21</td>
<td>12.9</td>
<td>61.5</td>
<td>D–16 TRPNNNT RKS I T L G P G R V E Y T T G D I I G D I R Q A H C</td>
<td>10.2</td>
<td>84.1</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Figure 2. Summary of the change in prevalence and FPR for the most common HIV-1 nucleotide sequence translated into amino acids from each of
the four patients with D/M HIV-1, before and after exposure to maraviroc (MVC). Amino acid substitutions between the most common sequence at the
first and last timepoints are noted; dashes represent no change at the position. Sequence prevalence, as a percentage of the number of useable reads
obtained from the sample, was calculated for each sequence as found at the pre-therapy and post-therapy timepoints. A variant with a g2p FPR of
<3.5% was considered to be X4. Amino acid positions marked by a square are associated with mutations characteristic of CXCR4-using virus.

The results here demonstrate a rapid replacement of the R5 population by non-R5 variants following the start of maraviroc treatment (Figure 3). The overall pVL increased, with a median of 0.36 log_{10} copies/mL by the final timepoint (Figure 1). Viral loads in patients A, B and D increased (0.27, 0.73 and 0.45 log_{10} copies/mL, respectively) after 26, 21 and 13 days of exposure, respectively. Patient C showed a decrease in viral load, with 1.32 log_{10} copies/mL after 21 days of maraviroc. When separated into R5 and non-R5 viral loads, it is evident that changes in the overall pVL are due to a large decrease in the R5 populations (median ≤ 3.5%, 3.5% ≤ FPR < 10%, 10% ≤ FPR < 20%, 20% ≤ FPR) according to their g2p FPR in order to estimate relative viral fitness in vivo in the presence of maraviroc. The frequency of the sequences was plotted for each bin at each timepoint, showing the change in FPR over time. The non-R5 variants with extremely low FPR values (<2%) had the most substantial increase in prevalence. R5 variants (FPR ≥ 3.5%) were represented by three bins, the higher being considered increasingly characteristic of R5 virus and most likely to respond to maraviroc. There was no corresponding increase in the proportion of virus with an FPR between 3.5% and 20%, thus implying the relative inhibition of these variants (Figure 4).

most recent sampling timepoint prior to the start of maraviroc treatment. The viral populations were composed of between <1% and 58% non-R5 virus at baseline (median 24%, IQR 6%–44%).

Even at the first timepoint following the start of maraviroc treatment, it was apparent that non-R5 virus was not inhibited by maraviroc as the percentage of non-R5 variants increased in all patients (Figure 1). In fact, the proportion of non-R5 populations increased by a median of 71% (IQR 49%–91%) after a median of 11 days of maraviroc exposure (Figure 1). We note an increase in the percentage of non-R5 virus in Patient A several days before the documented start of maraviroc; we could not find an explanation for this observation. When tested using ESTA, Patient C was determined to have R5 virus only at baseline (<1% non-R5 virus using deep sequencing). The result from the previous timepoint, 49 days prior to baseline, was determined as 11% non-R5 virus using deep sequencing. This non-R5 population subsequently increased to 99% after 21 days of maraviroc exposure. In all four patients, the predominant non-R5 variant contained a basic residue (Lys or Arg) at V3-loop positions 11 or 25 (Figure 2).
Based on the data collected, we employed a population genetic model to measure the relative fitness advantage under haploid selection (with a generation time of 1 day) of predicted X4 viral variants in the presence of maraviroc in vivo. The relative frequencies of these allelic classes over time were fitted to Wright’s haploid selection model to estimate four parameters of a flexible monotonic function relating relative fitness to FPR (Figure 5). The viral fitness of variants with an FPR >5% has little selective advantage in the presence of maraviroc, remaining relatively constant at a relative fitness of 0.2, where 1.0 is equal to the most-fit allele in the patient viral population. However, in variants with an FPR <5%, the relative fitness began to increase markedly. At an FPR between 2% and 0%, the relative fitness was shown to increase from ~0.65 to 1.0, representing an increase in fitness of ~0.2 units for every FPR unit (Figure 5).

The most common sequences from the pre-therapy and post-therapy samples tested were compared for each patient (Figure 2 and Table S1, available as Supplementary data at JAC Online). The prevalence of the most common sequences as a percentage of the total useable sequences obtained for the sample was calculated. For each patient, the most common sequence at the pre-therapy timepoint was always present at a pre-therapy timepoint; however, the most common sequence at the post-therapy timepoint was always present at a pre-therapy timepoint (Figure 2 and Table S1). In the case of Patient C, the most common sequence at the post-therapy timepoint was...
not found at the first pre-therapy timepoint but was found at baseline. With the exception of Patient B, the most common pre-therapy sequence was an R5 variant, whereas the post-therapy sequence was a non-R5 variant. The most common sequences for Patient B, pre- and post-therapy, were both non-R5 variants. Notably, the non-R5 variants that became the most common sequence all had extremely low FPR values (between 1.1% and 1.8%). For each patient, the most common sequence at the post-therapy timepoint had a greater prevalence than the most common sequence at the pre-therapy timepoint, showing a decrease in sequence variation and a selective advantage for those sequences with a low FPR in the presence of maraviroc (Figure 2).

Discussion

Using 454 deep sequencing, viral tropism was examined in plasma samples from patients with non-R5 HIV-1 who were exposed to maraviroc add-on therapy to attempt to obtain a potential clinical benefit. The R5 viral population in all patients responded to the presence of maraviroc, with a median decrease in R5 pVL of 2 log_{10} copies/mL within 21 days. However, consistent with the A4001029 study, the total viral population did not have a significant virological response to maraviroc. In fact, alongside the suppression of R5 virus, the non-R5 populations modestly expanded such that the total viral load remained nearly constant. Interestingly, the most successful non-R5 variants were those with an extremely low FPR using g2p, which suggests that maraviroc may inhibit HIV with a lower g2p FPR than previously thought.

It has previously been suggested that the emergence of non-R5 variants is due not to a switch in co-receptor use by variants but to the selective outgrowth of a pre-existing non-R5 reservoir in the presence of maraviroc or vicriviroc. This is consistent with the more recent data generated by applying the same deep sequencing screening method to a group of 24 individuals from Spain with non-R5 HIV populations who had been exposed to maraviroc for 8 days. Unlike most previous analyses using individuals participating in clinical trials of maraviroc and vicriviroc, patients here were given maraviroc despite having been screened as having non-R5 HIV by ESTA. In heavily pre-treated patients experiencing drug-related toxicity, such as those studied here, clinicians have to apply individually tailored empirical therapy regimens. Although this is not recommended, all four patients received maraviroc to assess its virological or immunological efficacy. This study was a retrospective analysis of four of these cases.

After a median of 21 days of maraviroc add-on therapy, patients with non-R5 virus at baseline had a dramatic increase in their non-R5 population, such that despite a minimum decrease of 1 log_{10} copies/mL in the median R5 pVL, the overall pVL remained nearly constant. In each patient from the study presented here, the most prevalent sequence after ~3 weeks of exposure to maraviroc was non-R5, and in agreement with the findings of Westby et al., these sequences had already been observed at a pre-therapy timepoint. Given the short amount of time that elapsed between samplings, it is unlikely that the mutations conferring non-R5 usage that had been observed in the pre-maraviroc samples had disappeared only to re-emerge de novo after the start of maraviroc therapy. The more parsimonious interpretation of our results is that non-R5 variants that had been present before maraviroc therapy continued to persist in the population until they gained a selective advantage at the start of maraviroc therapy.

There is an ongoing debate regarding the appropriate FPR cut-off point to apply in order to effectively predict a patient’s viral response to maraviroc. Genotypic tropism testing in British Columbia, Canada, uses a cut-off point of 5.75% in population-based sequencing methods and 3.5% in NGS methods, both of which have been retrospectively validated using populations from clinical trials of maraviroc. However, the European guidelines recommend a more conservative FPR cut-off point of 10%. Similarly, the German–Austrian guidelines recommend an FPR cut-off range of 5%–15% depending on the treatment options (www.daignet.de). Longitudinal analysis of the independent CCR5- and non-CCR5-using HIV populations shows that maraviroc selects for viruses with an extremely low FPR (between 0% and 2%), suggesting that the FPR cut-off points as high as 10% may exclude individuals who could respond to maraviroc.

This analysis provides another way in which to look at and interpret FPR cut-off points clinically. By composing a fitness map,
measuring the shape of a fitness function over the range of co-receptor-tropism predictions from g2p, there is a possibility of identifying results that are overlooked when making inferences based on outcome. The data are more consistent with the lower g2p cut-offs of the newer German–Austrian guidelines (as low as 5%) compared with the values of up to 20% suggested in older guidelines. Based on the results of this study and a retrospective analysis of clinical trials, a cut-off of 5% may be more applicable and should be explored further. However, due to our small sample size, this finding needs to be approached with caution, and oversampling is a concern as patients, with the exception of Patient C, generally had a high pVL following the start of maraviroc treatment. Unfortunately, we were unable to use the primer ID method more recently described by Jabara et al.,35 and therefore the viral input copy number as well as the effects of PCR and sequencing error are unknown.

In conclusion, when patients with D/M virus were exposed to maraviroc add-on therapy, selection for non-R5 populations caused them to expand to fill the space once occupied by R5 variants. Selection favoured non-R5 variants with an extremely low FPR, which may indicate that the antiretroviral activity of maraviroc may be effective in a broader range of HIV variants than currently suspected.

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

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