Primary resistance of CCR5-tropic HIV-1 to maraviroc cannot be predicted by the V3 sequence

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Objectives: Resistance of HIV-1 to CCR5 antagonists can occur without coreceptor switching by mutations in envelope glycoproteins that enable virus entry using the inhibitor-bound form of CCR5. We investigated whether mutations in the V3 region of HIV-1 from subjects naive to maraviroc could be associated with primary resistance to this drug.

Methods: The frequency of CCR5-tropic HIV-1 subtype B isolates harbouring putative V3 maraviroc resistance mutations was assessed among the HIV tropism database of Toulouse University Hospital, France. Phenotypic assessment of maraviroc susceptibility was performed for 14 isolates representative of the main mutation patterns and 14 controls. V3 mutations were reversed or introduced by site-directed mutagenesis.

Results: Ninety-three of 951 (9.8%) isolates harboured V3 mutations assumed to be associated with maraviroc resistance. Maraviroc completely blocked virus entry for all but 1 of the 14 isolates harbouring V3 mutations [IC50 8.6 nM; 95% CI (6.6–47.4)], as in the 14 control isolates [IC50 13.4 nM; 95% CI (7.7–50.3)] (P=0.24). Primary resistance to maraviroc, with a plateau in entry inhibition, was found in one isolate (harbouring a 20F/21I genotype). Site-directed mutagenesis showed that V3 mutations are necessary but not sufficient to induce maraviroc resistance.

Conclusions: The impact of V3 mutations depended on the env context in which they occurred. Simple assessment of the V3 genotype thus cannot accurately predict maraviroc resistance. Rather, phenotypic assessment of virus particles expressing the envelope glycoprotein as a whole is required. This approach revealed that primary resistance of CCR5-tropic HIV-1 subtype B isolates to maraviroc seems uncommon.

Keywords: receptors, HAART, maraviroc; drug resistance, env

Introduction

The process whereby HIV type 1 (HIV-1) gains entry to its target cells requires successive interactions between the virus envelope glycoproteins and the CD4 and CCR5 or CXCR4 molecules on the cell surface, acting respectively as receptor and coreceptors for the virus. The entry of CCR5-tropic HIV-1 can be inhibited by maraviroc, the first CCR5 antagonist to receive approval. Maraviroc binds to a hydrophobic pocket in the transmembrane helices of CCR5, inducing changes in the conformation of the CCR5 extracellular loops and thus causing allosteric inhibition of HIV-1 entry. This mechanism of resistance requires changes in the HIV-1 envelope glycoproteins to enable virus binding to and entry through CCR5 despite its modified conformation induced by maraviroc binding. When analysing the virus entry inhibition curves in vitro in the face of increasing concentrations of CCR5 antagonist, this resistance process usually leads to a plateau in entry inhibition, with only a part of the virus entry being blocked, even at supra-therapeutic maraviroc concentrations. Some mutations in the env gene of CCR5-tropic HIV-1 have been described in virological failure during maraviroc-based therapy. Most are located in V3 env, the key region of gp120 for interacting with the coreceptors. However, multiple patterns of V3 mutations have been described, with some variations from one patient to another and between HIV-1 subtypes.
Genotypic determinants located outside V3 have also been described\textsuperscript{17,18,23–26} and the genotype–phenotype correlations for maraviroc resistance remain unclear.

The structure–affinity relationships of gp120 to free or inhibitor-bound CCR5 are complex, and the impact of V3 mutations depends on the env context in which they occur.\textsuperscript{16,17,19,23,26} This cannot correctly be appreciated by the simple assessment of the V3 genotype.

On the basis of V3 genotyping, previous studies have claimed that 5%–10% of naive subjects could be infected by HIV-1 strains displaying primary resistance to maraviroc, as they harbour some patterns of V3 mutations assumed to be associated with resistance to maraviroc.\textsuperscript{27,28}

We investigated whether these V3 mutation patterns in subjects naive to maraviroc are really associated with primary phenotypic resistance to this drug. We also assessed the respective roles of V3 and extra V3 genotypic determinants in the phenotype of maraviroc resistance to demonstrate that V3 genotyping cannot correctly predict resistance to maraviroc.

**Methods**

**Patients and virus isolates**

The HIV tropism database of Toulouse University Hospital, France consisted in February 2012 of 1987 primary HIV-1 isolates successfully characterized for coreceptor usage both phenotypically by the Toulouse Tropism Test and genotypically by V3-based algorithms, as previously described.\textsuperscript{29} Among 951 isolates of HIV-1 subtype B phenotyped as pure CCR5-tropic, from subjects naive to maraviroc, we selected the isolates harbouring a pattern of mutations in V3 that had previously been reported in subjects with virological failure under maraviroc-based therapy while keeping a CCR5-tropic phenotype.\textsuperscript{20,21} Fourteen isolates representative of the various mutations patterns and 14 controls were then selected for further phenotypic assessment of resistance to maraviroc. Patient characteristics are shown in Table S1 (available as Supplementary data at JAC Online). The sequences encompassing the V3 region were given GenBank accession numbers JX993125 to JX993152.

**Phenotypic determination of the resistance of CCR5-tropic HIV-1 to maraviroc**

The phenotypic resistance of CCR5-tropic HIV-1 to maraviroc was assessed using an assay derived from the Toulouse Tropism Test. Recombinant virus particles were produced by homologous recombination between gp140 (gp120+ectodomain of gp41) env PCR products obtained from the challenged HIV-1-containing sample and an Nhel-linearized pNL43-Δgp140env-Luc2 vector cotransfected in 293T cells, as previously reported.\textsuperscript{23} The recombinant virus particles released into the supernatant were quantified by real-time quantitative RT–PCR (Roche) and a normalized input was used to infect U87 indicator cells bearing CD4 and an inducible expression upon tetracycline addition (the inducible expression of CCR5 gene) encoding the Tet repressor under blasticidin selection, and then transduced with pBABE-CMV-(TetO2)\textsuperscript{2}-CCR5-puro-SIN under puromycin selection (a map of the vector is shown in Figure S1, available as Supplementary data at JAC Online). This vector was constructed from the pBABE.CCR-5 vector (NIH AIDS Research and Reference Reagent Program from Dr Nathaniel Landau)\textsuperscript{31,32} and the T-REx system (Invitrogen). A clone of this U87 CD4+ CCR5\textsuperscript{plasm} stable cell line was selected based on the lowest basal expression of CCR5 in the absence of tetracycline and high-level expression upon tetracycline addition (the inducible expression of CCR5 measured by flow cytometry is shown in Figure S2, available as Supplementary data at JAC Online). For assessing the resistance of CCR5-tropic HIV-1 to maraviroc with high sensitivity, maximal CCR5 expression was induced by adding 50 ng/mL tetracycline 24 h before the infection. The infection of the U87 CD4+ CCR5\textsuperscript{plasm} cells was performed in the absence or presence of maraviroc at concentrations ranging from 0.1 pM to 10 μM. Virus entry was assessed at 48 h post-infection by measuring the luciferase activity as relative light units (RLUs) on a Glomax 96 microplate luminometer (Promega). The Bal strain and two virus clones isolated from subjects failing a maraviroc-based therapy (PFZ-1 and PFZ-4 clones, Pfzer) were used as CCR5-tropic maraviroc-susceptible and maraviroc-resistant controls, respectively (Figure S3, available as Supplementary data at JAC Online). All assays were repeated in at least three separate experiments and the median values are shown. Curves of entry inhibition related to the maraviroc concentrations were drawn using GraphPad Prism 4.0c.

**Clonal analysis**

Molecular clones from isolates harbouring V3 mutations assumed to be associated with maraviroc resistance were obtained using a TOPO-TA cloning kit (Invitrogen).

**Site-directed mutagenesis**

Site-directed mutagenesis (Quick Change II Kit, Agilent) was used to reverse V3 mutations in the resistant virus clone obtained from subject 14 and in PFZ-1 and PFZ-4 (clones 14-01, PFZ-1 and PFZ-4 to Bal, mutagenesis) and symmetrically to introduce mutations at the same positions in the envelope gene of a clone of the susceptible Bal strain (Bal to clones 14-01, PFZ-1 and PFZ-4 mutagenesis) (Table 3). For the resistant clone 14-01 from subject 14, we introduced F20L and I21Y mutations (clone 14-03 to Bal, mutagenesis) and symmetrically introduced L20F and Y21I mutations at the same positions in the susceptible Bal clone (Bal to clone 14-01 mutagenesis). For the resistant clone PFZ-1, we introduced T19A, F20L, A22T, D25E and V26I mutations (clone PFZ-1 to Bal, mutagenesis) and symmetrically introduced A19T, L20F, T22A, E25D and I26V mutations at the same positions in the susceptible Bal clone (Bal to clone PFZ-1 mutagenesis). For the resistant clone PFZ-4, we introduced T19A, F20L, A22T and D25E mutations (clone PFZ-4 to Bal, mutagenesis) and symmetrically introduced A19T, L20F, T22A and E25D mutations at the same positions in the susceptible Bal clone (Bal to clone PFZ-4 mutagenesis). The V3 region of the PFZ-4 clone differs from that of PFZ-1 only at position 26 (I residue in clone PFZ-4 versus V in clone PFZ-1).

**Production of chimeric virus particles for V3 or V1-V3 env**

To further differentiate between genotypic determinants located in and outside V3, chimeric viruses with changes restricted to V3 or V1–V3 were constructed using V3 or V1–V3 env PCR products and the pNL43-ΔV3env-Luc2 or pNL43-ΔV1V3env-Luc2 vectors, respectively. Phenotypic determination of the resistance of these chimeric virus particles to maraviroc was performed as described above for virus recombinant for the whole gp140.

**Statistical analyses**

Quantitative and categorical variables were compared using the Wilcoxon rank-sum test and Fisher’s exact test, respectively. The tests were two-sided, and P values < 0.05 were considered statistically significant. Statistical analyses were performed with Stata/SE 10.0.
Results

Frequency of CCR5-tropic HIV-1 isolates harbouring V3 mutations assumed to be associated with maraviroc resistance

Several mutation patterns have been reported in CCR5-tropic resistant viruses selected under maraviroc-based therapy. The most frequent patterns seem to be: 11S/26V; 20F/25D/26V; 19T/26V; 18G/22T; 19S/26V and 20F/21I. Among 951 isolates of HIV-1 subtype B phenotyped as CCR5-tropic by the Toulouse Tropism Test from subjects naive to maraviroc, 93 (9.8%) harboured one of these patterns of mutations in V3 (Table 1).

Phenotypic assessment of maraviroc susceptibility of CCR5-tropic isolates harbouring putative V3 mutations of resistance

We selected 14 CCR5-tropic HIV-1 isolates, representative of the main mutations patterns, and 14 controls for further phenotypic assessment of primary resistance to maraviroc. The V3 sequences are shown in Table 2. Complete inhibition of virus entry into the target cells was obtained by increasing maraviroc concentrations from 0.1 pM to 10 μM in all but 1 of the 14 isolates harbouring putative V3 mutations of resistance (Figure 1a), as in the 14 control isolates (Figure 1b). Entry inhibition of 50% (IC50) was obtained at a median maraviroc concentration of 8.6 nM (95% CI 6.6–47.4) in the group of isolates harbouring putative V3 mutations of resistance, and 13.4 nM (95% CI 7.7–50.3) in the control group (P = 0.24).

To confirm the genotype–phenotype correlations obtained at a virus population level, we performed clonal analysis for four isolates to obtain molecular clones harbouring the V3 mutations. The dose–response curves of the clones were similar to those of the whole isolates (data not shown).

Primary resistance to maraviroc was found in one isolate (subject 14), harbouring a 20F/21I genotype in V3 (Figure 1c). Even at a maraviroc concentration of 10 μM (i.e. >1000-fold higher than the mean IC50 of susceptible viruses) only 41% inhibition of entry was obtained, with a plateau in the dose–response curve, suggesting that this virus is able to utilize maraviroc-bound CCR5 for entry. This result was confirmed at a clonal level (clone 14-01) (Figure 2a).

Table 1. Frequency of CCR5-tropic HIV-1 isolates harbouring V3 mutations assumed to be associated with maraviroc resistance

<table>
<thead>
<tr>
<th>Genotypic pattern&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number (frequency)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>11S/26V</td>
<td>61 (6.4%)</td>
</tr>
<tr>
<td>20F/25D/26V</td>
<td>21 (2.2%)</td>
</tr>
<tr>
<td>19T/26V</td>
<td>7 (0.7%)</td>
</tr>
<tr>
<td>18G/22T</td>
<td>2 (0.2%)</td>
</tr>
<tr>
<td>19S/26V</td>
<td>1 (0.1%)</td>
</tr>
<tr>
<td>20F/21I</td>
<td>1 (0.1%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mutations are numbered according to their position in V3 from 1 to 35 along the BaL reference sequence.

<sup>b</sup>Among 951 isolates of HIV-1 subtype B phenotyped as CCR5-tropic from subjects naive to maraviroc.

Assessment by site-directed mutagenesis of the role of the V3 genotype on the phenotype of maraviroc resistance

We then investigated the role of the V3 mutations on maraviroc susceptibility by performing site-directed mutagenesis. We reversed mutations in V3 of resistant virus clones obtained from subjects 14, PFZ-1 and PFZ-4 (clones 14-01, PFZ-1 and PFZ-4 to BaL mutagenesis) and symmetrically introduced mutations at the same positions in the envelope gene of a clone of the susceptible BaL strain (BaL to 14-01, PFZ-1 and PFZ-4 mutagenesis). The V3 sequences and mutated residues in the BaL, 14-01, PFZ-1 and PFZ-4 clones are shown in Table 3.

For the resistant virus strain of subject 14, we reversed F to L and I to Y at V3 positions 20 and 21 of the 14-01 resistant clone (14-01 to BaL mutagenesis) and symmetrically introduced L to F and Y to I mutations at the same positions of the susceptible BaL clone (BaL to 14-01 mutagenesis). Recombinant virus particles for gp140 were then produced for the native and mutated clones of 14-01 and BaL. Curves of entry inhibition in response to increasing maraviroc concentrations showed that reversing F and I at V3 positions 20 and 21 in clone 14-01 almost completely restores its susceptibility to maraviroc, reaching entry inhibition of 90% (Figure 2a). By contrast, introducing F and I at V3 positions 20 and 21 of BaL did not lead to any resistance to maraviroc (Figure 2b). These results suggest that 20F and 21I in V3 are necessary but not sufficient to induce maraviroc resistance.

To further demonstrate that some of the genotypic determinants of maraviroc resistance are located outside V3, we performed additional site-directed mutagenesis on two other highly resistant virus clones isolated from subjects failing maraviroc-based therapy (clones PFZ-1 and PFZ-4, the V3 of which are identical except at position 26, which is V or I, respectively). We reversed mutations in V3 of the resistant clones PFZ-1 and PFZ-4 (PFZ-1 and PFZ-4 to BaL mutagenesis) and symmetrically introduced the same mutations in the susceptible BaL clone (BaL to PFZ-1 and PFZ-4 mutagenesis). For both PFZ-1 and PFZ-4 clones, reversing their V3 mutations restored full susceptibility to maraviroc (Figures 3a and 3b). The symmetrical introduction of these mutations in the context of the BaL envelope did not lead to any resistance to maraviroc for the BaL to PFZ-4 mutated clone (Figure 3d), while the addition of a V residue at position 26 in the BaL to PFZ-1 mutated clone led to some resistance to maraviroc with a plateau at about 50% entry inhibition (Figure 3c). These results thus further argue that V3 mutations are necessary to induce maraviroc resistance, as we have shown that the reversion of V3 mutations without any other change in env is sufficient to abolish maraviroc resistance. But, by contrast, V3 mutations associated with resistance to maraviroc only had a limited impact on the acquisition of resistance to maraviroc when being expressed in another env context.

Maraviroc susceptibility of viruses chimeric for the V3 or V1-V3 regions compared with viruses expressing the whole gp140

Lastly, to investigate the role of genotypic determinants of maraviroc resistance located outside V3 or even outside V1-V3, we compared the dose–response curves of chimeric clones harbouring the whole gp140, or only the V1-V3 or V3 env regions of the PFZ-1 and...
| Group | Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 |
| MUT\(^a\) | 1 | C | T | R | P | N | N | N | T | R | S | I | H | L | G | P | G | S | A | F | Y/F | T | T | G | E | V | I | G | N | I | R | Q | A | H | C |
| 2 | C | T | R | P | N | N | N | N | T | R | S | I | N | I | G | P | G | A | F | Y | A | T | G | A | V | I | G | D | I | R | Q | A | H | C |
| 3 | C | T | R | P | S | N | N | N | T | R | S | I | T | M | G | P | G | R | A | F | Y | A | T | G | A | V | T/A/V/I | G | D | I | R | K | A | H | C |
| 4 | C | T | R | P | N | N | N | N | T | R | S | V | H | V | G | P | G | R | A | I | Y | T | T | — | D | V | I | G | D | I | R | Q | A | H | C |
| 5 | C | T | R | P | N | N | N | N | T | R | S | N | N | I | A | P | G | R | A | F | Y | A | T | G | D | V | I | G | D | I | R | Q | A | H | C |
| 6 | C | T | R | P | N | N | N | N | T | R | R/K | S | I | N | I | A | P | G | R | A | F | Y | A | T | G | D | V | I | G | D | I | R | Q | A | H | C |
| 7 | C | T | R | P | N | N | N | N | T | R | R | S | I | H | I | A | P | G | K | A | F | Y | A | T | G | D | V | I | G | D | I | R | Q | A | H | C |
| 8 | C | T | R | P | N | N | N | N | T | R | S | I | N | I | G | P | G | A | F | Y | A | T | G | D | V | I | G | D | I | R | Q | A | H | C |
| 9 | C | V | R | P | N | N | N | N | T | R | S | I | P | L | G | P | G | K | T | F | Y | A | G | — | E | V | I | G | D | I | R | Q | A | H | C |
| 10 | C | T | R | P | N | N | N | N | T | R | S | I | H | I | G | P | G | R | T | F | Y | T | T | G | E | V | I | G | D | I | R | Q | A | H | C |
| 11 | C | T/A | R | P | N | N | N | N | T | R | S | I | H | M | G | P | G | K | T | L | F | T | T | — | E/D | I/V | I | G | D | I | R | Q | A | H | C |
| 12 | C | T | R | P | N | N | N | N | T | R | R | G | I | H | M | G | P | G | G | A | F | Y | T | T | G | E | V | I | G | D | I | R | K/Q | A | H | C |
| 13 | C | T | R | P | N | N | N | N | T | R | S | I | N | I | G | P | G | K | S | F | Y | T | T | — | N | V | I | G | D | I | R | Q | A | H | C |
| 14 | C | T | R | P | N | N | N | N | T | R | S | I | P | I | G | P | G | R | A | F | I | A | T | G | D | I | I | G | D | I | R | Q | A | H | C |
| 15 | C | T | R | P | N | N | N | N | T | R | G | I | H | M | G | P | G | A | I | Y | A | T | — | D | I | I | G | D | I | R | K | A | Y | C |
| 16 | C | T | R | P | N | N | N | N | T | R | G | V | H | I | G | P | G | R | A | F | Y | T | T | — | D | I | I | G | N/D | I | R | Q | A | Y | C |
| 17 | C | T | R | P | N | N | N | N | T | R | G | I | H | M | G | P | G | R | A | F | Y | T | T | G | D | I | I | G | D | I | R | Q | A | H | C |
| 18 | C | T/I | R | P | S/G | N | N | N | T | R | G | I | H | M | G | P | G | K/R | A | F | Y/F | T/A | T | G | Q/E | I | T | G | D | I | R | K | A | H | C |
| 19 | C | T | R | P | N | N | N | N | T | R | K/N | S/G | I | H | I | A/P | P | G | G | A | F | Y | A | T | G | D | I | I | G | D/N | I | R | Q | A | H | C |
| 20 | C | T | R | P | N | N | N | N | T | R | S/G | I | H | I | G | P | G | S | T/A | L | Y | A | T | G | E | I | I | G | D | I | R | Q | A | H | C |
| 21 | C | T | R | P | N | N | N | N | T | R | S | I | H | I | G | P | G | S | T/A | L | Y | A | T | G | E | I | I | G | D | I | R | Q | A | H | C |
| 22 | C | T | R | P | N | N | N | N | T | R | S | I | P/S | I/L | G | P | G | R | A | W | Y | A | T | G | D | I | I | G | D | I | R | Q | A | Y | C |
| 23 | C | T | R | P | N | N | N | N | T | R | S | I | P | S | I/L | G | P | G | R | A | W | Y | A | T | G | D | I | I | G | D | I | R | Q | A | Y | C |
| 24 | C | T | R | P | N | N | N | N | T | R | S | I | H | I | G | P | G | R | A | F | Y | T | T | G | E | I | I | G | D | I | R | K | A | H | C |
| 25 | C | T | R | P | N | N | N | N | T | R | S | I | H | I | A | P | G | R | A | F | Y | T | T | G | E | I | I | G | D | I | R | Q | A | H | C |
| 26 | C | T/I | R | P | N | N | N | N | T | R | S | I | H | I | A | P | G | R | A | F | Y | A | T | G | D | I | I | G | D | I | K/R | Q | A | Y | C |
| 27 | C | T | R | P | N | N | N | N | T | R | S | S | I | G | P | G | R | A | F | F | A | T | — | D | I | I | G | D | I | R | Q | A | H | C |
| 28 | C | T | R | P | N | N | N | N | T | R | S | I | H | M | G | P | G | K | T | F | F | T | T | — | D | I | I | G | D | I | R | Q | A | H | C |

Residues associated with resistance to maraviroc are highlighted in bold; dashes show gaps inserted to maintain alignment; slashes indicate amino acid position related to a mixed virus population.

\(^a\)MUT, group of patients harbouring putative V3 mutations of resistance to maraviroc.

\(^b\)WT, control group.
PFZ-4 maraviroc-resistant clones. We found that the entry of the recombinant virus harbouring the whole gp140 of PFZ-1 was poorly inhibited by maraviroc, with an almost flat curve and a plateau of entry inhibition at 13%. By contrast, the recombinant virus harbouring only V1-V3 of PFZ-1 was less resistant to maraviroc, with a plateau at 52%, while the clone harbouring only V3 of PFZ-1 was fully susceptible to maraviroc (Figure 4a). Similar results were obtained for the PFZ-4 clone, with a plateau of entry inhibition at 14% when the whole gp140 is expressed, mild resistance (80% entry inhibition) of the virus recombinant for V1-V3, and full susceptibility to maraviroc of the virus recombinant only for V3 (Figure 4b). The comparison between the dose–response curves of viruses chimeric for the V3 and V1-V3 regions of the PFZ-1 and PFZ-4 clones suggests that some determinants of resistance to maraviroc are located in the V1-V2 or C2 regions. But other determinants located outside V1-V3 also appear to play a significant role.

![Graphs showing dose–response curves for maraviroc susceptibility](https://example.com/graph.png)

**Figure 1.** Phenotypic assessment of maraviroc susceptibility of CCR5-tropic isolates harbouring or not harbouring putative V3 resistance mutations. Dose–response curves showing the percentage of HIV-1 entry inhibition in response to increasing maraviroc concentrations. (a) Isolates harbouring putative V3 mutations of resistance (n = 13; sample 14 is shown in (c)). (b) Control isolates (n = 14). Each curve represents a different isolate. (c) Dose–response curve showing a plateau in HIV-1 entry inhibition even at supra-therapeutic maraviroc concentrations for isolate 14, harbouring a 20F/21I genotype. The median of at least three independent experiments for each isolate is shown.

**Table 3.** Changes in V3 residues induced by site-directed mutagenesis between BaL and the 14-01, PFZ-1 and PFZ-4 clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>V3 Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35</td>
</tr>
<tr>
<td>BaL</td>
<td>C T R P N N N N T R K S I H I G P G R A</td>
</tr>
<tr>
<td>14-01</td>
<td>C T R P N N N N T R K S I P I G P G R A</td>
</tr>
<tr>
<td>BaL</td>
<td>C T R P N N N N T R K S I H I G P G R A</td>
</tr>
<tr>
<td>PFZ-1</td>
<td>C T R P N N N N T R K S I H I G P G R T</td>
</tr>
<tr>
<td>BaL</td>
<td>C T R P N N N N T R K S I H I G P G R A</td>
</tr>
<tr>
<td>PFZ-4</td>
<td>C T R P N N N N T R K S I H I G P G R T</td>
</tr>
</tbody>
</table>

Mutated residues are highlighted in bold.
in maraviroc resistance, as chimeric viruses for V1–V3 are much less resistant than those expressing the full-length gp140.

Taken together, these results suggest that the V3 genotype alone cannot predict the resistance of CCR5-tropic HIV-1 to maraviroc because the impact of V3 mutations may depend on the env context in which they occur, involving extra V3 genotypic determinants.

### Discussion

For most of the drugs currently used for HIV-1 therapy, clear genotype–phenotype correlations have been established and well-known mutation patterns in the reverse transcriptase, protease, integrase and gp41 genes are predictive of HIV-1 phenotypic resistance to their cognate drugs. Simple genotyping is thus routinely used for monitoring HIV-1 resistance. 33

By contrast, the genotype–phenotype correlations remain poorly known regarding CCR5-tropic HIV-1 resistance to CCR5 antagonists, notably maraviroc, the only one approved for HIV-1 therapy. Some mutation patterns in V3 have been identified in isolates from subjects with virological failure under maraviroc-based therapy that keep a CCR5-tropic phenotype. 20, 21 These genotypes can also be found in isolates from subjects naive to maraviroc, and primary resistance of these isolates to maraviroc has thus been expected. 27, 28 However, the env gene is characterized by a high genetic diversity, particularly in the variable regions. The global context of the HIV-1 envelope in which some V3 mutations occur is thus very different between subjects, and this could lead to various phenotypes. 26

In the large HIV-1 tropism database of Toulouse University Hospital, France (n=1987 primary HIV-1 isolates characterized both genotypically and phenotypically), we found that 93 (9.8%) of the 951 isolates of subtype B phenotyped as CCR5-tropic from subjects naive to any CCR5 antagonist harbour some mutation patterns in V3 assumed to be associated with maraviroc resistance. The most frequently found genotypes were 11S/26V (6.4%) and 20F/25D/26V (2.2%), in agreement with previous reports. 21, 27, 28 However, all but one of these isolates display full susceptibility to maraviroc, similar to the control isolates, when assessed by a phenotypic assay. Phenotypic resistance to maraviroc was found in only one virus harbouring a 20F/21I genotype with a pure CCR5-tropic phenotype, which is unique in the database. Eight other viruses in the database with a 20F/21I genotype were CXCR4-tropic; all were of subtype B.

Site-directed mutagenesis revealed that reversing this 20F/21I genotype almost completely restores susceptibility to maraviroc. Similarly, reversing the V3 mutations of PFZ-1 and PFZ-4 clones also abolishes maraviroc resistance, suggesting that V3 mutations are necessary for inducing maraviroc resistance. But when we symetrically assessed the impact on maraviroc susceptibility of introducing V3 mutations, we found discordances regarding their impact, depending on the env context in which they are expressed. In the env context of BaL, the V3 mutations of PFZ-1 resulted in some resistance to maraviroc, albeit at a lower level than that obtained in the native PFZ-1 env context. But in the env context of NL4-3, the V3 of PFZ-1 did not result in any resistance to maraviroc. For the PFZ-4 clone, the V3 genotype did not lead to any resistance when being expressed in the BaL or in the NL4-3 env context. These results suggest that V3 mutations have different consequences depending on the env context in which they occur, arguing for a role in maraviroc resistance of genotypic determinants located outside V3. This is in agreement with previous studies reporting the selection of mutations outside V3 in emerging resistance to other CCR5 antagonists, such as vicriviroc and TAK-779. 17, 18, 23, 24, 26 Recently, a mutation in the CD4 binding site in the C4 region of gp120 has also been reported to play a role in maraviroc resistance. 25 This mutation seems to impact the interaction of gp120 with CD4 and leads to a shift in 50% inhibitory concentrations rather than in a reduction of the maximal plateau inhibition, as usually described in maraviroc resistance. Previous work has underlined the need for cooperative effects of mutations located inside and outside V3 for a virus to acquire full resistance to CCR5 antagonists, in agreement with our results, which show that mutations in V3 are necessary but not sufficient to induce full resistance to maraviroc. 16, 17, 19, 23, 26 In a few cases, V3 mutations introduced by site-directed mutagenesis appeared to be sufficient to induce full maraviroc resistance. 34 However, in these cases, V3 mutations were introduced in a virus clone isolated at baseline from the same subject, thus sharing a
close env context. Introducing the same V3 mutations in a completely different env context, as in our work, where V3 mutations were introduced in the env context of the BaL strain, could have produced different results.

V3 mutation patterns found in CCR5-tropic viruses resistant to CCR5 antagonists can vary between HIV-1 subtypes. Here, we chose to focus on isolates of subtype B. In addition, the residues of V3 involved in resistance to maraviroc were significantly different from those involved in resistance to other CCR5 antagonists such as vicriviroc. We cannot exclude that different results in genotype–phenotype correlations might have been obtained for other HIV-1 subtypes or other CCR5 antagonists.

Further complexity in the resistance of CCR5-tropic HIV-1 to CCR5 antagonists lies in the influence of the density of CCR5 on target cells. Viruses resistant to CCR5 antagonists can use either the free or inhibitor-bound forms of CCR5. But the entry process is usually less efficient when the virus uses the inhibitor-bound form of CCR5. At high concentrations of CCR5 antagonist, all the CCR5 molecules are theoretically in an inhibitor-bound conformation, without free CCR5 molecules. The reduced entry efficiency of resistant HIV-1 using the inhibitor-bound form of CCR5 can be compensated for if the density of CCR5 is increased at the surface of the target cells. We thus used an engineered cell line expressing a high level of CCR5 upon tetracycline induction and a normalized virus inoculum in each experiment to detect maraviroc resistance with maximum sensitivity. We found that maraviroc resistance of the virus from subject 14 was difficult to detect if the phenotypic assay was performed with cells bearing a low density of CCR5, as is the case for the original U87 CD4+ CCR5+ cell line (data not shown). The resistance of HIV-1 to CCR5 antagonists can also be assessed in peripheral blood mononuclear cells, but such assays are hampered by an important inter-individual variability. Differences in the degree of maraviroc resistance between the various subsets of CD4+ T cells could also be expected as CCR5 expression increases following cellular activation and differentiation towards effector memory cells. The level of HIV-1 resistance to entry inhibition by CCR5 antagonists could thus vary in vivo from one CD4+ T cell subset to another, being theoretically maximal in CCR5^high cells such as mucosal effector memory CD4+ T cells.

Taken together, our findings suggest that the genotypic determinants of maraviroc resistance are complex. V3 mutations seem necessary but not sufficient to induce maraviroc resistance, and their impact may depend on the env gene context in which

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**Figure 3.** Impact on maraviroc susceptibility of reversing the V3 mutations of PFZ-1 and PFZ-4 clones or introducing the same mutations in the BaL strain. (a) Restoration of maraviroc susceptibility by introducing T19A, F20L, A22T, D25E and V26I reverse mutations in clone PFZ-1 by site-directed mutagenesis (PFZ-1 to BaL mutagenesis). (b) Restoration of maraviroc susceptibility by introducing T19A, F20L, A22T and D25E reverse mutations in clone PFZ-4 by site-directed mutagenesis (PFZ-4 to BaL mutagenesis). (c) Induction of some maraviroc resistance by introducing A19T, L20F, T22A, E25D and I26V mutations in BaL by site-directed mutagenesis (BaL to PFZ-1 mutagenesis). (d) No impact on maraviroc susceptibility by introducing A19T, L20F, T22A and E25D mutations in BaL by site-directed mutagenesis (BaL to PFZ-4 mutagenesis). The median of three independent experiments is shown.
they occur. Simple assessment of the V3 genotype thus cannot accurately predict resistance to maraviroc. Rather, phenotypic assessment of virus particles expressing the envelope glycoprotein as a whole is required. Such an approach reveals that primary resistance of CCR5-tropic HIV-1 subtype B isolates to maraviroc seems uncommon.

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None to declare.

Supplementary data
Table S1 and Figures S1 to S3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


