Evolution of proviral DNA HIV-1 tropism under selective pressure of maraviroc-based therapy

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Received 8 November 2011; returned 18 December 2011; revised 10 January 2012; accepted 27 January 2012

Objectives: To evaluate the evolution of HIV-1 coreceptor tropism in proviral DNA of patients during maraviroc-based therapy.

Methods: Fourteen heavily high active antiretroviral therapy (HAART)-treated patients with a CCR5 Trofile profile were monitored over a 24 month period from the start of maraviroc therapy. Whole-blood samples were obtained at different timepoints, and coreceptor tropism was determined for proviral DNA from the V3-loop region sequence using the Geno2Pheno algorithm [false positive rate (FPR): 20%].

Results: At the start of maraviroc treatment, 13/14 patients were viraemic (median: 4.33 log copies/mL). Concordance in R5 tropism (R5/R5) was observed between circulating HIV-RNA (Trofile) and HIV-DNA provirus in 10/14 patients (median FPR = 54.0%), while 4 patients showed a CXCR4-tropic R5/X4 variant in their provirus (FPR: 5.8%, 5.7%, 16.6% and 1.1%, respectively). All R5/R5 patients showed a stable HIV-1 DNA coreceptor usage. Two out of four R5/X4 patients showed a tropism shift in their archived provirus and, after 6 months a prevalence of R5-tropic virus was detected in DNA. The other two R5/X4 patients harboured the 11/25 genotype, and maintained X4 tropism in provirus during the study. Virological response did not reveal differences in RNA decay and CD4+ cell recovery in patients with discordant tropism.

Conclusions: A relatively good correlation between RNA and DNA tropism was observed at baseline. Proviral DNA tropism remained stable over 24 months of maraviroc-based therapy, indicating that determination of proviral DNA V3 sequence could be used in tropism prediction in clinical practice. The data also confirm the importance of the 11/25 rule in predicting viral tropism.

Keywords: HIV-1 coreceptor, CCR5 inhibitors, V3 genotypetype, highly active antiretroviral therapy

Introduction

The HIV type 1 (HIV-1) virion requires a primary receptor, CD4, and a secondary chemokine receptor (such as CCR5 or CXCR4) to start cell infection. Over recent years the CCR5 entry coreceptor has become the target of a new class of inhibitors, with a novel drug, maraviroc, approved for clinical use. Because maraviroc targets the human coreceptor and not viral proteins, it was originally assumed that resistance acquisition by HIV would be slower. However, viral use of CCR5 in the presence of the pharmacological coreceptor blocker, and the emergence of preexisting minority CXCR4-tropic variants have been described.

Previous studies, both in vitro and in clinical trials, have described viral strains carrying different maraviroc-resistance-associated mutations within the third variable (V3) region of the viral gp120 gene. In these clinical trials, about half of the individuals with therapy failure showed a coreceptor switch from CCR5 (R5) to CXCR4 (X4). The detectable presence of dual mixed-tropic or X4 viruses has been associated with therapeutic failure under treatment with CCR5 antagonists.

The emergence of viral variants using the CXCR4 coreceptor is considered worrying by many clinicians because of its association with accelerated disease progression and reduced survival in untreated individuals.
Since maraviroc activity is restricted to patients harbouring exclusively R5-tropic variants, a phenotypic analysis for coreceptor usage is mandatory before prescription.\textsuperscript{10} The Trofile test, based on a recombinant phenotypic assay, is the most commonly used, but genotypic methods have also recently been proposed and validated.\textsuperscript{11,12} Coreceptor usage can be determined by bioinformatic prediction models based on analysis of the sequence of the HIV gp120 V3-loop region, which is the principal determinant of tropism.\textsuperscript{13,14} Such genotypic approaches have been developed for both plasma (amplification of viral RNA) and peripheral blood mononuclear cells (PBMC; amplification of proviral DNA). This approach is particularly important to determine viral tropism in patients with undetectable viral load, and could represent a valid tool to investigate the prevalence of virus tropism in archived viral genomes. It is important to note that tropic shift can occur in patients receiving highly active antiretroviral therapy (HAART) for 5 years with undetectable viral load.\textsuperscript{15}

Previous studies have found a concordance between HIV-RNA and HIV-DNA,\textsuperscript{16–18} even if the percentage of X4-tropic viruses proved enriched in cell-associated provirus.\textsuperscript{7,18}

Herein we present a study on V3-loop sequencing using whole blood of treatment-experienced patients who started maraviroc-optimized therapy and were monitored for 24 months. Our goal was to evaluate the evolution of provirus tropism in PBMC HIV-DNA of patients under the pressure of a CCR5 antagonist and its role as a tool to predict virological response.

**Methods**

The present study is part of an ongoing multicentre observational study on the use of new antiretroviral inhibitors in long-term treatment-experienced patients with HIV-1 (the NIA-ISS study). Patients had documented resistance or intolerance to the three main drug classes (nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs)) and were prescribed maraviroc (150 mg twice daily) in a context of salvage regimens. All patients included in this study had HIV coreceptor usage examined in plasma samples using the enhanced sensitivity Trofile assay (ESTA; Monogram Biosciences, South San Francisco, CA, USA), which is one of the most common diagnostic tests to determine HIV-1 coreceptor usage.

Clinical course and immunological and virological status were evaluated every 3 months, and biological samples were collected. Analysis of the V3 loop from whole blood was performed at entry (before initiation of maraviroc-based therapy) and at two or more subsequent timepoints. The study was conducted in compliance with the requirements of the sites’ Institutional Review Boards/Ethics Committees.

**Protease and reverse transcriptase resistance testing**

RT-PCR and sequencing were performed at clinical sites using commercial kits, according to the manufacturer's instructions. The genotypic susceptibility score (GSS) was calculated using the Stanford HIV Resistance Database.\textsuperscript{19} and was assigned to each drug included in the salvage regimen (0 for drugs to which the virus was considered to be resistant, 0.5 for partially resistant, 1 for no resistance). The sum of the scores for the individual drugs provided the global GSS of the antiretroviral regimen. Based on treatment history, when patients were naïve for a new drug with a new mechanism of action (i.e. enfuvirtide, raltegravir and maraviroc) the drug was assumed to be fully active and GSS = 1 was assigned.\textsuperscript{20} The number of drugs with an individual GSS score of 1 (fully active) was recorded for each regimen.

**Viral amplification and HIV-1 gp120 V3 region sequencing**

For sequencing the HIV-1 gp120 V3 domain, HIV-1 DNA was extracted from 200 μL of whole blood using a QIAamp Blood extraction kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

The HIV-1 env fragment (2.8 kb) including the V3 region was amplified by PCR using GP-160 primers and the Trugene core reagents kit (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA). The conditions for amplification were: one cycle at 94°C for 2 min, 37 cycles (94°C for 30 s, 60°C for 30 s and 68°C for 2 min) and a final step at 68°C for 7 min. PCR-amplified products were sequenced in both 5’ and 3’ directions using the V3-F2 forward primer Cy5 GGA CAATGTACACATGGAAAT (HXB2 nt 6959–6980) and the V3-RE2 reverse primer Cy5 AAAATTCCTCCCTACAATA (HXB2 nt 7352–7371)\textsuperscript{21} and the Trugene core reagents kit. The sequencing conditions were: one cycle at 94°C for 5 min, 30 cycles (94°C for 20 s, 54°C for 20 s and 70°C for 1.5 min) and a final step at 70°C for 5 min. Sequencing products were visualized on the OpenGene™ System automated sequencer and analysed using the sequence analysis software, with the limit for recognizing minority variants in a nucleotide mixture set to 20%.

**Genotypic determination of HIV-1 coreceptor usage**

Tropism prediction was performed with the clonal geno2pheno (G2P) prediction algorithm,\textsuperscript{22} with the false positive rate (FPR) set to 20%. The determination of the FPR at 20% was based on recommendations of European Guidelines for HIV-1 tropism, which suggest increasing the false-positive rate to 20% when only one sequence from a DNA sample can be generated for tropism analysis.\textsuperscript{12} The reported FPR was used as quantitative input. The FPR indicates the probability of falsely classifying an R5 variant as X4. The sample tropism was expressed as the proportion of non-R5 sequences within the sample’s viral population. Patients harboring < 20% non-R5 variants in PBMC were classified as having non-R5 virus. The presence of positively charged amino acids (arginine or lysine) at positions 11 and/or 25 was also evaluated.\textsuperscript{23,24}

**Statistical analysis**

Differences were evaluated using the Fishers’s exact test for categorical variables and Wilcoxon test for continuous variables. Significance levels were set at 0.05. All the analyses were performed using SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA).

**Results**

**Patients**

The population characteristics are reported in Table 1. Patients had a median age of 44.0 years and 85.7% were Caucasian. Of the 14 patients, 12 were infected with clade B virus. Most had a long history of HIV infection (median 18 years, range 2–23), with a median of 15 years of previous antiretroviral treatment, and more than 40% were in clinical stage C of the U.S. Centers for Disease Control and Prevention (CDC) classification. At the beginning of the maraviroc-based regimen all patients apart from one (patient 238) had a detectable viral load (median: 4.33
HIV-RNA log copies/mL) and a median CD4 cell count of 209 cells/mm³.

**Antiretroviral therapy**

Before starting maraviroc, regimens were based on a median of 4 drugs (range 1–6). Three patients were taking only lamivudine, and 9/14 were taking an NRTI-based regimen plus PIs. Five patients were taking enfuvirtide. All patients reported resistance to at least one drug of the main antiretroviral classes (NRTIs, NNRTIs and PIs).

The introduction of maraviroc was accompanied by marked changes in treatment; most of the new regimens included drugs from four different classes with a median of 5 drugs (range 3–7). Eight patients (57.1%) had co-administration of raltegravir; PIs and/or NRTIs were present in 71.4% of regimens and NNRTIs were present in 42.9%. Four patients (28.6%) were also taking enfuvirtide. Overall, the median GSS score, including maraviroc, was 3.75 (range 1.5–5.0) and the number of fully active drugs was 3 (1–5; Table 2).

**Virus tropism in plasma and whole blood at baseline**

Based on phenotypic HIV-RNA Trofile analysis, all patients harboured a CCR5-tropic virus and were thus eligible for maraviroc. V3-loop sequences were obtained from 14 of the 17 proviral DNA samples available at baseline, a success rate of 82.4% for amplification and sequencing. Only patients whose V3 regions were sequenced at baseline were included in the study.

According to Geno2pheno, most patients (71.4%, n=10) demonstrated CCR5-tropic viruses in their archived HIV-DNA (median FPR: 54.0%, range 44.2–89.3), before starting maraviroc-based therapy. Four patients (patients 052, 053, 238 and 251) showed CXCR4-tropic virus in their provirus (FPRs: 5.8%, 5.7%, 1.1% and 16.6%, respectively). Two R5/X4 patients (patients 238 and 251) presented the 11/25 genotype in the DNA V3-loop sequence (Table 2).

**Evolution of virus tropism in proviral HIV-DNA under maraviroc-based HAART**

Coreceptor usage was determined at two or more timepoints during a follow-up period of 24 months. The median interval between two subsequent analyses was 7 months (range 4–18 months), and the median number of timepoints for V3-loop sequencing was 4 per patient (range 2–4).

All patients harbouring R5-tropic virus at baseline maintained a stable V3-loop proviral genotype (less than 50% variation with respect to baseline levels, Table 2). The fluctuations in FPR are indicated in Figure 1. Of the four patients harbou
a prevalence of X4-tropic virus in proviral DNA at baseline, two (patients 052 and 053) showed a change of tropism from month 6, with subsequent dominance of R5-tropic proviral variants (Figure 1). The FPR values of these patients showed marked fluctuations during maraviroc-based therapy, even in the absence of detectable viral replication. Patients 238 and 251, who presented the 11/25 genotype, had a clear prevalence of the X4-tropic variant in HIV-DNA.

Virological response

Virological response did not differ between patients harbouring R5- or X4-tropic virus. After 3 months of maraviroc-based therapy, most patients had undetectable (<50 HIV-RNA copies/mL) plasma HIV-RNA (69.2%). No difference in decay of plasma RNA was observed between patients harbouring R5- or X4-tropic virus ($P=0.188$; Figure 2). At month 6, one patient (patient 054) had virological failure (plasma RNA >4.0 log copies/mL), and at month 12 exited the study. His tropism profile in DNA did not change over time, and from the V3-loop sequence analysis we could exclude resistance to maraviroc. Viral suppression persisted in most patients, and after 24 months of maraviroc-based therapy only three patients had detectable HIV-RNA plasma levels ($P<0.002$). All viraemic patients had predominance of the R5-tropic variant in archived DNA.

A positive trend in CD4-cell recovery was observed throughout the study period ($P=0.007$; Figure 3). The median cell recovery was +79 CD4 cells/mm$^3$ (range −48 to +218) after 3 months and +271 after 24 months. No differences in CD4 recovery were observed between patients harbouring X4 variants and patients with R5-tropic provirus.

Discussion

In this study viral tropism prediction was analysed using the web-based tool, geno2pheno[coreceptor], which predicts coreceptor usage from the V3 region of HIV-1 gp120. This bioinformatic software has been validated by many clinical studies that found a high concordance with the Trofile test.25–30 Although this study did not aim to compare geno2pheno[coreceptor] and Trofile for the prediction of coreceptor usage, our baseline data showed 71.4% concordance between the two approaches, which is consistent with data from analogous studies.17,31,32 According to G2P analysis, four patients harboured an X4 variant. A higher prevalence of X4 variants in PBMCs than in plasma has already been observed by other authors, for patients in both early17 and chronic phases of HIV infection,18 and this is consistent with the observation that the archived virus population may not correspond to the most prevalent variant in plasma.25,26
CXCR4 variants in provirus of maraviroc-treated patients

Figure 3. CD4 cell recovery during 24 months of maraviroc-based therapy. Black circles indicate patients harbouring a prevalence of X4-tropic variants at baseline.

CCR5 usage in proviral DNA was stable over time in all individuals harbouring R5-tropic variants. In most of our patients we observed highly conserved HIV-DNA V3-region sequences during the 24 months on maraviroc-based therapy. Switches over time in HIV-DNA tropism under suppressive HAART are rare;11,35 our findings argue against a selective pressure towards X4 evolution under suppressive therapy, thereby confirming that changes in tropism occur with low frequency in maraviroc-treated patients.36 Moreover, changes in the cellular reservoir could tend to be less frequent in patients with undetectable viraemia. The two patients carrying the 11/25 genotype harboured the X4-tropic virus throughout the follow-up period. It has been observed that the base changes at position 11 and 25 are neither necessary nor sufficient for a phenotypic switch37 and our data confirms that the 11/25 rule is one of the most important genotypic predictors of X4-tropic virus.18

Conversion from X4 to R5 tropism in HIV-DNA was observed in the other two R5/X4 patients with a prevalence of X4-tropic provirus at baseline. The conversions occurred in the first 6 months of maraviroc-based therapy, and the R5 variant remained prevalent in the following months. Why and under which circumstances the X4→R5 shift occurs is not well understood. However, consistent with our findings, previous studies reported that conversion from X4 to R5 variants is generally observed shortly after the initiation of antiretroviral therapy, before complete suppression of plasma viraemia.15,39

This small study confirms the efficacy of maraviroc-based therapy in multidrug-experienced HIV patients.6,60 In our study, most patients introduced to maraviroc-based therapy had a good virological response, and plasma viraemia was undetectable as early as month 3. The low number of HIV-DNA X4-tropic patients did not permit statistical analysis, but the presence of X4 variants in archived virus did not seem to influence immunovirological response, as already observed in a previous study.61 One patient with R5-tropic provirus exited the study because of virological failure (probably due to scarce adherence, data not shown), and no variation in the HIV-DNA V3-loop sequence was recorded. During the clinical monitoring, 3 patients (all with baseline R5-tropic provirus) remained viraemic and R5-tropic during follow up. Unfortunately we were unable to verify the DNA-RNA tropism concordance because no plasma samples were available to analyse RNA tropism.

The major limitation of our study is the small number of patients. However the longitudinal analysis of proviral HIV-DNA V3 loop demonstrated stability of the V3 region under maraviroc-selective pressure. In aviremic patients, the evaluation of HIV-DNA coreceptor usage might be important to investigate whether administration of maraviroc selects for HIV variants that prefer the CXCR4 coreceptor. Our results suggest that DNA V3-loop sequencing could be a useful tool to predict shift in virus tropism, and this study suggests that determination of the HIV-1 V3 loop in DNA and RNA populations may provide different yet complementary information.

Acknowledgements
We thank Tonino Sofia for his help in revising the final manuscript, Mrs Stefania Donnini and Alessandra Mattei for secretarial help and all the patients who participated in this study. We also thank P. Cocco and F. Costa for technical support. We are grateful to Mr Stefano Lucattini for computer support.

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Division of Infectious Disease University of Bari, Bari, Italy (G. Angarano, N. Ladisa, L. Monno and A. Volpe); Division of Infectious Diseases, Department of Experimental Medicine, University of Perugia, Perugia, Italy (F. Baldelli, D. Francisci, A. Mercuri and S. Bostianelli); Department of Infectious Diseases and Hepatology, Azienda Ospedaliera di Parma, Parma, Italy (C. Ferrari, A. Degli Antoni and A. Cavalli).

Funding
This work was supported by a grant from the National Program on Research on AIDS 2009–2010. No funding was received for this work from any of the following organizations: National Institutes of Health (NIH); Wellcome Trust; and the Howard Hughes Medical Institute (HHMI).

Transparency declarations
None to declare.

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