Virological failure of patients on maraviroc-based antiretroviral therapy

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Objectives: Virological failure (VF) in patients on maraviroc-based treatment has been associated with altered HIV tropism and resistance to maraviroc. This multicentre study aimed to characterize VF in patients treated with maraviroc.

Methods: We analysed 27 patients whose treatment failed between 2008 and 2011. They had been screened for HIV tropism before maraviroc initiation using population-based V3 genotyping. HIV-1 tropism and resistance of R5 viruses to maraviroc at VF and at baseline were determined retrospectively using an ultrasensitive recombinant virus assay (RVA).

Results: Viruses from 27 patients given maraviroc on the basis of the R5 genotype were characterized at the time of treatment failure. The RVA indicated that 12 patients harboured CXCR4-using viruses and 15 (56%) had pure R5 viruses at failure. One-third of those harbouring CXCR4-using viruses (4/12) were infected with R5X4/X4 viruses according to the RVA before maraviroc initiation. We analysed the phenotypic resistance to maraviroc of four patients harbouring R5 viruses at failure; two harboured viruses whose maximum percentage inhibition was reduced by 65%–90%, while the other two were infected with susceptible viruses. All patients had effective concentrations of drugs.

Conclusions: Half of the maraviroc-treated patients who experienced VF harboured CXCR4-using viruses at failure, one-third of them were detected by a phenotypic method before maraviroc initiation. Phenotypic assessment of R5 virus resistance to CCR5 antagonists at failure could help optimize antiretroviral therapy.

Keywords: HIV-1 tropism determination, entry, phenotypic assay, resistance to maraviroc, CXCR4

Introduction

HIV tropism must be determined prior to antiretroviral treatment with a CCR5 antagonist to indicate cell entry exclusively via the CCR5 coreceptor (R5 virus), CXCR4 alone (X4 virus) or both receptors (RSX4 virus). HIV tropism can be determined genotypically or phenotypically, based on analysing the gp120 virus envelope. European guidelines for clinical use of the first CCR5 antagonist maraviroc rely on the genotypic approach. Maraviroc-based antiretroviral treatment may fail due to selection of pre-existing minor CXCR4-using viruses or resistance of CCR5-using viruses that can enter cells via maraviroc-bound CCR5. Mutations in the envelope gene of CCR5-tropic HIV-1 have been described in patients who experienced virological failure while on maraviroc-based therapy. Most are located in V3 of Env, the key region of gp120 for interacting with the coreceptors. However, the patterns of V3 mutations vary from one patient to another and between HIV-1 subtypes. Thus, the reference assay for determining the susceptibility to maraviroc of HIV in patients who experience treatment failure is phenotypic assessment of
Virological failure of maraviroc treatment

virus particles bearing the envelope glycoprotein as a whole. However, little is known about the relative importance of resistance mechanisms when maraviroc therapy fails in clinical practice.

We used an ultrasensitive recombinant virus assay to characterize the virological failure of 27 patients treated with maraviroc by retrospectively determining HIV-1 tropism and resistance of R5 viruses to maraviroc at the time of failure and at baseline.

Methods

Study population and samples

We studied 27 HIV-1-infected patients from 13 centres throughout France who were being treated with maraviroc and an optimized background therapy. They had all experienced treatment failure between 2008 and 2011. Inclusion criteria were HIV-1 infection, age ≥18 years and therapeutic failure while on maraviroc with plasma RNA >50 copies/mL at >6 months after maraviroc initiation. All the patients had been treated previously and had experienced virological failure. The patients had been screened for HIV tropism before maraviroc initiation using population-based V3 sequencing and the geno2pheno algorithm with a 10% false-positive rate and the combined 11/25 and net charge rule for HIV-1 non-B subtypes. Geno2pheno is available at http://coreceptor.geno2pheno.org. Phenotypic characterization of HIV-1 coreceptor usage

We sequenced the complete V3 loop of virus extracted from plasma samples obtained before maraviroc initiation and at the time of failure. The PCR primers and conditions and the sequencing primers are described in the ANRS consensus technique (http://www.hivfrenchresistance.org). The geno2pheno algorithm with a 10% false-positive rate and the combined 11/25 and net charge rule for HIV-1 non-B subtypes. Geno2pheno is available at http://coreceptor.geno2pheno.org.

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

The phenotypic resistance of CCR5-tropic HIV-1 to maraviroc was assessed by retrospectively determining HIV-1 tropism and resistance of R5 viruses to maraviroc. The median CD4 cell count at the time of maraviroc initiation was 185 (95% CI 7–50.3) in CCR5 high cells. The Bal strain and two virus clones isolated from subjects whose maraviroc-based therapy (Pfizer) had failed were used as CCR5-tropic maraviroc-sensitive and maraviroc-resistant controls. All assays were performed at least three times and median values are shown. Curves of entry inhibition versus maraviroc concentrations were drawn using GraphPad Prism 4.0c. Entry inhibition of 50% (IC50) was obtained previously at a median maraviroc concentration of 13.4 nM (95% CI 7–50.3) in CCR5 antagonists’ naïve patients.

Results

Patient characteristics

Patients experiencing virological failure while on a maraviroc-based regimen were recruited from 13 virological centres participating in the ANRS resistance study group. The 419 patients on a maraviroc-based regimen between 2008 and 2011 included 56 (13%) who experienced virological failure. These 56 patients included 6 whose samples taken before treatment initiation were missing and 23 others whose samples taken at the time of failure or at maraviroc initiation could not be amplified mainly because the virus load was too low to amplify a 2200 nt fragment. Thus, we studied 27 patients whose virus had an R5 genotype before maraviroc initiation. All patients were treatment experienced and were given maraviroc plus an optimized background regimen. The median CD4 cell count at the time of maraviroc initiation was 247 cells/mm3. Most patients were infected with subtype B (20/27; 74%) or CRF02-AG (4/27; 15%) (Table 1). Two patients had undetectable viremia at maraviroc initiation; they were switched to maraviroc for toxicity reasons. The other 25 patients had a mean virus load of 3.58 log copies/mL before threshold after 6 months of maraviroc treatment. The TT4 phenotypic assay was used to determine HIV-1 tropism; this assay has been accredited according to the International Organization for Standardization 15189 standards. Briefly, a fragment of virus RNA encoding gp120 and the ectodomain of gp14 was amplified by RT–PCR using HIV-1 RNA isolated from plasma or by PCR using HIV-1 DNA isolated from blood cells. The PCR products were then amplified by nested PCR. Two amplifications were performed in parallel from each sample and pooled to prevent sampling bias. The phenotype of HIV-1 coreceptor usage was determined using a recombinant virus entry assay with the pNL43-Denv-Luc2 vector. 293T cells were co-transfected with NheI-linearized pNL4.3-Denv-Luc2 vector DNA and the product of the nested PCR obtained from the HIV-1-containing sample. The chimeric recombinant virus particles released into the supernatant were used to infect U87 indicator cells bearing CD4 and either CCR5 or CXCR4. Virus entry was assessed by measuring the luciferase activity in lysed cells (as relative light units; RLU). Minor X4 variants were detectable when present at a rate of ≥0.5%.

Phenotypic characterization of HIV-1 coreceptor usage

The samples from the 13 virological centres were phenotyped at the Virolögy Laboratory, Toulouse University Hospital, Toulouse, France. The Toulouse tropism test (TTT) was performed on samples taken at two times: one prior to maraviroc initiation and one at virological failure, defined as the first sample with a plasma virus load above the detection threshold after 6 months of maraviroc treatment. The TTT phenotypic assay was used to determine HIV-1 tropism; this assay has been accredited according to the International Organization for Standardization 15189 standards. Briefly, a fragment of virus RNA encoding gp120 and the ectodomain of gp14 was amplified by RT–PCR using HIV-1 RNA isolated from plasma or by PCR using HIV-1 DNA isolated from blood cells. The PCR products were then amplified by nested PCR. Two amplifications were performed in parallel from each sample and pooled to prevent sampling bias. The phenotype of HIV-1 coreceptor usage was determined using a recombinant virus entry assay with the pNL43-Denv-Luc2 vector. 293T cells were co-transfected with NheI-linearized pNL4.3-Denv-Luc2 vector DNA and the product of the nested PCR obtained from the HIV-1-containing sample. The chimeric recombinant virus particles released into the supernatant were used to infect U87 indicator cells bearing CD4 and either CCR5 or CXCR4. Virus entry was assessed by measuring the luciferase activity in lysed cells (as relative light units; RLU). Minor X4 variants were detectable when present at a rate of ≥0.5%.

Genotypic prediction of HIV-1 coreceptor usage

We sequenced the complete V3 loop of virus extracted from plasma samples obtained before maraviroc initiation and at the time of failure. The PCR primers and conditions and the sequencing primers are described in the ANRS consensus technique (http://www.hivfrenchresistance.org). The geno2pheno algorithm with a 10% false-positive rate and the combined 11/25 and net charge rule for HIV-1 non-B subtypes. Geno2pheno is available at http://coreceptor.geno2pheno.org.
maraviroc initiation. The GSSs of the optimized treatment without maraviroc were 1–4.5 (median 2). The mean time to virological failure on maraviroc was 7 months and the mean virus load at failure was 3.26 log copies/mL.

**HIV-1 tropism in patients failing maraviroc treatment**

TTT was used successfully to determine the HIV-1 tropism in 27 patients who underwent virological failure while on maraviroc treatment. Over half of them (15; 56%) harboured pure R5 viruses and 12 (44%) harboured CXCR4-using viruses (9 R5X4 and 3 X4) at the time of failure (Table 2). Virus phenotypes were also determined before maraviroc initiation. The 12 patients failing with CXCR4-using viruses included 4 (33%) infected with R5X4 or X4 viruses before maraviroc initiation. All the patients experiencing virological failure with an R5 phenotype were infected with pure R5 viruses before treatment. Therefore, all viruses had a predicted R5 genotype before maraviroc initiation, but 4/27 (15%) were phenotyped R5X4/X4. The R5 genotype was predicted using the geno2pheno algorithm with a false-positive rate of 10% (R5 viruses thus have V3 scores between 11 and 100). The closer the score is to 10, the greater the risk of mispredicting the virus tropism. The geno2pheno scores of the four viruses with a discordant R5 genotype were between 52 and 62.

**Causes of virological failure with R5 phenotype viruses during treatment with maraviroc**

The 15 patients harbouring R5 viruses at the time of failure included 11 whose sensitivity to maraviroc was not investigated because their RNA virus loads at failure were too low (mean 2.5 log copies/mL) for successful amplification and no further samples were available. Thus, the sensitivities to maraviroc of four patients (Patients 1, 5, 8 and 15) were investigated, together with the concentrations of antiretroviral drugs. Patients 1 and 5 harboured viruses that were sensitive to maraviroc before treatment and at failure (Figure 1a). The virus from Patient 1 was very sensitive to maraviroc at baseline, as a maraviroc concentration of 195 ng/mL for Patient 1 were in the expected range for concentrations of darunavir (4985 ng/mL), etravirine (136 ng/mL) and maraviroc at baseline, as a maraviroc concentration of 10 nM. The concentrations of antiretroviral drugs were measured at both times of analysis (Figure 1b). The virus was still sensitive inhibited virus entry 100%. The V3 sequences were identical at both times of analysis (Figure 1b). The virus was still sensitive inhibited virus entry 100%. The V3 sequences were identical
the nucleos(t)ide inhibitors is not recommended, but abacavir, lamivudine and tenofovir (648, 1419 and 75 ng/mL, respectively) were measured in the sample from Patient 5. The concentrations of saquinavir (1611 ng/mL) and maraviroc (803 ng/mL) for Patient 5 were in the expected range for recommended dosages.

Patient 8 was infected with a resistant R5 virus before treatment and at the time of failure. This primary resistance reduced the maximum percentage of inhibition by 90%. The V3 sequence at virological failure showed the emergence of mutations, as previously described in patients failing a maraviroc-based treatment: 19T (threonine), 20F (phenylalanine) and 26V (valine) (Figure 1b). Patient 15 was infected with a resistant R5 virus before maraviroc initiation. The maximum percentage of inhibition was reduced by 65%. The R5 virus infecting Patient 15 was no longer resistant to maraviroc at virological failure (verified in two independent experiments). The V3 sequences from Patient 15 changed slightly between maraviroc initiation and the time of failure. The concentrations of antiretroviral drugs were measured 1 week after the treatment of Patient 8 was changed and maraviroc was no longer part of the regimen. The concentrations of abacavir (10 ng/mL), lamivudine (107 ng/mL) and maraviroc (117 ng/mL) were effective in the sample from Patient 8, while that of darunavir (1137 ng/mL) was probably ineffective.

**Discussion**

We characterized virological failure in treatment-experienced patients on a maraviroc-based regimen. Phenotypic assays performed at the time of failure showed that 44% of the patients harboured CXCR4-using viruses. Although the genotypic assay did not predict X4 viruses in these patients before maraviroc initiation, the phenotypic assay retrospectively detected CXCR4-using variants in one-third of them. The viruses found in some of the patients harbouring R5 viruses at failure were more or less insensitive to maraviroc prior to treatment.

We believe that this is the first large study of virological failure while on a maraviroc-based regimen that is not part of maraviroc clinical trials. The estimated number of patients given maraviroc during the study period was 419, 56 of whom experienced virological failure (13%). The percentage of failure was much lower than expected.
lower than in the MOTivate (55%) and MERIT (30%) studies. Maraviroc was approved by the EMA for use in pre-treated patients but was often used to replace the third antiretroviral drug for tolerance or toxicity reasons in patients in virological success unlike those in the MOTivate study. This could be why 87% of the maraviroc-treated patients were in virological success. The patients experiencing virological failure were heavily pre-treated and harboured viruses resistant to multiple drug classes. Half of the patients were treated with three or fewer active drugs after adding maraviroc, which undoubtedly favoured the virological failure.

The TTT phenotypic assay showed that 44% of the patients were infected with CXCR4-using viruses at the time of failure. Others have obtained results similar to ours. CXCR4-using viruses were detected in 57% of the MOTivate patients at the time of failure and in 46% of those in the maraviroc expanded-access programme (A4001050). The presence of CXCR4-using viruses in patients given maraviroc could be due to selection of pre-existing minor CXCR4-using viruses or to a change in HIV-1 tropism enabling the virus to use CXCR4 for entry, probably after mutations in the envelope gene. The 12 of our patients failing with R5X4 viruses included four infected with CXCR4-using viruses at baseline according to the TTT phenotypic assay. Low plasma virus loads may induce PCR sampling bias, but the virus load was >3 log copies/mL in 10/12 samples at baseline. However, the other two samples with a viral load <3 log copies/mL were R5 and the phenotypic assay may not have been sensitive enough to detect minor X4 variants. CXCR4-using viruses emerged in the other eight patients after 4–11 months of treatment, probably from cellular reservoirs of HIV under CCR5 antagonist pressure. It is also possible that there was a tropism switch due to mutations in the HIV-1 envelope, but studies suggest that this is rare even under CCR5 antagonist selection pressure. Moreover, only one of the nine patients who continued treatment with maraviroc for 10 months after the first failure experienced a tropism switch from R5 to R5X4 after 12 months of replication. DNA templates were used for the phenotypic assay when RNA amplification failed. The tropism determination had no influence because CXCR4-using variants were detected using DNA templates for two

### Figure 1. Assessment of maraviroc susceptibility of CCR5-tropic isolates from four patients. (a) Phenotypic assessment of maraviroc sensitivity. Dose–response curves showing the percentage inhibition of HIV-1 entry by increasing maraviroc concentrations. Each curve represents a different sample. PFZ04 cl02 A is a maraviroc-resistant control. (b) V3 genotype of the samples assessed for maraviroc susceptibility. V3 amino acid sequence alignments were obtained by bulk sequencing env PCR products from the samples phenotyped at baseline and at failure of maraviroc treatment. Dots indicate identity with amino acid baseline sequence.
patients for whom CXCR4-using variants were detected in plasma at failure.

Four CXCR4-using viruses were not detected at baseline using the population-based sequencing and genotypic prediction. The population-based sequencing detects viruses accounting for >20% of the virus population, while TTT detects minor variants accounting for <1% of the virus population. European guidelines indicate that the genotypic method can be used to predict tropism but the panel of experts advised us to use triplicate PCR amplification and sequencing testing to maximize assay sensitivity. We determined virus genotypes at baseline using single PCRs and sequencing, which could be less sensitive for detecting low percentages of X4 variants. Thus, in our study, the use of a phenotypic assay rather than a population-based genotypic assay to determine tropism may have prevented 15% of treatment failures. The detection of minor CXCR4-using variants could be improved by shifting from population-based sequencing to ultra-deep sequencing, whose sensitivity approaches that of a phenotypic assay. The performance of the genotypic approaches depends also on the accuracy of the genotypic algorithms; these may be inadequate for non-B subtypes of HIV-1. However, the four patients with genotype–phenotype discordance were infected with subtype B viruses and tropism was predicted using geno2pheno with a 10% false-positive rate previously validated for this subtype.

We investigated the antiretroviral drug concentrations and resistance to maraviroc of the R5 phenotype virus in four patients who experienced virological failure. At failure, the virus of one patient was resistant to maraviroc using a phenotypic assay that showed a reduction in the maximum percentage of inhibition, while the other three viruses were sensitive to maraviroc and had effective plasma maraviroc concentrations. Over half (54%) of the patients who experienced virological failure in the maraviroc expanded-access programme (A4001050) and had an R5 phenotyped virus were infected with viruses resistant to maraviroc. Too few patients were characterized for phenotypic resistance for us to evaluate the frequency of resistance to maraviroc at failure. The patient whose virus was resistant to maraviroc at failure also harboured resistant virus prior to initiating treatment. Primary resistance to maraviroc is thought to be rare but could be more common in patients experiencing virological failure during treatment with maraviroc. The evolution of the maraviroc susceptibility in Patient 15 was uncommon since the phenotypic resistance observed before treatment could not be demonstrated at the time of failure despite a plasma viral load >3 log copies/mL. The ineffective concentration of darunavir may have contributed to the virological failure of Patient 15, together with the intermediate resistance to the nucleoside inhibitors that were part of the regimen.

Resistance to maraviroc follows mutations in the virus envelope gene (env) to give CCR5-using viruses that can use maraviroc-bound CCR5 for entry. The amino acid patterns in patients failing maraviroc treatment have been described, but each virus harboured different patterns. Our previous studies have shown that these motifs are not sufficient to confer maraviroc resistance in naive patients. The V3 genotypes at the time of failure were analysed, but the determinants associated with maraviroc failure or resistance were not identified. The virus of Patient 8 developed mutations in the V3 region, but they were not detected before treatment although the phenotype showed primary resistance to maraviroc.

Two patients failed the maraviroc-based treatment although their viruses were sensitive to maraviroc and the drug concentrations were effective at failure. The patients were all highly treatment experienced and may have acquired viruses less sensitive to the antiretrovirals. The virus from Patient 1 had intermediate resistance to etravirine that was associated with darunavir and maraviroc; therefore, the efficacy of the whole ART regimen was suboptimal. The IC50 of maraviroc for the virus from Patient 1 underwent a great shift but maraviroc completely inhibited virus entry, suggesting the existence of viruses hypersensitive to maraviroc as previously described. This observation should be confirmed by further phenotypic analyses of maraviroc susceptibility. The IC50 of maraviroc in Patient 5 varied a little, which could be attributed to assay variability or to PCR sampling bias in the sample at failure due to low plasma virus load. This patient received three nucleoside inhibitors against which the virus resistance was intermediate. Thus, only saquinavir and maraviroc were fully active in the regimen.

In conclusion, half of the patients previously treated with antiretroviral drugs whose maraviroc-containing regimen failed harboured CXCR4-using viruses at failure. Direct sequencing and genotyping did not detect these viruses before treatment, but one-third of them would have been detected using a phenotypic assay. We also identified the primary resistance of R5 viruses to maraviroc that may explain virological failure. Further studies are now needed to evaluate the prevalence of primary resistance to maraviroc. Characterization of viruses at the time of failure indicates that sensitive tropism assays should be used before maraviroc treatment to prevent the emergence of CXCR4-using viruses. In addition, patients with R5 viruses who experience virological failure may benefit from a phenotypic assay to detect resistance to maraviroc.

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None to declare.
Author contributions
S. R. and J. I. designed the study, analysed the data and wrote the manuscript. D. Descamps coordinates the ANRS AC11 Resistance Study Group. A. M., C. A., D. Descamps, M. A. T., D. Desbois, P. B., C. D., C. S. and A. G. M. performed virology analyses and provided clinical samples. G. P. performed pharmacology analyses.

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