Maraviroc is able to inhibit dual-R5 viruses in a dual/mixed HIV-1-infected patient

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Objectives: Maraviroc is the first licensed chemokine co-receptor 5 (CCR5) co-receptor antagonist in clinical practice. It is currently being used in patients harbouring exclusively CCR5-tropic virus. The objective of the study was to investigate the impact of maraviroc on viruses with different co-receptor preferences in a patient with a dual/mixed (D/M) infection.

Methods: We present a case report of an HIV-1 patient infected with a D/M virus population. Co-receptor tropism was determined by phenotypic and genotypic tests. Biological clones from pre- and post-maraviroc therapy were generated. Tropism of these infectious clones was investigated in U373-MAGI cells expressing CD4+CCR5+ or CD4+CXCR4+. Maraviroc susceptibility and viral replication were determined using donor peripheral blood mononuclear cells (PBMCs).

Results: In-depth clonal genotypic analysis revealed the presence of both R5-tropic variants and X4-tropic viruses before the start of maraviroc. During maraviroc therapy all R5-predicted viruses were suppressed. Phenotypic analyses revealed that all biological clones before maraviroc therapy could infect both CCR5- and CXCR4-bearing U373-MAGI cells, demonstrating dual tropism. The baseline biological clones preferentially infected the CCR5 cell line and were fully susceptible to maraviroc in PBMCs (dual-R5). In contrast, during maraviroc therapy the dual-R5-tropic viruses were replaced by more X4-tropic viruses (dual-X4), which could not be inhibited by maraviroc.

Conclusions: This case report demonstrates that dual-tropic viruses, capable of using both co-receptors in phenotypic assays, can be inhibited by maraviroc if they have a CCR5 co-receptor preference in vivo.

Keywords: tropism, co-receptor, CCR5 antagonist

Introduction

HIV-1 entry into host cells requires binding of the viral envelope protein to the CD4 receptor and subsequently to a chemokine co-receptor (CCR5, CXCR4).1,2 Viral populations in an infected patient can be categorized by phenotypic tests as R5-tropic, X4-tropic or dual/mixed (D/M); use of both co-receptors by one virus (dual) and/or a mixture of CCR5- and CXCR4-using (X4) viruses (mixed).3 Genotypic tests predict viral co-receptor tropism based on the sequence of the viral envelope by means of interpretation algorithms (R5 or X4 prediction). Inhibition of co-receptor usage is a new antiretroviral strategy and multiple compounds are now being studied. Maraviroc is the first licensed CCR5 antagonist and in clinical trials demonstrated potent activity in patients in whom only CCR5-tropic viruses were detected.4,5 In general, no added value of maraviroc with respect to viral efficacy was observed in patients harbouring D/M-tropic viral populations, which are capable of using the CXCR4 co-receptor.6 Nevertheless, detailed genotypic analysis demonstrated that virological response could be achieved in a subset of these patients with <10% of X4-predicted viruses in their viral population.7 Here we present a report of a patient with a D/M viral population where maraviroc inhibited not only R5-tropic viruses but also a range of dual-tropic viruses.

Methods

The patient participates in the AIDS Therapy Evaluation in the Netherlands (ATHENA) observational cohort, which has been approved by local and national institutional review boards.
Genotypic analysis

Viral RNA was isolated from 200–1000 μL of plasma/serum as described previously. The V3 region of the envelope was amplified using primers V3-1 (5’-TATCCTTGGCAACATTCCACAT-3’) and V3-2 (5’-CACTGGAAAATTCCTCGTACCA-3’) (Superscript-III One-Step Platinum Taq, Invitrogen). Nested PCR using primers V3-3 (5’-AATCCTGGCAACATTCAAAAGTGG-3’) and V3-4 (5’-AGACTGAAATTCCTCGTACCA-3’) was performed (Expand High Fidelity PCR System, Roche). PCR-amplified products were ligated (pGEM-T Easy Vector; Promega) and sequenced using nested primers. Viral co-receptor tropism was predicted using Geno2PhenoCo-receptor [R5 prediction, >10%; and X4, ≤10% false positive rate (FPR)] and Web PSSM (where PSSM stands for position-specific scoring matrices) (R5 prediction, >6.69; and X4, ≤2.88; the 11/25 rule was applied at intermediate values). Genotypic sensitivity scores (GSSs) were calculated using the Stanford HIVdb algorithm.

Phenotypic analysis

Cells

MT-2 cells were maintained in culture medium [CM; RPMI1640 with L-glutamine (BioWhittaker), 10% fetal bovine serum (FBS; Biochrom AG) and 10 mg/L gentamicin (Gibco)]. U373-MAGI cell lines were maintained as recommended by the NIH AIDS Research and Reference Reagent Program. Donor peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll–Paque density gradient centrifugation of heparinized blood from five HIV-seronegative donors (CCR5-homozygous). The mix was stimulated for 2–3 days with phytohaemagglutinin (2 mg/L) in CM. Cells were incubated at 37°C and 5% CO₂.

MT-2 cell culture and generation of biological clones

Patient-derived PBMCs (1 × 10⁶) prepared by Ficoll–Paque density gradient centrifugation were co-cultured in triplicate with 1 × 10⁶ CXCR4-CCR5-MT-2 cells in CM. Viral cultures were maintained for 3 weeks and monitored for syncytium formation. Positive viral cultures were used for the generation of biological clones by infecting 4 × 10⁴ MT-2 cells/well in a 96-well plate in a 5-fold dilution series. In cases where less than one-third of viral cultures were positive, virus was harvested. These biological clones were expanded by infecting 1 × 10⁶ MT-2 cells. Supernatant was harvested, and p24 was measured and sequenced. No nucleotide differences were observed after expansion and the biological clones corresponded to the dominant viral population at the specific timepoints. The 50% tissue culture infective dose (TCID₅₀) was determined on donor PBMCs. These biological clones were subsequently used in phenotypic analysis.

Co-receptor usage and inhibition in U373 cells

At day 0, 1 × 10⁶ cells/well of U373-MAGI-CCR5 or U373-MAGI-CXCR4, expressing CD4+, CCR5+, and CXCR4+, and CD4+ CCR5–CXCR4+, respectively, were plated into a 96-well plate in 100 μL of Dulbecco’s modified Eagle’s medium (DMEM; BioWhittaker) with 10% FBS and 10 μg/mL gentamicin. Subsequently, at day 1, medium was discarded and replaced by 150 μL DMEM with 10% FBS and 10 μg/mL gentamicin, with a final concentration of 10 μM maraviroc, 1 μM AMD-3100 (a CXCR4 inhibitor) or no inhibitor. This was incubated for 1 h at 37°C. Subsequently, 1 ng of p24 of the biological clones, Bal or HXB2 suspended in 50 μL of CM was added and incubation was continued for 2 days at 37°C. Subsequently, luminescence was measured using the Galacto-Star™ β-Galactosidase Reporter Gene Assay System for Mammalian Cells (Applied Biosystems) according to the manufacturer’s protocol using 20 μL of lysis buffer and 60 μL of reaction buffer. Background activity (cells without virus in the absence or presence of inhibitor) was subtracted from the activity of the wells containing virus.

Analysis of maraviroc and AMD-3100 susceptibility in PBMCs

Donor PBMCs were infected with a biological clone using a multiplicity of infection (MOI) of 0.001 (PBMC titration) in CM with 5 U/mL IL-2 and incubated for 2 h at 37°C, after which cells were washed twice. Subsequently, 0.2 × 10⁶ cells/well were plated into a 96-well plate with 5 U/mL IL-2 in CM containing increasing concentrations of maraviroc or AMD-3100. p24 was analysed on days 0 and 7.

Analysis of viral replication capacity in PBMCs

Donor PBMCs (5 × 10⁶) were infected with a biological clone (50 ng of p24) in 1 mL of CM, incubated for 2 h at 37°C and washed twice. Cells were cultured in 10 mL of CM with 5 U/mL IL-2. Cells were incubated for 14 days and p24 was analysed daily. Viral replication of all biological clones was comparable.

Results

A 51-year-old man was diagnosed with HIV-1 subtype B infection in 1992. Zidovudine monotherapy was initiated and he was subsequently treated with multiple antiretroviral regimens, including integrase and fusion inhibitors. Initial antiretroviral monotherapy and add-on therapy together with intolerability to enfuvirtide, darunavir and other drugs resulted in frequent virological failure and selection of multidrug-resistant HIV (cumulative resistance profile: RT, 41L-675-69del-741V-98G-103N-118I-Y181C-184V-190A-210W-215Y-219E; PR, 10I-20I-361-43T-46I-54V-62V-63P-71V-73S-82C-84V-90M; and IN,

Image Description:

Figure 1. Schematic representation of HIV-1 tropism and corresponding phenotypic and genotypic test results. OTA, original Trofile assay; ESTA, enhanced sensitivity Trofile assay; NSI, non-syncytium-inducing (correlates to CCR5 usage); SI, syncytium-inducing (correlates to CXCR4 usage).
Q95K-V151I-N155H). Due to a lack of alternative treatment options, sustained virological suppression was never achieved and immunological deterioration was observed (CD4+ cell count <50 cells/mm³). In 2008, the patient received tenofovir, raltegravir and tipranavir/ritonavir and presented with a CD4+ cell count of 4 cells/mm³ and plasma HIV-RNA of 5.6×10⁴ copies/mL. Efforts to design an effective antiretroviral regimen included determination of viral co-receptor tropism using phenotypic assays, namely Trofile (original Trofile assay and enhanced sensitivity Trofile assay); Monogram Biosciences] and MT-2.¹¹ These assays demonstrated that the viral population was capable of using the CXCR4 co-receptor (D/M and

![Figure 2](https://academic.oup.com/jac/article-abstract/66/4/890/724727)

**Figure 2.** (a) Plasma HIV-RNA levels and CD4+ cells/mm³ during maraviroc (MVC) therapy. (b) V3 loop sequence analysis at start of MVC (t0) and subsequent timepoints (t1–t3) during MVC therapy. Amino acid (AA) positions associated with HIV tropism are boxed, and amino acid changes as selected in the patient associated with X4 prediction are depicted in red italics. Genotypic interpretation of co-receptor tropism by Geno2Pheno[co-receptor using an FPR of 10% and Web PSSM is indicated (R5 prediction is given in green and X4 prediction is given in red italics). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
Figure 3. (a) Co-receptor preference in the CCR5 and CXCR4 cell lines (n=3). (b) Entry inhibition in the CCR5 and CXCR4 cell lines with no inhibitor, maraviroc (MVC) or AMD-3100. Standard error of the mean is depicted (n=3). (c) Representative MVC susceptibility assay in donor-derived PBMCs. Biological clones pre-MVC therapy (t0-I and t0-II), biological clones obtained during MVC therapy (t2-I, t2-II and t2-III) and the reference strains HXB2 and Bal were used. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
CCR5 co-receptor, as indicated by full inhibition by a CXCR4
mainly used the CXCR4 co-receptor and not maraviroc-bound
viral entry in this cell line (Figure 3b). Interestingly, in PBMCs
be inhibited by AMD-3100, whereas maraviroc did not inhibit
(Figure 3c). These maraviroc-resistant biological clones (t2)
exposure (t2) were only partly inhibited by maraviroc
biological clones obtained during subsequent maraviroc
clones could be fully inhibited by maraviroc, indicating their
which both co-receptors were expressed, all baseline biological
entry efficacy in the CXCR4 cell line as compared with the CCR5
clones obtained at t2 (t2-I, t2-II and t2-III) showed a higher
compared with the CXCR4 cell line. In contrast, the biological
were observed in reverse transcriptase and protease.
Co-receptor usage of the biological clones at baseline (t0-I
and t0-II) and at t2 (t2-I, t2-II and t2-III) was assessed in X4
and R5 cell lines. The biological clones at t0 (t0-I and t0-II)
demonstrated a higher entry efficacy in the CCR5 cell line as
compared with the CXCR4 cell line. In contrast, the biological
clones obtained at t2 (t2-I, t2-II and t2-III) showed a higher
entry efficacy in the CXCR4 cell line as compared with the CCR5
cell line (Figure 3a). Since all biological clones were able to use
both co-receptors, we asked why the dominant viral population
in the patient shifted from R5- to X4-predicted viruses after
3 weeks of treatment intensification no clear effect on HIV-RNA
concentration or CD4
3 weeks of treatment intensification no clear effect on HIV-RNA
central nervous system (CNS) usage of the CCR5 co-receptor and are typed as R5, X4 or
dual-R5-tropic, whereas others use the CXCR4 co-receptor
more efficiently (X4 > R5 and dual-X4) in PBMCs. In our
case, the patient harboured a viral population that was reported
to be D/M in the Trofile assays and syncytium-inducing in the
MT-2 assay, but R5-tropic in two genotypic prediction algorithms.
In vitro experiments demonstrated that this dominant baseline
viral population could be considered R5 > X4 or dual-R5 since it
was capable of using the CXCR4 co-receptor, but preferentially
used the CCR5 co-receptor in cell lines and natural target cells
(PBMCs). Furthermore, entry of these dual-R5 viruses in the
CCR5 cell line and in PBMCs could be inhibited by maraviroc.
Also, in vivo these dual-R5 variants were suppressed by mara-
viroc, demonstrating their preferential usage of the CCR5
coreceptor.

Unfortunately, in our patient no viral efficacy of maraviroc was
observed, which could be explained by the presence of a dual-X4
minority at baseline that was rapidly selected in the absence of
an active backbone regimen.

These results indicate that viruses capable of using both co-
receptors in vitro may be inhibited by maraviroc. Further
research is warranted to establish whether maraviroc in combi-
nation with an active backbone might be of added value in
patients harbouring dual-tropic virus.

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