Design and validation of new genotypic tools for easy and reliable estimation of HIV tropism before using CCR5 antagonists

Eva Poveda1*, Eduardo Seclé1, María del Mar González1, Federico García2, Natalia Chueca2, Antonio Aguilera3, Jose Javier Rodríguez3, Juan González-Lahoz1 and Vincent Soriano1

1Department of Infectious Diseases, Hospital Carlos III, Madrid, Spain; 2Department of Microbiology, Hospital San Cecilio, Granada, Spain; 3Department of Microbiology, Hospital CHUS-Conxo, Santiago de Compostela, Spain

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Background: Genotypic tools may allow easier and less expensive estimation of HIV tropism before prescription of CCR5 antagonists compared with the Trofile® assay (Monogram Biosciences, South San Francisco, CA, USA).

Methods: Paired genotypic and Trofile® results were compared in plasma samples derived from the maraviroc expanded access programme (EAP) in Europe. A new genotypic approach was built to improve the sensitivity to detect X4 variants based on an optimization of the webPSSM algorithm. Then, the new tool was validated in specimens from patients included in the ALLEGRO trial, a multicentre study conducted in Spain to assess the prevalence of R5 variants in treatment-experienced HIV patients.

Results: A total of 266 specimens from the maraviroc EAP were tested. Overall geno/pheno concordance was above 72%. A high specificity was generally seen for the detection of X4 variants using genotypic tools (ranging from 58% to 95%), while sensitivity was low (ranging from 31% to 76%). The PSSM score was then optimized to enhance the sensitivity to detect X4 variants changing the original threshold for R5 categorization. The new PSSM algorithms, PSSMX4R5-8 and PSSMSINSI-6.4, considered as X4 all V3 scoring values above 8 or 6.4, respectively, increasing the sensitivity to detect X4 variants up to 80%. The new algorithms were then validated in 148 specimens derived from patients included in the ALLEGRO trial. The sensitivity/specificity to detect X4 variants was 93%/69% for PSSMX4R5-8 and 93%/70% for PSSMSINSI-6.4.

Conclusions: PSSMX4R5-8 and PSSMSINSI-6.4 may confidently assist therapeutic decisions for using CCR5 antagonists in HIV patients, providing an easier and rapid estimation of tropism in clinical samples.

Keywords: maraviroc, tropism, Trofile, genotypic algorithm, PSSM

Introduction

HIV co-receptor antagonists have recently entered the therapeutic armamentarium against HIV infection and represent the second class of entry inhibitors, along with enfuvirtide, a fusion inhibitor, to gain regulatory approval. In the second step of the HIV entry process, the CD4–gp120 complex interacts with a chemokine co-receptor exposed on the cell surface, typically CCR5 or CXCR4. Accordingly, HIV isolates are classified as R5-tropic, X4-tropic or as dual/mixed-tropic, depending on their co-receptor use. The term dual/mixed refers to viral isolates that may contain truly dual tropic viruses, which may use any chemokine co-receptor, or mixtures of virus isolates exclusively using either CCR5 or CXCR4, giving the whole virus population a dual tropic character.

Maraviroc represents the first CCR5 antagonist and the only oral HIV entry inhibitor approved for clinical use. Several other compounds designed to block the gp120–CCR5 interaction are in advanced stages of clinical development (e.g. vicriviroc) or still in preclinical or early clinical stages. Maraviroc exclusively inhibits...
the replication of R5-tropic HIV variants by an allosteric mechanism after binding to the transmembrane CCR5 co-receptor cavity.\textsuperscript{6,7} The presence of detectable dual-tropic X4 viruses or mixtures that contain CXCR4-using variants along with R5 viruses has been associated with therapeutic failure using maraviroc.\textsuperscript{8,9} Since the activity of CCR5 antagonists is limited to patients in whom only R5-tropic viruses can be detected, viral tropism determination is required before maraviroc prescription. Several assays have been developed to determine HIV tropism in clinical samples.\textsuperscript{10} The Trofile\textsuperscript{w} assay (Monogram Biosciences, South San Francisco, CA, USA), which uses a recombinant virus assay,\textsuperscript{11} has been the most widely used to date to provide tropism information. Although phenotypic assays such as Trofile\textsuperscript{w} are considered to be very reliable, they are expensive and require special facilities and expertise. HIV-1 co-receptor usage can, alternatively, be predicted using the amino acid sequence of the V3 region of gp120, which is the main determinant of viral tropism.\textsuperscript{12,13} Indeed, several genotypic algorithms have been developed to predict HIV co-receptor usage based on V3 genetic sequences, and many of them are available at publicly accessible web sites.\textsuperscript{10}

Following the approval of maraviroc, the interest in knowing the accuracy of, and concordance between, genotypic and phenotypic assays in determining HIV tropism in clinical samples has been on the rise. Although early comparisons noticed relatively poor concordances, mainly due to low sensitivity to detect X4 variants using genotypic algorithms (<45%),\textsuperscript{14} more recent studies have shown a better sensitivity for the detection of X4 variants using certain genotypic tools.\textsuperscript{15–18}

The prediction of HIV co-receptor usage applying genotypic approaches needs further research, improvements and preferably clinical validation before being used in the clinic. Here, we report the results of a comparative study of HIV tropism using the Trofile\textsuperscript{w} assay and several genotypic tools in samples derived from antiretroviral-experienced patients included in the maraviroc expanded access programme (EAP) in Europe. Following optimization of the webPSSM algorithm, further validation was carried out on a different set of samples.

Materials and methods

Study population

A total of 266 plasma specimens were collected from HIV-infected individuals enrolled in the maraviroc EAP in Europe. Inclusion criteria required age older than 16 years, virological failure under antiretroviral therapy, plasma HIV-RNA >1000 copies/mL and resistance mutations to drugs belonging to two or more antiretroviral families. All specimens were tested for HIV tropism using the Trofile\textsuperscript{w} assay (Monogram Biosciences). Duplicates of these samples were sent to the Molecular Biology Laboratory at Hospital Carlos III in Madrid, Spain, where V3 sequences were obtained and distinct genotypic tools to estimate viral tropism were examined. A separate set of 148 plasma samples collected from HIV-1-infected patients included in the ALLEGRO trial were then tested using a modification of the PSSM score to estimate HIV tropism. The ALLEGRO trial was a multicentre study conducted in Spain during 2007, which assessed the prevalence of R5 variants in HIV-1-infected antiretroviral-experienced patients in Spain. To be included in the trial, HIV-1 patients had to harbour plasma HIV-RNA above 1000 copies/mL under any antiretroviral regimen. Plasma specimens were sent to Monogram Biosciences to test viral tropism using the Trofile\textsuperscript{w} assay. Stored samples were tested in parallel using V3 genotypic tools.

Determination of HIV co-receptor usage based on V3 sequences

The env V3 region was amplified and sequenced from plasma HIV-RNA as described previously.\textsuperscript{15} Sequence analyses were performed using Seqscape v2.5 (Applied Biosystems, Foster City, CA, USA). Nucleotide mixtures were considered when the second highest peak in the electropherogram was above 25%. V3 sequences harbouring nucleotide mixtures were translated into all possible amino acid permutations.\textsuperscript{14} Sequences with eight or more nucleotide mixtures within the V3 region were excluded from subsequent analyses, given the enormous diversity (>512) of possible amino acid sequences generated. HIV-1 subtyping was performed by phylogenetic analyses.

In samples belonging to the European maraviroc EAP, co-receptor usage was estimated for each V3 sequence using several bioinformatics tools freely available at three web sites: Wetcod,\textsuperscript{19} which uses C4.5, C4.5p8-p12, PART, SVM and charge rule; webPSSM,\textsuperscript{20} which uses both X4R5 and SINSI matrices; and geno2phen\textsubscript{co-receptor},\textsuperscript{21} which uses 1%, 5%, 10%, 15% and 20% of false-positive rates (FPRs) for the detection of X4 variants. Co-receptor usage was also estimated using other simple rules such as 11/25,\textsuperscript{22} 11/24/25\textsuperscript{23} and net charge.\textsuperscript{24} All HIV-1 variants were classified as R5- or X4-tropic. For samples containing nucleotide mixtures, a sample was labelled as X4 if any permutation of the V3 sequence was identified as X4.

PSSM is a simple bioinformatic method that estimates the propensity of V3 amino acid sequences to use CXCR4. The PSSM is a matrix of numbers whose columns represent amino acid positions in the V3 loop and whose rows represent the possible residues. A sequence is given a score by summing the cells in the matrix that correspond to the particular residue present in the sequence at each V3 position. The X4 and R5 threshold values originally established were −2.88 and −6.96, respectively. Accordingly, V3 sequences with values below the R5 threshold (−6.96) will be considered as being R5, while sequences with values above −2.88 will be considered as predictive of X4. Intermediate scores are recommended to be interpreted using the 11/25 rule.\textsuperscript{20} Since PSSM provides a report with a single score for each V3 sequence, it is possible to increase the sensitivity to detect X4 variants modifying the threshold originally established to classify a sample as R5 or X4. Given that all samples had been tested using the Trofile\textsuperscript{w} assay, those results were taken as reference for optimizing PSSM.

Statistical analyses

Descriptive results are expressed as median values and interquartile ranges (IQRs). Comparisons were performed using the Student’s t-test for continuous variables, and the Pearson, \(\chi^2\) or Fisher’s exact tests for categorical variables. Receiver operating curves (ROCs) were performed for assessing the accuracy/concordance between distinct PSSM thresholds and Trofile results. The best PSSM discriminatory thresholds for X4R5 and SINSI matrices increasing the sensitivity to detect X4-variants were identified. For samples with V3 sequences containing nucleotide mixtures, the highest PSSM score obtained in the different V3 sequences generated was considered for prediction of co-receptor usage. All statistical analyses were made using the SPSS software package v15.0 (SPSS Inc., Chicago, IL, USA).
Results

Concordance between V3 genotypic tools and Trofile®

A total of 266 specimens from the maraviroc EAP in Europe were tested. Paired genotypic/phenotypic results could be obtained in 202 (76%). Failure to produce genotypic results occurred in 20 (7.5%) specimens, due to the presence of more than 8 nucleotide mixtures in 12 (4.5%) or repeated problems in the amplification and/or sequencing process in 8 (3%). Phenotypic results could not be produced in 46 samples (17.3%). In samples with both genotypic and phenotypic results, concordance ranged from 64% using geno2pheno 20%-FPR to 80% using PSSMX4R5 (Table 1).

The sensitivity and specificity for the detection of X4 variants using genotypic-based algorithms compared with Trofile® are depicted in Figure 1. The sensitivity was generally low, ranging from 31% for geno2pheno 1%-FPR to 76% using geno2pheno 20%-FPR. In contrast, the specificity was generally high, ranging from 58% for geno2pheno 20%-FPR to 95% using geno2pheno 1%-FPR or C4.5 and C4.5p8-p12.

Table 1. Concordance between Trofile® and genotypic results in clinical specimens belonging to the maraviroc EAP in Europe

<table>
<thead>
<tr>
<th>Genotypic algorithms</th>
<th>Concordance with Trofile® (%)</th>
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<tbody>
<tr>
<td>11/25</td>
<td>75</td>
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<tr>
<td>11/24/25</td>
<td>75</td>
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<tr>
<td>C4.5</td>
<td>76</td>
</tr>
<tr>
<td>C4.5p8p12</td>
<td>75</td>
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<tr>
<td>PART</td>
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<td>SVM</td>
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<tr>
<td>Charge rule</td>
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<tr>
<td>PSSMX4R5</td>
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<tr>
<td>PSSMINSI</td>
<td>79</td>
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<tr>
<td>Geno2pheno 1%</td>
<td>75</td>
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<tr>
<td>Geno2pheno 5%</td>
<td>76</td>
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<tr>
<td>Geno2pheno 10%</td>
<td>71</td>
</tr>
<tr>
<td>Geno2pheno 15%</td>
<td>67</td>
</tr>
<tr>
<td>Geno2pheno 20%</td>
<td>66</td>
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</table>

The discordant results between genotypic methods and Trofile® could not be attributed to genetic subtype diversity due to the low prevalence of non-B subtypes in the study population (<1%). All but two specimens belonged to clade B carriers; these two samples were from subtypes F and D, respectively. For the subtype F specimen, all genotypic interpretation systems were in agreement with Trofile® results (R5-tropic). In contrast, discordant results between Trofile® and genotypic tools were noticed testing the clade D specimen.

Improvement of the PSSM score to detect X4 viruses

The sensitivity/specificity to detect X4 variants using PSSMX4R5 was 59%/89%, and 61%/87% using PSSMINSI (Figure 1). The accuracy between them and Trofile® was assessed using ROC analyses. The areas under the ROC values were 0.81 (95% confidence interval: 0.74–0.87) and 0.80 (95% confidence interval: 0.73–0.86) for PSSMX4R5 and PSSMINSI, respectively. In order to enhance the sensitivity to detect X4 variants, the PSSM score was optimized using ROC analyses to categorize samples as X4. For PSSMX4R5, the best PSSM threshold was −8, increasing the sensitivity to detect X4 variants to 81% with a specificity of 68%. For PSSMINSI, the best PSSM threshold was −6.4, increasing the sensitivity to detect X4 variants to 80% with a specificity of 69%. The PSSM algorithms using the new PSSM thresholds were re-named as PSSMX4R5-8 and PSSMINSI-6.4. Accordingly, samples were considered as X4 when the PSSMX4R5 matrix gave values above −8, and were otherwise interpreted as R5. Likewise, samples were interpreted as X4 when the PSSMINSI matrix gave values above −6.4.

Validation of PSSMX4R5-8 and PSSMINSI-6.4

The new PSSM thresholds for estimating HIV-1 co-receptor usage were tested in a separate set of 148 plasma specimens belonging to HIV-1-infected patients included in the ALLEGRO trial. These subjects had a median plasma HIV-RNA of 4.2log copies/mL (IQR: 4.04–4.66), and their median CD4 count was 269 cells/mm³ (IQR: 148–362). Co-receptor usage for each V3 sequence was estimated using both PSSMX4R5-8 and PSSMINSI-6.4. Paired genotypic/phenotypic results could be obtained in 118 (80%) of the samples. Genotypic results could not be produced for three samples (2.0%) due to failures during amplification and/or sequencing processes. On the other hand, Trofile® did not produce results in 21 specimens (14.2%). The concordance between Trofile® and PSSMX4R5-8 and PSSMINSI-6.4 was 75% and 76%, respectively. The sensitivity/specificity to detect X4 variants using PSSMX4R5-8 was 93%/69%, 97% being the negative predictive value (NPV) and 49% the positive predictive value (PPV). For PSSMINSI-6.4, the sensitivity/specificity to detect X4 variants was 93%/71%, with 97% NPV and 51% PPV (Figure 1).

Estimated rate of R5 and X4 variants using genotypic tools or Trofile®

The rate of X4 variants in samples from the maraviroc EAP in Europe was 31.7% using Trofile®. In this population, an overestimation of X4 variants was seen using both PSSMX4R5-8 and PSSMINSI-6.4, which gave rates of 48% and 47%, respectively. Similarly, an overestimation of X4 variants was seen in the set.
of 148 samples belonging to the ALLEGRO trial. The estimated rate of X4 variants was 25% using Trofile® and increased to 46% and 44% using PSSM\textsubscript{X4R5-8} and PSSM\textsubscript{SINSI-6,4}, respectively (Figure 2).

It is noteworthy that non-reportable results using genotypic tools occurred less frequently than Trofile® (7.5% versus 17.2%, respectively). In the set of samples from the maraviroc EAP in Europe in which Trofile failed to report results \((n=44)\), PSSM\textsubscript{X4R5-8} results in 48% of them \((n=21)\) as R5.

### Discussion

The use of genotypic tools may be an easier, faster and less expensive alternative approach to assess HIV-1 tropism compared with using the Trofile® assay, which is currently considered the gold standard.\textsuperscript{10} However, the reliability of genotypic methods has been under debate, with generally poor performance observed in particular when testing samples derived from antiretroviral-experienced patients, who are otherwise currently the most frequent candidates for maraviroc therapy.

The results of our study testing a set of 202 clinical specimens from patients enrolled in the maraviroc EAP in Europe showed that the sensitivity to detect X4 variants using different currently available genotypic algorithms is generally low, ranging from 31% to 76%, taking Trofile® as reference, which is in agreement with previous reports.\textsuperscript{14-27} This low sensitivity was noticed even considering as X4 those samples containing any nucleotide mixtures in the V3 sequences that lead to a single permutation interpreted as X4. In order to enhance the sensitivity to detect X4 variants, we introduced a simple modification in the webPSSM algorithm, shifting the threshold established for considering the V3 sequences as R5, based on ROC analyses. The new PSSM algorithms named as PSSM\textsubscript{X4R5-8} and PSSM\textsubscript{SINSI-6,4} categorized samples as X4 when V3 scores were above −8 or −6.4, respectively. These new approaches increased the sensitivity to detect X4 variants to above 80%.

The reliability of the new PSSM approaches was then validated in a separate set of 148 samples collected from patients enrolled in the ALLEGRO trial. In this dataset, the sensitivity/ specificity for detecting X4 variants using PSSM\textsubscript{X4R5-8} was 93%/69%, with 97% being the NPV and 49% the PPV. For PSSM\textsubscript{SINSI}, the sensitivity/specificity to detect X4 variants was quite similar, 93%/70%, with 97% being the NPV and 51% the PPV. Thus, 97% of clinical samples classified as R5 by genotypic tools were also considered as R5 phenotypically, and only 3% were misclassified. Thus, the possibility of prescribing maraviroc in patients with Trofile® reported X4 variants based on a false-negative prediction by the new PSSM scores was low.

Along with the improved sensitivity to detect X4 variants, the PPV diminished to ~50%. This means that using PSSM\textsubscript{X4R5-8} and/or PSSM\textsubscript{SINSI-6,4}, half of the samples reported as X4 could be R5 by Trofile®. However, this low specificity for X4 variants would most likely be lower using the new enhanced version of Trofile®, which is 10- to 100-fold more sensitive for X4 minor populations.\textsuperscript{28} On the other hand, it is noteworthy that Trofile® consistently considers as non-reportable more than 15% of tested samples, while failure to produce results is seen in <7.5% of cases using genotypic tools.\textsuperscript{29,30} Altogether, the proportion of HIV patients to whom maraviroc could wrongly be denied based on the overestimation of X4 variants using V3 genotypic tools could compensate for the lack of reportable R5 specimens using Trofile®. Moreover, samples considered initially as X4 using PSSM\textsubscript{X4R5-8} and PSSM\textsubscript{SINSI-6,4} could be confirmed using Trofile® to minimize overestimation of X4 variants. In this way, genotypic tools could be used for rapid screening, allowing maraviroc prescription when reporting R5 variants, requiring further Trofile® testing only for those samples interpreted as X4.

In summary, our results highlight the feasibility of employing the genotypic algorithms PSSM\textsubscript{X4R5-8} and PSSM\textsubscript{SINSI-6,4} to assist in therapeutic decisions on the use of CCR5 antagonists in HIV patients in routine clinical practice, providing an easier and more rapid determination of viral tropism. The new PSSM algorithms could be performed in laboratories familiar with virus sequencing with no other specific technological requirements, avoiding the overseas shipment of plasma specimens.

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### Transparency declarations

None to declare.
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