Correlation between genotypic (V3 population sequencing) and phenotypic (Trofile ES) methods of characterizing co-receptor usage of HIV-1 from 200 treatment-naïve HIV patients screened for Study A4001078

Simon Portsmouth, Srinivas Rao Valluri, Martin Däumer, Bernhard Thiele, Hernan Valdez, Marilyn Lewis, Charles Craig, Alexander Thiel, Ian James, James Demarest, Jayvant Heera

Pfizer Global Research and Development, New York, NY, USA
Institute of Immunology and Genetics, SEQ.IT GmbH, Kaiserslautern, Germany
Pfizer Global Research and Development, Sandwich, UK
Max-Planck-Institute for Informatics, Saarbrücken, Germany
ViiV Healthcare, Research Triangle Park, NC, USA
Pfizer Global Research and Development, Groton, CT, USA

Article info
Article history:
Received 17 February 2012
Revised 11 October 2012
Accepted 7 November 2012
Available online 16 November 2012

Keywords:
CXCR5
Genotype
HIV-1
Maraviroc
Phenotype

Abstract
Assessment of HIV-1 co-receptor usage is essential to identify patients who are likely to respond to maraviroc (MVC)-containing regimens. Co-receptor usage of plasma virus from all treatment-naïve patients screened for a MVC clinical trial was assessed using phenotypic and genotypic methodologies to evaluate concordance between testing methods and to assess the quantity of CXCR4-using (non-R5) virus in samples giving discordant results. Co-receptor usage was prospectively measured using the enhanced sensitivity Trofile assay (Trofile ES) to screen patients for enrollment in Study A4001078. Population and deep sequencing methodologies were utilized retrospectively to analyze all screening samples, with co-receptor usage determined using the geno2pheno algorithm. Concordance between methods was explored using descriptive statistics. The quantity of non-R5 virus in all samples was measured using deep sequencing. Trofile ES and matched genotype results were obtained for 199 screening samples. Concordance of Trofile ES with population genotyping (5.75% false-positive rate [FPR]) and deep sequencing (3.5% FPR; 2% non-R5 threshold) was 91.7% and 89.6%, respectively. Population genotype data were available for all samples with non-reportable Trofile ES results; the distribution of co-receptor usage in this set was consistent with that in the overall population: 75% (12/16) R5 and 25% (4/16) non-R5. The majority of samples contained non-R5 plasma HIV-1 RNA estimated at either <1 log10 (62.0%) or ≥4 log10 (30.5%) copies/mL; the absolute amount of detectable non-R5 virus remained stable between screening and baseline visits. Samples originally classified as non-R5 by Trofile ES but R5 by population sequencing had a relatively low absolute amount of non-R5 virus (mean 2.1%, median 0.1%). The determination of co-receptor usage using either Trofile ES or genotyping methodologies showed similar frequencies of R5 and non-R5 virus in this treatment-naïve study population. For both concordant and discordant samples, population sequencing appropriately identified R5 samples with low levels of non-R5-using virus.

1. Introduction
The prevalence of CCR5-using (R5) HIV-1 is greater in treatment-naïve individuals (80–90%) compared with treatment-expe-rienced individuals (50–55%), more of whom have CXCR4-using or dual/mixed virus (Hoffmann, 2007). As maraviroc (MVC), a CCR5 antagonist, inhibits CCR5-dependent HIV-1 cell entry (Dorr et al., 2005), determination of HIV-1 co-receptor usage is required before commencing treatment. The original Trofile assay (Monogram Biosciences), based on recombinant virus technology, was the assay most widely used for prospective determination of co-receptor usage in clinical trials of the first CCR5 antagonists, including the registrational Phase 3 trials for MVC (Whitcomb et al., 2007). However, it has since been superseded by an enhanced sensitivity Trofile assay, denoted here
as Trofile ES, which is more sensitive for detecting minor CXCR4-using (dual, and/or X4; non-R5) populations in vitro (Reeves et al., 2009). HIV-1 co-receptor usage is largely determined by the third variable region (V3) of the HIV envelope glycoprotein (Hwang et al., 1991). Genotypic methods, such as population genotyping or deep sequencing, are based on sequencing the V3 loop and analyzing the sequence using bioinformatic algorithms to infer likely co-receptor usage.

The determination of HIV-1 co-receptor usage using Trofile ES and genotypic methodologies has been previously shown to predict clinical response to MVC with similar degrees of accuracy (McGovern et al., 2010a,b; Swenson et al., 2011). Retrospective analyses of viral co-receptor usage in the MVC registrational studies MERIT (Portsmouth et al., 2010) and MOTIVATE (McGovern et al., 2010b) showed a relatively high degree of concordance, albeit in samples from patients enrolled in these studies. Co-receptor usage in screening samples from a randomized trial of MVC-based therapy (not preselected for R5 virus and prior to study enrollment and MVC administration) has not been assessed previously.

The objectives of this study were: to assess concordance between results obtained using Trofile ES and genotypic methodologies in screening samples from the MVC clinical trial Study A4001078 (Portsmouth et al., 2011); to quantify, using deep sequencing methods, the amount of non-R5 virus in all samples, particularly in those giving discordant results between genotypic and phenotypic assays; and to determine the co-receptor usage of virus from samples with a non-reportable (NR) result using Trofile ES, comparing the composition of this subset with distribution in the overall patient population.

2. Materials and methods

Study A4001078 was a randomized, open-label, two-arm, international Phase 2b study (Clinicaltrials.gov identifier NCT00827112) conducted at 33 centers in Germany, Spain, and the US. The study was conducted in accordance with the ethical principles set out in the Declaration of Helsinki, and the good clinical practice guidelines established by the International Conference on Harmonisation. All patients provided informed written consent for participation in Study A4001078 and retrospective determination of co-receptor usage.

Treatment-naive patients infected with R5 HIV-1, as determined at the screening visit using Trofile ES, were randomized to receive atazanavir/ritonavir (ATV/r; 300/100 mg once daily [QD]) with either MVC (150 mg QD) or tenofovir/emtricitabine (Truvada; 300/200 mg QD) for 48 weeks.

Plasma HIV-1 RNA from each patient screened for study entry was re-assessed retrospectively for viral co-receptor usage at screening and baseline using both population and deep sequencing methodologies. Amplicons of 420 base-pairs, which included the encoding region for the entire V3 loop of gp120, were generated using the single nested RT-PCR product of viral RNA extracted from plasma. The population genotype of each sample was determined using standard Sanger sequencing (single sample) and co-receptor usage was assigned using the geno2pheno (co-receptor) algorithm (g2p) (Sing et al., 2007) with a false-positive rate (FPR, i.e. predicted frequency of classifying R5 virus as non-R5 virus) of 5.75% (or other FPRs as indicated).

The viral co-receptor usage composition of each sample was assessed using the GS FLX Titanium (454 Life Sciences/Roche) amplicon sequencing protocol. The HIV-1 V3 deep sequencing approach of the samples achieved an average ± standard deviation (range) of 11,490 ± 4646 (1148–27,714) reads per sample. Low numbers of viral input templates in the reverse transcription and subsequent PCR may have resulted in oversampling leading to pseudohomogeneity virtually not present in the virus population (Jabara et al., 2011; Vandenbroucke et al., 2010). The average viral load was 100,998 copies/mL (median: 46,500 copies/mL; range: 1650–750,000 copies/mL). Since HIV-1 RNA was extracted from 500 µl of plasma (QIAamp Viral RNA Mini-Kit, Qiagen, Hilden, Germany [protocol slightly modified]), the arithmetical range of input RNA molecules was 825–375,000.

Sequences were extracted directly from the Standard Flowgram Format (sff) files, which store the sequencing trace data produced by the 454 GS FLX System, analyzed and processed for full-length V3, and the co-receptor usage of each individual sequence inferred using g2p with an FPR of 3.5%. Samples were classified as non-R5 if at least 2% of individual sequences were inferred as non-R5. The non-R5 viremia (copies/mL) was estimated as the overall screening plasma HIV-1 RNA concentration (copies/mL) multiplied by the proportion of non-R5 sequences, as determined using deep sequencing. The quality of individual V3 loops was assessed using a 95-percentile cut-off as implemented in the g2p-454 algorithm (Sing et al., 2007).

Concordance between Trofile ES, population genotyping, and deep sequencing was explored using descriptive statistics; the proportion of non-R5 virus in screening and baseline samples both concordant and discordant, comparing genotype and phenotype, was evaluated using population and deep sequencing methodologies.

3. Results

Of the 220 patients who were screened for entry to Study A4001078, 200 patients had prospective Trofile ES screening data available (Supplementary Fig. 1). Screening samples from 20 patients were not included in this analysis due to patients not meeting entry criteria (n = 4), patients no longer willing to participate (n = 6), or other reasons (n = 10). Matched genotype data were obtained retrospectively for 199/200 patients (99.5%). Demographic data are not available for patients that screen-failed, although for the 199 patients with matching Trofile ES and genotypic data, the median plasma HIV-1 RNA concentration was 46,500 copies/ml (range 1670–750,000 copies/ml). One-hundred-and-twenty-one patients infected with R5 HIV-1, determined by Trofile ES, were randomized to study treatment (Supplementary Fig. 1).

Co-receptor usage results for the 199 patients with Trofile ES and genotype data are presented in Fig. 1A. A greater percentage of patient samples were characterized as R5 by both population and deep sequencing when compared with Trofile ES. As expected, the relative percentage of samples classified as non-R5 increased with higher FPRs; more viruses classified as R5 using lower FPRs were classified as non-R5.

Fewer patients had NR screening results using either genotypic method when compared with Trofile ES. Population genotypes were successfully determined for all 16 patients with an NR Trofile ES result at screening. Consistent with the composition of the overall population, as determined using genotyping and phenotyping, the subset of patients with NR Trofile ES results was found to contain 75% (12/16) R5 and 25% (4/16) non-R5 virus.

Samples classified as R5 by Trofile ES were compared with co-receptor usage predicted by the g2p algorithm using either population sequencing (5.75–10% FPR) or deep sequencing (3.5% FPR and 2% non-R5 threshold); concordance was in the range 83–92% (Fig. 1B). Concordance with population genotype decreased from 91.7% (at 5.75% FPR) to 83.3% (at 10% FPR); this is consistent with more viruses being classified as non-R5 at a higher FPR.
Fig. 1. (A) Overall HIV co-receptor usage results by Trofile ES and by retrospective population sequencing and deep sequencing in patients screened for Study A4001078 (N = 199). (B) Concordance of R5 results by prospective Trofile ES testing and retrospective genotypic testing in screened patients (N = 199). (C) Estimated levels of non-R5 plasma HIV-1 RNA among screened patients derived using deep sequencing. n, number of concordant R5 samples by method indicated. Population data were included for both the 5.75% FPR cut-off previously investigated in maraviroc studies and the 10% cut-off suggested by some co-receptor usage testing reports. ES, enhanced sensitivity; FPR, false-positive rate; NR, non-reportable.
Analysis in this treatment-naive patient population showed that the majority of samples had calculated non-R5 HIV-1 RNA levels of \(<1.0 \log_{10} (64.5\%) \) copies/mL of plasma (Fig. 1C, Supplementary Fig. 2) using the GS FLX Titanium amplicon sequencing protocol. The amount of non-R5 virus for all patients enrolled and randomized in Study A4001078 (N = 121) remained stable between the screening (median non-R5 sequences 0%, mean 0.7% and baseline visits (median non-R5 sequences 0%, mean 2.08%), as assessed by deep sequencing.

The proportion of non-R5 virus in samples whose genotypic results were both concordant and discordant with Trofile ES is shown in Table 1. Fifteen samples originally classified as non-R5 by Trofile ES, but as R5 with population genotyping, were found to contain >2% detectable non-R5 virus; this value is the deep sequencing threshold used to define a non-R5 sample (n = 19; median 0.1% non-R5 sequences; mean 2.08%), as assessed by deep sequencing.

<table>
<thead>
<tr>
<th>Trofile ES result</th>
<th>Population genotype result (5.75% FPR)</th>
<th>Deep sequencing result</th>
</tr>
</thead>
<tbody>
<tr>
<td>R5</td>
<td>Non-R5</td>
<td>NR(^a)</td>
</tr>
<tr>
<td>n = 122(^b)</td>
<td>n = 8</td>
<td>n = 4</td>
</tr>
<tr>
<td>N = 144</td>
<td>mean 0%</td>
<td>mean 0%</td>
</tr>
<tr>
<td></td>
<td>(0.3, 1.7)</td>
<td>(13.2, 76.6)</td>
</tr>
<tr>
<td>Non-R5</td>
<td>n = 19(^a)</td>
<td>n = 2</td>
</tr>
<tr>
<td>N = 39</td>
<td>mean 2.1%</td>
<td>mean 0.54%</td>
</tr>
<tr>
<td></td>
<td>(0.1, 4.3)</td>
<td>(36.7, 69.6)</td>
</tr>
<tr>
<td>NR</td>
<td>n = 12</td>
<td>n = 0</td>
</tr>
<tr>
<td>N = 16</td>
<td>mean 0.07%</td>
<td>mean 44.5%</td>
</tr>
<tr>
<td></td>
<td>(–0.1, 0.2)</td>
<td>(–24.4, 123.4)</td>
</tr>
</tbody>
</table>

Results are shown as median and mean (95% CI) percentage non-R5 sequences.

\(^a\) NR results for genotyping refer to unamplifiable samples or samples with non-evaluable Sanger traces.

\(^b\) Three patients with R5 Trofile ES and population results had >2% non-R5 virus by deep sequencing (4.7%, 5.6%, and 66.9%).

\(^c\) Four patients with non-R5 Trofile ES and R5 population results had >2% non-R5 virus by deep sequencing (3.4%, 6.9%, 10.1%, and 14.4% X4 virus).

4. Discussion

Several studies have compared phenotype- and genotype-based methodologies for the determination of co-receptor usage. In a study carried out by Poveda and colleagues of 266 samples, 72% concordance was reported between the original Trofile assay and population genotypic methods (Poveda et al., 2009). Retrospective analysis of treatment-naive patient samples (n = 705) in the MERIT trial showed >80% concordance between the population genotypic method and Trofile ES (McGovern et al., 2010a). A similar analysis of the MOTIVATE trials in treatment-experienced patients (N = 1164) demonstrated 90% concordance between the same population genotypic method and the original Trofile assay; Trofile ES data were not available for this analysis (McGovern et al., 2010b). In a study carried out by Saliou et al. to assess concordance between deep sequencing and the Trofile and Toulouse phenotypic assays, deep sequencing was found to be 67% concordant with phenotypic methods and was in the same range of sensitivity (deep sequencing: 0.4%; phenotypic methods: 0.3–0.5%) for the detection of minor CXCR4-using variants (Saliou et al., 2011).

The results described here using screening samples from Study A4001078 showed that Trofile ES was similar in its ability to assess co-receptor usage, when compared with genotypic methods of population sequencing (5.75% FPR) and deep sequencing (3.5% FPR and 2% non-R5 threshold). Concordance between Trofile ES and genotypic methods was particularly high for R5 virus samples, and it is recognized that this treatment-naive population was rich in R5 virus, which may partly explain this observation. Concordance was less for non-R5 samples, which is explained in part by the higher rates of NR results from Trofile ES. These data included samples from patients who were originally excluded from enrollment in the clinical study due to detection of non-R5 virus or NR results by Trofile ES in addition to other reasons for screen failure. This differs from the previous studies mentioned above, which included only those patients with a reportable Trofile result and who enrolled in the clinical trial (Gulick et al., 2008; McGovern et al., 2010a,b). The data also highlight how changing the FPR may affect the degree of concordance reported (Fig. 1A, Fig. 1B). In addition to the FPR settings, other technical parameters (e.g. viral fitness for Trofile or the presence of multiple mixtures of sequences for genotyping) can affect the ability of a given assay to detect non-R5 virus. Thus full concordance between assays would not be expected. Indeed, the most appropriate measure of performance in a comparison between assays is the prediction of clinical outcome.

Consistent with this concept, despite differences in concordance and performance characteristics, the previous analyses of the MOTIVATE and MERIT trials demonstrated the ability of both phenotypic and genotypic methods to identify responders and non-responders to a MVC-based regimen (Cooper et al., 2010; Fäktenheuer et al., 2008; McGovern et al., 2010a,b; Swenson et al., 2011). These data also highlighted some of the limitations of both phenotypic and genotypic methods. For example, a greater sensitivity for detecting minor populations of CXCR4-using variants was associated with a better prediction of clinical response when comparing Trofile ES with the original Trofile assay, in particular for the MERIT population (Cooper et al., 2010). The reported sensitivities of some genotypic methods for the detection of CXCR4-using variants range from approximately 30% to 80% (Low et al., 2007; McGovern et al., 2010b; Poveda et al., 2009), a range that can be influenced by FPR settings, the use of triplicate PCR in the initial steps of the assay, or other factors. Retrospective analyses of MOTIVATE and MERIT using deep sequencing showed that the presence of >2% non-R5 virus is predictive of response to treatment with MVC (Swenson et al., 2010, 2011). Indeed, while the thresholds for detection of non-R5 virus differ across phenotypic or genotypic methodologies, the ability to predict a clinical response to a MVC-based regimen in the current analysis was comparable. Furthermore, in the MOTIVATE trials, the significance of co-receptor usage determination (R5 vs non-R5) decreased as the number of active agents in the optimized background therapy.
increased (Fätkenheuer et al., 2008), indicating that co-receptor usage is one of several factors associated with response to a MVC-containing regimen.

In addition to the relative proportion of non-R5 virus detected by deep sequencing (0% to >70%, even in patients classified as R5 by Trofile ES), we estimated the contribution of these variants to the viral load surrogate, plasma HIV-1 RNA. Approximately 19% of patients (n = 38) screened for Study A4001078 had estimated non-R5 HIV-1 RNA in excess of 1000 copies/mL of plasma; 11 of these were classified as R5 by Trofile ES and four patients had non-R5 plasma HIV-1 RNA >10,000 copies/mL. The clinical relevance of this estimated value remains unknown and, as such, should be viewed strictly as a research observation. Interestingly, only two patients in each study arm experienced protocol-defined treatment failure by Week 48; in each of the four instances, virus was classified as R5 by Trofile ES at screening and remained R5 when assessed retrospectively. Given the relatively small number of patients enrolled in this pilot study that went on to receive the MVC regimen and the success rate thus far in both treatment arms, it is difficult to provide any formal analysis of the relationship of co-receptor usage (R5 or non-R5, % non-R5, and/or non-R5 copies/mL) to virologic outcome.

As reported here, Trofile ES methodology can result in a considerable proportion of NR results; virus polymorphisms may affect the efficiency of the cloning procedure; inherently inefficient reverse transcription of the env-coding region of the HIV genome used in the Trofile assay, sample handling, and other factors may impact the success rate for obtaining a result using Trofile ES. In this study, samples with an NR result using Trofile ES were successfully amplified and genotyped, and the distribution of co-receptor usage in this set was found to be consistent with that in the overall population. This suggests a potential benefit to the use of genotypic methods for successfully determining co-receptor usage.

5. Conclusions

In summary, the analysis of screening samples from Study A4001078 showed a similar ability of phenotypic and genotypic methods to assess co-receptor usage. The data also suggest that V3 genotyping using either population or deep sequencing methodologies is subject to fewer NR results and may provide an additional option for the identification of patients who could benefit from treatment with MVC.

Role of the funding source

This research was conducted by Pfizer Inc and funded by Viiv Healthcare. Employees of the study sponsor were involved in the study conception, design, and conduct, and in the data collection and analysis. All authors had full access to the study data and had final responsibility to submit for publication.

Disclosures

Drs. Portsmouth, Valluri, Valdez, Lewis, Craig, and Heera are employees of Pfizer Inc, and hold stock/stock options in Pfizer Inc. Dr. Thiele has acted as a consultant for Pfizer Inc. Dr. James was employed by Pfizer Inc at the time of the study and holds stock/options in Pfizer Inc. Drs. Dämert and Thiele have no conflict of interest.

Acknowledgments

The authors would like to thank the patients, study investigators, clinical site staff, and the study team involved in Study A4001078. Editorial support was provided by Karen Irving at Complete Medical Communications and was funded by Viiv Healthcare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.antiviral.2012.11.002.

References


