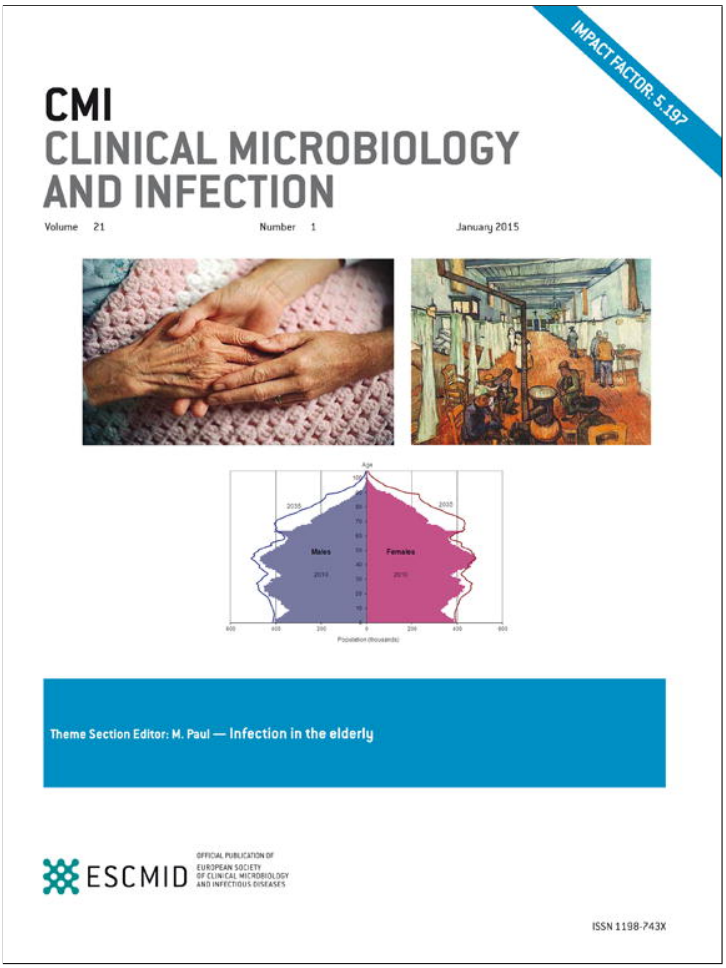

Effect of maraviroc on non-R5 tropic HIV-1: refined analysis of subjects from the phase IIb study A4001029

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Abstract

We characterized maraviroc susceptibility of dual/mixed tropic viruses from subjects enrolled onto phase IIb study A4001029. Maraviroc baseline plasma samples from 13 multidrug-experienced subjects were sequenced and the *HIV-1-env* gene cloned into pNL4.3Δenv to obtain recombinant viruses. The V3 region was sequenced by the Sanger method and ultradeep sequencing. By analysing subjects having a weighted optimized background therapy susceptibility (wOBT) score of <1, 3/7 subjects were characterized by good *in vivo* and *in vitro* response to maraviroc therapy. Molecular docking simulations allowed us to rationalize the maraviroc susceptibility of dual/mixed tropic viruses. A subset of subjects with dual/mixed tropic viruses responded to maraviroc. Further investigations are warranted of CCR5 antagonists in subjects carrying dual/mixed tropic virus that explore the feasible use of maraviroc in subjects that is potentially larger than those infected with a pure R5 virus.

Introduction

Human immunodeficiency virus type 1 (HIV-1) strains can be phenotypically classified according to their ability to use CCR5 and/or CXCR4 coreceptors [1,2]. Special attention should be paid to dual/mixed tropic viruses, which can be divided into those more efficient in using the CCR5 coreceptor ($R5^+/X4$), those using CXCR4 ($R5/X4^+$) more efficiently and those using both coreceptors (R5/X4) with similar efficiency [3–5].

Maraviroc, the only U.S. Food and Drug Administration–approved CCR5 antagonist, demonstrated potent activity in subjects infected by CCR5 tropic viruses [6]. Nevertheless, it has been suggested that maraviroc could also be used to improve therapy efficacy in subjects infected with dual/mixed tropic viruses [3,4,7,8]. The A4001029 study is a unique exploratory, randomized, double-blind, multicenter trial designed to assess the use of maraviroc in treatment-experienced subjects infected with non-R5 viruses which has proved the efficacy of the regimen in 27% of subjects in the maraviroc twice-daily arm [8].

Thus, to deeply characterize the genotypic and phenotypic coreceptor usage of dual/mixed tropic viruses and to investigate their *in vitro* susceptibility to this drug, we analysed the maraviroc effect against HIV from 13 subjects enrolled onto the A4001029 study.

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Methods

Subjects

Thirteen plasma samples randomly selected from HIV-1-infected subjects with available baseline samples were analysed. All subjects had dual/mixed tropic viruses at screening by standard Trofile assay and were treated with maraviroc according to the study protocol [8].

Sequencing

For each baseline plasma sample, the V3 region was amplified and sequenced by Sanger sequencing and by ultradeep sequencing (UDPS; Roche 454-GS-FLX) [9]. The Geno2Pheno algorithm set at <10% false-positive rate (FPR) was used to define the non-R5 viruses.

Production of recombinant viruses

Infectious recombinant viruses were obtained by cotransfecting 2 μg of gp160 polymerase chain reaction product obtained from each baseline plasma sample and 3 μg of pNL4.3-Δenv plasmid.

Phenotypic tropism determination

Phenotypic tropism of recombinant viruses was evaluated by a multiple replication cycle assay on U87MG-CD4$^+$/CCR5$^+$/CXCR4$^+$-astroglioma-expressing cells [3,7].

Phenotypic activity of maraviroc

Susceptibility to entry inhibitors (maraviroc 0.02–10 000 nM, AMD3100 1.3–65 000 nM) was investigated in human peripheral blood mononuclear cells.

Structural analysis

Structural analysis was performed using the CCR5–maraviroc–gp120 binding complex recently developed [10].

Detailed information about all methodologies are available in the online supplementary material.

Results and discussion

Subject characteristics

Data from 13 multidrug-experienced subjects, enrolled onto the phase IIb study A4001029, treated with maraviroc-containing regimens, all harbouring dual/mixed tropic viruses, were analysed. Most of them (8/13) had a wOBT score of $\leq$1. For all subjects, recombinant viruses were successfully obtained by cloning the entire gp160 from baseline plasma samples into a pNL4.3Δenv plasmid (Table 1).

Genotypic characterization

V3 population sequencing was performed for all 13 pairs of plasma samples and corresponding recombinant viruses. The tropism prediction was concordant for 12/13 samples (92%), with a CXCR4 tropism prediction for 11 samples (FPR < 10%). The only discordant subject was subject 10 (plasma FPR 3.4% vs. recombinant virus FPR 27.0%) (Table 1). By UDPS analysis, non-R5-using species were found in all plasma samples, with a median prevalence ranging from 3.7% to 99.6%. Interestingly, in 5/11 subjects with plasma and recombinant virus V3 population FPR of <10%, UDPS revealed R5 species with a prevalence of >25% (Table 1).

Phenotypic tropism characterization

By using U87MG-CD4$^+$-CCR5$^+$/CXCR4$^+$-expressing cells, 12/13 (92.3%) viruses replicated in both CXCR4$^+$ and CCR5$^+$ cell lines, and thus were defined as dual/mixed tropic viruses, while one recombinant virus (ID#3) replicated only in CXCR4$^+$ cells, showing pure X4 tropism (Table 1). No recombinant virus replicated solely in CCR5$^+$ cells. According to p24 production, three recombinant viruses were defined as R5/X4 tropic for similar p24 production in supernatants of either U87MG-CD4$^+$/CCR5$^+$ or U87MGCD4$^+$/CXCR4$^+$ cells. Four other viruses were defined as R5$^+$/X4 for p24 production higher in U87MG-CD4$^+$/CCR5$^+$ cells than in U87MG-CD4$^+$/CXCR4$^+$-cells. The latter five viruses were defined as R5/X4$^+$ tropic for p24 production higher in U87MG-CD4$^+$/CXCR4$^+$ cells than in U87MG-CD4$^+$/CCR5$^+$ cells.

Antiviral activity of maraviroc *in vitro*

As expected, maraviroc was not active against the pure X4 recombinant virus (ID#3; viral replication inhibition 0% at 200 nM/10 000 nM). At a concentration of 200 nM (close to the minimal concentration achieved in plasma of maraviroc-treated subjects with the common dose of 300 mg) maraviroc was able to significantly inhibit 12 dual/mixed tropic viruses (median (interquartile range, IQR) inhibition = 31.5% (15.5–54.5)). According to phenotypic tropism characteristics, viral inhibition (maraviroc 200 nM) and drug concentration causing 50% inhibition (IC_{50}) of R5$^+$/X4 were considerably different from those observed for R5/X4 and R5/X4$^+$ viruses (median (IQR) inhibition = 77% (45–86.5) vs. 16% (15–30.5) vs. 26% (19–37), respectively; IC_{50} 0.7 nM vs. >1000 nM vs. >1000 nM, respectively; Supplementary Table 1). Only R5$^+$/X4 viruses responded to maraviroc *in vitro* similarly to the pure R5 virus control (HIV BaL), reaching approximately 90% of viral inhibition at 20 nM (Fig. 1(A)). In contrast, X4 inhibitor AMD3100 (1300 nM) efficiently inhibited the pure X4 recombinant virus (95% inhibition) and partially inhibited 9/12 dual/mixed tropic viruses (median (IQR) inhibition = 43.5% (6.8–59),

TABLE 1. Genotypic and phenotypic characterization

Subject ID	Phenotypic tropism	G2P FPR	UDPS % Non-R5[b] FPR, median (IQR)	UDPS % R5 FPR, median (IQR)	V3 mutations	MVC IC_{50} (nM)[a]
1_RV	**R5/X4**	1.7			R9S **S11R I12I/V** H13S **F20W** Y21R T22A **G24del** E25D/N/S	333
1_Plasma		1.5	99.6 1.7 (1.6–1.8)	0.4 25.2 (19.5–33.0)	R9S **S11R** H13S **F20W** Y21R T22A **G24del** E25D/N/S	
2_RV	**R5/X4**	2.8			K10R S11G **I12I/V** G24D **E25R** I27V D29N I30I/V	>10 000
2_Plasma		2.8	99.4 2.6 (1.7–2.7)	0.6 73 (27.1–96.2)	K10R S11G **I12I/V** G24D **E25R** I27I/V D29N I30I/V	
3_RV	**X4**	0.2			N5S N6S **N7K** T8K/N/S/Q/H/R/E/D/G **R9T/I** K10T **S11K/R** Y21H **G24K** E25S I27T G28E D29G I30L	>10 000
3_Plasma		0.9	88.7 0.2 (0.1–0.7)	11.3 27.3 (13–48.9)	N5S N6S **N7K** T8K/N/S/Q/H/R/E/D/G **R9R/I** K10T **S11K/R** Y21H **G24K** E25S I27T G28E D29G I30L	
4_RV	**R5/X4**$^+$	0.2			**T2M N7Y R9K**/N/S **S11R I12L** H13S Y21D/V/F T22A T23A **G24R** E25S	266
4_Plasma		1.7	72.4 0.1 (0.0–0.7)	27.6 92.3 (73.3–95.8)	**T2I/M N7Y R9K**/N/S **S11R I12V/L** H13P/S Y21F/V T22A T23A/T **G24G/R** E25E/N/D/S	
5_RV	**R5/X4**$^+$	0.2			T8T/R **R9R/K S11R H13T** I14I/L **A19A/V** F20I/F **G24K** I27D **Q32K/R**	>1000
5_Plasma		1.7	8.9 1.7 (0.2–6.8)	91.1 38.1 (25.5–46.0)	**S11S/R H13T A19A/V F20I/F Y21Y/F G24G/K**	
6_RV	**R5/X4**$^+$	0.7			T2I K10R S11A H13G A19T F20V T22A T23A G24D **E25K**	>1000
6_Plasma		0.7	99.5 0.7 (0.5–1.1)	0.5 18.3 (15.0–20.9)	T2I K10R S11A H13G A19T F20V T22A T23A G24D **E25K**	
7_RV	**R5/X4**	1.1			R9S K10Q **S11R** H13S T22A T23S **G24R E25R** I27T	>1000
7_Plasma		1.7	70.6 1.7 (1.1–3.8)	29.4 15.6 (15.6–21.2)	R9R/S K10E/Q **S11G/R** H13S T22A T23S **G24R E25R** I27T	
8_RV	**R5/X4**$^+$	1.7			K10R **S11R H13T** I14M **A19V F20Y** E25D **Q32K**	>1000
8_Plasma		1.7	77.3 1.1 (0.7–5.3)	22.7 37.7 (18.3–38.0)	K10R **S11R H13T** I14M **A19V F20Y** E25D **Q32K**	
9_RV	**R5/X4**$^+$	0.1			T2E N7T S11T **H13R** I5–17GHlins **A19S** F20L E25S D29N Q32E	>1000
9_Plasma		6.8	3.7 1.7 (1.6–2.8)	96.3 95.8 (95.2–96.2)	T2T/K/E N7N/T S11S/T I12V/I **H13R**/P **A19A/S** F20L T22T/P/A E25E/S D29D/N Q32E	
10_RV	**R5**$^+$**/X4**	27			N5S S11G E25Q I27T	4.6
10_Plasma		3.4	72.2 1.8 (1.7–2.6)	27.8 27.1 (24.0–37.4)	N5S T8T/I S11G G24G/E **E25Q/K** I27T	
11_RV	**R5**$^+$**/X4**	1.7			T2E K10K/Q **S11R** I12I/L H13S T22A T23T/R **G24G/R** E25N/D A33A/S	2600
11_Plasma		1.7	96 1.7 (0.8–5.8)	4 14.3 (13.1–16.4)	T2E K10K/Q **S11R** I12I/L H13S T22A T23T/R **G24G/R** E25N/D A33A/S	
12_RV	**R5**$^+$**/X4**	17			S11G A19T F20I T22A E25Q	0.03
12_Plasma		27.9	18.7 1.7 (1.1–5.2)	81.3 27.9 (23.5–39.8)	S11G A19A/T F20I T22A E25Q	
13_RV	**R5**$^+$**/X4**	4.1			N5G K10R **H13R** R18G **F20Y** Y21F T22R E25D **Q32K**	0.7
13_Plasma		4.7	98.4 4.1 (2.8–4.7)	1.6 17.2 (11.4–34.6)	N5G K10R **H13R** R18R/S/G **F20Y** Y21F T22R G24G/A E25E/D I30T/I R31K/R **Q32Q/K**	

_Plasma indicates virus from subject plasma; _RV, recombinant virus (pNL4.3Δenv + gp160 from subject). V3 mutations (in bold) are found associated with X4 or dual tropism [3]. Underlined mutations were found associated with MVC or VCV resistance. Consensus B was used as reference sequence. Phenotypic tropism was evaluated on U87MG-CD4+/CXCR4+/CCR5+. Genotypic tropism was evaluated by the Geno2Pheno (G2P) algorithm using FPR at 10%.
FPR, false-positive rate; MVC, maraviroc; IC_{50}, drug concentration causing 50% inhibition; IQR, interquartile range; PBMC, peripheral blood mononuclear cells.
[a]MVC IC_{50} was evaluated by measuring the p24 production of recombinant viruses after 7 days after infection in PBMC cultures in presence of maraviroc. IC_{50} for control strain BaL was 0.2 nM; that for IIIB was >10 000 nM.
[b]Percentage of non-R5 species by UDPS was calculated setting G2P FPR at 10%. Similar results were obtained setting FPR at 3.75% and 5.75%, with the exception of samples 7, 8, 11 and 13.

Supplementary Table 1). These results confirm that dual tropic viruses represent a swarm of different viruses with different characteristics and different sensitivity to R5/X4 inhibitors.

Comparison between *in vitro* and *in vivo* response to maraviroc

By comparing the *in vitro* and *in vivo* maraviroc response in a subgroup of subjects (n = 7) with available viroimmunological follow-up and with wOBT score of <1 (indicating a very limited efficacy of the backbone drugs), we found in 6/7 subjects a good correlation between the two maraviroc responses. In particular, three subjects (subjects 4, 10, 12) with >25% of R5 species in plasma at UDPS exhibited good *in vivo* (Fig. 1(B)) and *in vitro* responses (Table 1) (median (IQR) change in viral load = −2.1 log copies/mL (−2.05; −2.75) at 8 weeks, HIV RNA<50 copies/mL at 24 weeks and median maraviroc IC_{50} 4.6 nM). Conversely, 3 subjects (subjects 1, 2, 11), infected by a viral population with >95% of X4 species were characterized by exhibiting no or very low response *in vivo* and *in vitro*. For only one subject (subject 13), with 98.4% of X4 species in plasma but characterized by a R5$^+$/X4 phenotype *in vitro* were the 2 maraviroc responses were not concordant, with no/low response exhibited *in vivo* and excellent activity exhibited *in vitro*.

Even if few subjects have been analysed, our results suggest that subjects having >25% of R5 species in plasma at UDPS and characterized by a good *in vitro* response to maraviroc (IC_{50} < 300 nM) could potentially respond to a maraviroc-containing regimen, even in the setting of a low wOBT score.

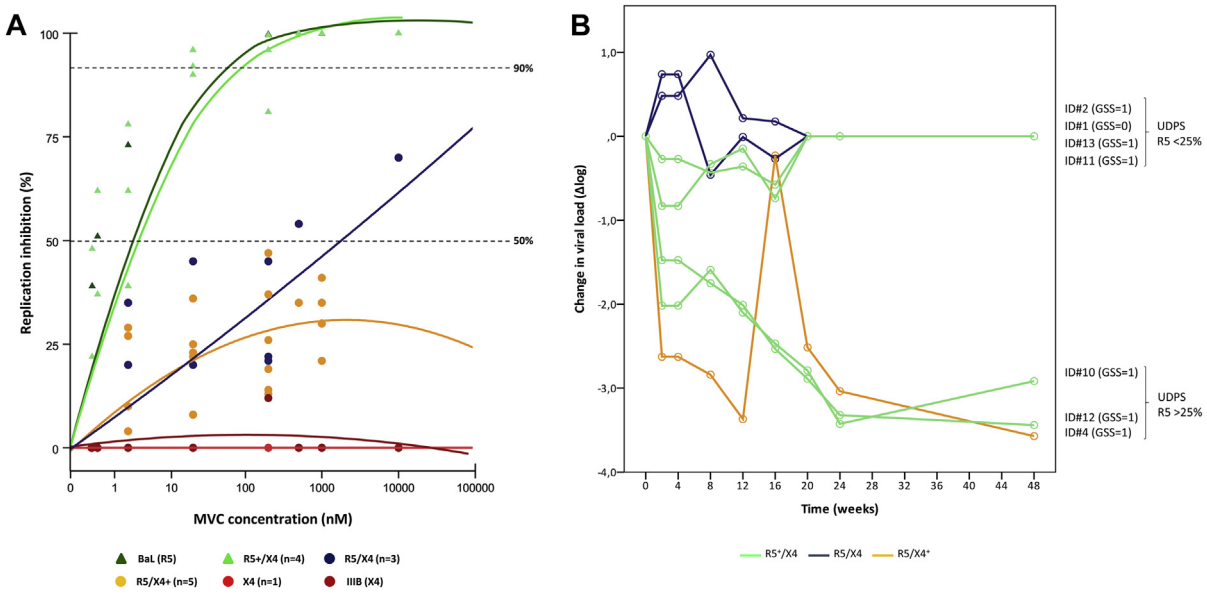

FIG. 1. *In vitro* and *in vivo* virological response to maraviroc. (A) Dose–response curves for maraviroc (MVC)-dependent inhibition of R5$^+$/X4, R5/X4, R5/X4$^+$ and X4 human immunodeficiency virus type 1 (HIV-1) strains in primary peripheral blood mononuclear cells. Dots represent the percentage of inhibition at 0.02, 0.2, 2, 20, 200, 500, 1000 and 10 000 nM MVC concentration for each virus. (B) Virological response in HIV-1-infected subjects with weighted optimized background therapy susceptibility score of <1 receiving antiretroviral therapy including MVC 300 mg twice daily.

This finding is important from a clinical point of view. Despite recent studies demonstrated the ability of CCR5 antagonist to inhibit dual/mixed tropic viruses both *in vivo* and *in vitro* [3,4,7,8], maraviroc treatment is recommended today only for subjects having a pure R5 viral population [11]. Of note, extending our V3 analysis at UDPS to all subjects, irrespective of wOBT score, the cutoff of R5 species found associated with *ex vivo* maraviroc response was 5% (6/8 subjects with >5% R5 species had HIV RNA<50 copies/mL at week 24 vs. 0/5 with <5% R5 species, p 0.02). Other recent studies reported that a prevalence of >2% of X4 species is sufficient to negatively affect the decline in viral load during maraviroc regimen [12]. In these studies, however, it is important to mention that a cutoff FPR of 3.5% was consistently used, therefore suggesting that samples with viral mixture with low X4% by UDPS are the ones in which it is reasonable to expect a good *in vivo* response and phenotypic susceptibility. Overall, UDPS studies with a higher number of subjects infected with non-pure-R5 viruses are strongly suggested; indeed, our results may support the use of V3 UDPS, in the particular setting of subjects with few therapeutic options, as a rapid method to identify subjects for whom maraviroc can be a suitable option [12,13].

Correlation between maraviroc activity and structure analysis

Among all subjects analysed, two (subjects 2 and 11) having at baseline very high percentage of non-R5 sequences at UDPS (>95%) and very low FPR value by population sequencing (2.8% and 1.7%, respectively) and phenotypically characterized by a R5/X4 and R5$^+$/X4 virus, respectively, responded neither *in vivo* nor *in vitro* to maraviroc, and, interestingly, not even to AMD3100 *in vitro* (Supplementary Table 1).

Both plasma and recombinant viruses of these two subjects carried key mutations required for CXCR4 binding (e.g. S11R, I12V, H13S, I27V, G24R, E25R) but also for CCR5 binding (e.g. E25D, G24D) [14]. For these reasons, we raised the question of whether the mutations observed in these nonresponsive subjects may have an impact on interaction with the coreceptors. Because of the absence of a CXCR4 crystallographic model, we focused on the interaction network of CCR5 with gp120, or with maraviroc in the adopted HIV-1 CCR5–maraviroc–gp120 binding structure complex [10,15]. After molecular dynamic simulations of CCR5–gp120 complexes in the presence of maraviroc, the productive interactions V3 sequences in subjects 2 and 11 with CCR5 increased or were superimposable to those observed for the reference YU2-WT-V3-complex (Supplementary Table 2). Differently, in the CCR5–maraviroc–gp120 binding complex, the productive interactions of the drug remarkably decreased (465 = ID#2, 426 = ID#11S1 and 444 = ID#11S2 vs. 478 = WT), with a consequent binding destabilizing effect. Thus, these data confirmed the phenotypic results, demonstrating the ability of these viruses to efficiently bind the CCR5 coreceptor in presence of maraviroc as well. Our results are concordant with

other studies suggesting that maraviroc-resistant viruses may develop an increased dependence on the CCR5/N-terminus, most likely signaling a shift in gp120 binding to a region of CCR5 not modified by the antagonist binding [16].

In conclusion, by using an integrated genotypic, phenotypic and structural approach, this study provides evidence for the existence of a wide variety of HIV-1 dual tropic viruses, with some of them showing a substantial susceptibility against maraviroc. This is clinically important since dual/mixed viruses are frequently observed in subjects' viral population [17–19], and the emergence of X4/dual mixed variants correlates with a worse clinical outcome [20]. For these reasons, this work highlights the importance of further investigations of the activity of CCR5 antagonists in subjects carrying dual/mixed tropic virus, exploring a feasible use of maraviroc as a therapeutic option in a subset of multidrug-failed subjects, particularly in those with R5 species present in >25% of the viral population by UDPS and with susceptible *in vitro* maraviroc phenotype.

Transparency Declaration

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Appendix A. Supplementary data

Supplementary data related to this article can be found at doi:10.1016/j.cmi.2014.08.002.

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