Antiviral Chemistry and Chemotherapy
2021, Vol. 29: 1–12

DOI: 10.1177/20402066211030380
journals.sagepub.com/home/avc
SAGE

V3-Loop genotypes do not predict maraviroc susceptibility of CCR5-tropic virus or clinical response through week 48 in HIV-1–infected, treatment-experienced persons receiving optimized background regimens

ME Lewis[1,2], P Simpson[1,3], J Mori[1,4], B Jubb[1], J Sullivan[1,5], L McFadyen[6], E van der Ryst[1,2], C Craig[1,2], DL Robertson[7] and M Westby[1,8]

Abstract

Viruses from 15 of 35 maraviroc-treated participants with virologic failure and CCR5-tropic (R5) virus in the MOTIVATE studies at Week 24 had reduced maraviroc susceptibility. On-treatment amino acid changes were observed in the viral envelope glycoprotein 120 third variable (V3)–loop stems and tips and differed between viruses. No amino acid change reliably predicted reduced susceptibility, indicating that resistance was genetic context–dependent. Through Week 24, poor adherence was associated with maraviroc-susceptible virologic failure, whereas reduced maraviroc susceptibility was associated with suboptimal background regimen activity, highlighting the importance of overall regimen activity and good adherence. Predictive values of pretreatment V3-loop sequences containing these Week 24 mutations or other variants present at >3% in pretreatment viruses of participants with virologic failure at Week 48 were retrospectively assessed. Week 48 clinical outcomes were evaluated for correlates with pretreatment V3-loop CCR5-tropic sequences from 704 participants (366 responders; 338 virologic failures [83 with R5 virus with maraviroc susceptibility assessment]). Seventy-five amino acid variants with >3% prevalence were identified among 23 V3-loop residues. Previously identified variants associated with resistance in individual isolates were represented, but none were associated reliably with virologic failure alone or in combination. Univariate analysis showed virologic-failure associations with variants 4L, 11R, and 19S ($P < 0.05$). However, 11R is a marker for CXCR4 tropism, whereas neither 4L nor 19S was reliably associated with reduced maraviroc susceptibility in R5 failure. These findings from a large study of V3-loop sequences confirm lack of correlation between V3-loop genotype and clinical outcome in participants treated with maraviroc.
Clinical trial registration numbers (ClinicalTrials.gov): NCT00098306 and NCT00098722

Keywords

HIV entry, V3-loop genotype, maraviroc susceptibility

Date received: 2 April 2021; accepted: 14 June 2021

Introduction

HIV-1 enters cells through a series of conformational changes in its envelope glycoprotein homo-trimer complex of the viral envelope glycoprotein 120 (gp120)/viral envelope glycoprotein 41 (gp41), mediated by interactions with the CD4 cell receptor and a coreceptor (C-C chemokine receptor type 5 [CCR5] or C-X-C

[1]Pfizer Global Research and Development, Sandwich Labs, Sandwich, Kent, UK
[2]The Research Network Ltd, Sandwich, Kent, UK
[3]AstraZeneca, Cambridge, UK
[4]hVIVO, Queen Mary BioEnterprise Innovation Centre, London, UK
[5]Cytel, London, UK
[6]Pfizer Inc, Pharmacometrics, Sandwich, UK
[7]MRC-University of Glasgow Centre for Virus Research, Glasgow, UK
[8]Centauri Therapeutics Limited, Discovery Park, Kent, UK

Corresponding author:
Charles Craig, IPC 600 Discovery Park Enterprise Zone, Ramsgate Road, Sandwich, Kent CT13 9NJ, UK.
Email: charles.craig@pfizer.com

chemokine receptor type 4 [CXCR4]).[1,2] Coreceptor binding is critical to the ordered cascade of conformational changes leading to viral entry into cells. The predominant coreceptor used by virus on transmission and early infection is CCR5.[3] However, CXCR4-using viruses can naturally arise during the course of infection in untreated individuals.[4–6]

Maraviroc is a CCR5 inverse agonist (ie, it stabilizes CCR5 in an inactive form) that acts to prevent the interaction between gp120 and CCR5 on the target cell surface.[7,8] It is an allosteric inhibitor, binding in a deep pocket within the CCR5 transmembrane region.[9,10] The CCR5-tropic (R5) virus may acquire maraviroc resistance through adaptation of its gp120 so that it can bind to maraviroc-bound CCR5, thereby undergoing the required conformational changes for viral entry.[11] This adaptation results in reduced capacity for saturating concentrations of maraviroc to fully inhibit viral entry (maximum percent inhibition [MPI] <95%)[12] and, in rare instances, viruses might develop dependency on the presence of maraviroc during prolonged treatment in virologic failure (MPI <0%).[13] Maraviroc is not active against CXCR4-using virus, so appropriate testing for viral tropism is required prior to its use. Thus, susceptibility to maraviroc may be influenced by 2 mechanisms: reduced inhibition of R5 viral entry to maraviroc-bound CCR5 ("reduced maraviroc susceptibility") or by selection of preexisting minority CXCR4-using variants.[10] The focus of the current study is on maraviroc susceptibility reduction.

Two gp120 third variable (V3)–loop amino acid variants, A19T and I26V, confer reduced maraviroc susceptibility in one R5 virus strain (CC1/85) selected during serial in vitro passage.[12] These and other changes have been identified following virologic failure in a small number of V3-loop sequences of R5 viruses in participants treated with maraviroc.[11,12] However, no correlation was observed between V3-loop sequence changes and maraviroc susceptibility in these small studies.[14]

HIV sequence database searches have found combinations of these variants potentially associated with reduced susceptibility to maraviroc in a proportion of viruses from CCR5 antagonist treatment-naive participants, raising concerns that naturally occurring resistance to maraviroc could occur;[15–17] however, these studies did not include phenotypic susceptibility testing. In addition, in the absence of information on maraviroc treatment outcomes, virologic and clinical significance have not been determined. Herein we examine the V3-loop sequences and maraviroc susceptibility of viruses from 704 participants receiving maraviroc in relation to clinical outcome, background regimen antiretroviral activity, and adherence in the MOTIVATE 1 and 2 studies.

Methods

Study populations and sample testing

Participants in the MOTIVATE trials were prescreened for CCR5 tropism using a phenotypic tropism assay (Trofile; Monogram Biosciences, South San Francisco, CA, USA). Of the 1049 treated participants in the MOTIVATE studies, 840 received maraviroc.[18] The study populations for analysis were identified at Weeks 24 and 48 using the time to loss of virologic response <50 copies/mL (TLOVR50) algorithm. At Week 24, there were 35 maraviroc-treated virologic-failure participants identified with R5 virus at the time of virologic failure. By Week 48, a total of 725 participants, including those with treatment failure by Week 24, had met a virologic outcome using the TLOVR50 algorithm (response, nonresponse, or rebound); for 704 of these individuals, the V3 loops were successfully sequenced pretreatment (Screening) and were included in the analysis of V3-loop variants and association with virologic outcome (Supplementary Figure 1 and Table 1).[19]

The Week 24 analysis examined the genotypic correlates of phenotypic maraviroc susceptibility in a clonal manner, whereas the larger population at Week 48 was used to examine the potential for using genotype to predict virologic outcome.

Study protocols were approved by institutional review boards or independent ethics committees at study centers. Written informed consent was obtained from all participants. The studies were performed in accordance with International Conference on Harmonisation Good Clinical Practice guidelines and applicable local regulatory requirements and laws. Both trials were registered on ClinicalTrials.gov with identifiers NCT00098306 and NCT00098722. An independent data and safety monitoring board was responsible for oversight of the progress of the studies, the study data, and safety considerations.

Tropism testing

Analysis of tropism at Screening, Day 1, and at treatment failure was performed using a phenotypic tropism assay. Tropism was initially assigned using the Original Trofile assay (Monogram Biosciences) and later repeated using the Enhanced Sensitivity Trofile assay (Monogram Biosciences). To retrospectively evaluate genotypic tropism, selected V3-loop sequences were assessed using the Geno2Pheno algorithm (https://coreceptor.geno2pheno.org; Max Planck Institute for

Table 1. Changes in V3-loop sequences associated with plateaus in MPI <95% in 15 participants failing with R5 virus and reduced susceptibility to maraviroc through week 24.

		Phenotypic susceptibility		
Pat no.	Visit	MPI pool (clones), %[a]	Clones with MPI <95% n (clones)	V3 loop consensus sequence (10 / 20 / 30)
1	Pre-T	100 (94-100)	1 (11)	CTRPNNNTRKSIXLG PGSAFYATGDIIGDIRQAHC
	Wk 16	95[b] (85-100)	7 (12)	.....K..HX. .G.....Q...
2	Pre-T	100 (99-100)	0 (12)	CXRPNNNTRKSISIG PGRAFYATGEVIGDIRQAYC
	Wk 20	13 (20-30)	11 (11)[c]	.T.....G..RA.....D.I.....Y.
3[d]	Pre-T	100 (96-100)	0 (12)	CTRPNNNTRKSIPIG PGRAFYATGDIIGDIRQAHC
	Wk 24	30 (36-77)	11 (11)[c]	.....S..A.....I..I.....
4	Pre-T	100 (100)	0 (12)	CVRPNNNTRRSIXIG PGRAFYGT-DIIGXIRQAHC
	Wk 8	77 (67-96)	11 (12)	.....N.....IN.. A.....D..Q...
5	Pre-T	96 (86-96)	11 (12)	CTRPSNNTSKGIHMG PGGAFYATDRIIGDIRQAHC
	Wk 20[e]	73 (54-77)	3 (3)	.....K..... ..-.....D.....
		73 (50-100)	5 (6)	.....K..... ....L.....R....
		73 (73-82)	2 (2)	.....K..... .....X.....
		73 (27)	1 (1)	.....R..N.. .....T.....
6	Pre-T	99 (99-100)	0 (12)	CTRPGNNTRRGIHXG PGXAMYTNN-IXGDIRRAHC
	Wk 8	65 (63-94)	12(12)	.....F. ..G.L....I.I.....
7	Pre-T	99 (97-100)	0 (12)	CTRPGNNTRKSIHIG PGRAFYTTGDIIGDIRQAHC
	Wk 8	91 (90-98)	10 (12)	.....S.... ...S..A..DV.....Q...
8[d]	Pre-T	100 (98-100)	0 (12)	CTRPGNNTRKSIHMG PGSSIYATGAIIGDIRQAHC
	Wk 24	84 (61-99)	10 (12)	.....N..... .GX.F....DV.....
9	Pre-T	100 (99-100)	0 (12)	CSRPNNNTRKSINIG PGRAFYAT-DIIGDIRQAHC
	Wk 20	51 (28-52)	7 (7)	.....S..... A.....
		51 (68-100)	4 (5)	..... A.....X.X.
10	Pre-T[f]	100 (98-100)	0 (9)	CVRPNNNTRKSINIG PGRAWYAT-DIIGDIRQAYC
		100 (97-100)	0 (3)	....G....N....H.
	Wk 8[e]	83 (80-99)	8 (11)	.....SIH.. .....G....N..Q.H.
		83 (100)	0 (1)	....-.....
11[d]	Pre-T	100 (95-100)	0 (12)	CXRPNNNTRKXINIG PGXAWYTTXDIIGDIRQAHC
	Wk 8	80 (51-70)	6 (6)	.T.....T..S.H.. ..K...A.G.....
		80 (57-91)	6 (6)	.T.....T..S.H.. ..KA....G.....
12[d]	Pre-T	100 (95-100)	0 (12)	CXRPNNNTRKGIHIG PGRSFYATGDIIGDIRQAHC
	Wk 8	85 (21-92)	12 (12)	.I.....S.... .....DV..D...A..
13	Pre-T	100 (99-100)	0 (12)	CTRPNNNTRKSIPVG PGSSFYATGDIIGDIRQAHC
	Wk 16	92 (66-96)	11 (12)	.....S.H.. .....H.
14	Pre-T	98 (80, 95-100)	1 (11)	CTRPXNNTIRSIHMG PGXAFSTTGDIIGDIRKAHC
		98 (99)	0 (1)	....S....K..... ..R.....ERV..N..H...
	Wk 16	92 (68-94)	12 (12)	....N...IR..... P.K..S..GDI..D..K.H.
15	Pre-T	100 (100)	0 (12)	CTRPNNNTRKSISFG PGSTIYATGDIIGDIRQAHC
	Wk 16[e]	78 (81-90)	4 (4)	....GA..T.....
		78 (96, 97)	0 (2)	.....C.... ..GA..T.....
		78 (79-96)	5 (6)	.....X.. ...AFI.....

Note: Amino acids in **bold** were present in all clones from an individual time point or subset of clones. Other amino acids shared >80% conservancy between clones. X indicates <80% conservancy between clones. Amino acids in red represent changes from Day 1 in >1 maraviroc-resistant clone and not in any susceptible clones for each set of individual Pat's isolates. Amino acids in blue represent changes from Day 1 seen in >1 maraviroc-resistant clone in addition to ≥1 susceptible clone for each set of individual Pat's isolates. Detailed information, including MPI, susceptibility, and sequences of the clones, are provided in the Supplementary Table.

MPI: maximum percent inhibition; Pat: participant; Pre-T: pretreatment.

[a]Values from the bulk virus clones (pool) are provided with the range of MPI values for the clones in parentheses.

[b]This Pat had their viral isolate from the protocol-defined virologic failure treatment time point at Week 16 included. At this time point there was a 5% reduction in MPI (95%) vs baseline virus (100%). Later treatment with open-label maraviroc showed a virus with MPI of 83% at Week 40 using the standard maraviroc susceptibility assay.

[c]One clone had no maraviroc susceptibility data because the analysis was unsuccessful. Sequence data are provided in the Supplementary Table.

[d]Phenotypic and genotypic results for Pat 3, Pat 8, Pat 11, and Pat 12 have been presented previously,[20] and site-directed mutagenesis confirmed the role of the V3 mutations in maraviroc resistance in these 4 Pats.

[e]More than one genetic pathway to resistance was observed in viruses from Pat 5, Pat 10, and Pat 15.

[f]Two viral genotypes were observed pretreatment in the virus from Pat 10.

Informatics, Saarbrücken, Germany), with a false-positive rate of 10%.

Phenotypic susceptibility testing

Phenotypic susceptibility to maraviroc was determined with the PhenoSense Entry assay (Monogram Biosciences) and viruses classified as either maraviroc$_{sus}$ or maraviroc$_{res}$ based on an MPI cut-off value of 95%.[20]

V3-Loop sequencing and analysis

Week 24. For the initial participants identified with R5 virologic failure (TLOVR50) and with reduced susceptibility to maraviroc through Week 24, clonal analysis of maraviroc susceptibility and gp160 sequencing was performed on viral isolates obtained at pre- and post-treatment time points (Monogram Biosciences). For each participant, clonal sequences were aligned using a multiple alignment sequence program (MUltiple Sequence Comparison by Log-Expectation),[21] and the sequences for the V3-loop region were extracted and compared. Any sequence variation between pre- and post-treatment that was also observed in the virus with reduced maraviroc susceptibility was used in subsequent analysis.

Week 24 sequence data have previously been submitted to GenBank (National Institutes of Health, Bethesda, MD, USA), accession numbers KT452108 to KT452130, KT452143 to KT452286, KT452311 to KT452358, and KT452383 to KT452526.

Week 48. For the retrospective assessment of V3-loop sequences, a database was prepared to associate clinical outcomes from Week 48 with Screening sequence data (Week 48 database). HIV-1 RNA was extracted from frozen plasma samples, and triplicate nested reverse transcription polymerase chain reaction methods were used to amplify and subsequently sequence the V3 loop of gp120 of the HIV-1env encoding region from the RNA extracts, as described by McGovern et al.[19] The replicate nucleotide sequences for each virus were translated, and the sequences were aligned against a reference V3-loop amino acid sequence detailed by LaRosa[22] using BLASTX (National Library of Medicine, Bethesda, MD, USA) to identify codons corresponding to positions 1 to 35 of the reference sequence (Figure 1). Codons containing ambiguous nucleotides were translated to provide all possible predicted amino acids, each of which were included in the analysis. Numbers of every amino acid predicted at each codon position and of all indels were obtained for the whole population. The most common amino acid at each position defined the consensus sequence. The MOTIVATE consensus sequence was compared with another consensus sequence generated from the Los Alamos dataset of 391 subtype B sequences by Patel et al.[23] Positions with >3% variants were also compared.

Assessment of correlation of V3-loop sequence with virologic outcome and maraviroc susceptibility

Variants associated with reduced maraviroc susceptibility in the Week 24 analysis, together with those previously associated with maraviroc resistance in individual viruses, and all additional amino acid variants, insertions, or deletions that occurred in ≥20 participants (>3%) in the Week 48 database, were used to query the pretreatment V3-loop sequences in relation to the incidence of virologic failure in participants with a given residue compared with participants without that residue. Fisher's exact test was performed with the α level of significance set to 0.05. The stratified Cochran–Mantel–Haenszel test was also applied to control for the effects of variable background regimen activity. Corrections for multiple testing or confounding factors were not included in these exploratory analyses.

Specific pretreatment residues found to be associated with virologic failure were then queried against a subset of the database comprised of pretreatment V3-loop sequences from the 83 participants who failed at Week 48 with an R5 virus and had maraviroc susceptibility data. Participants whose virus on Day 1 had residues known to be associated with virologic failure

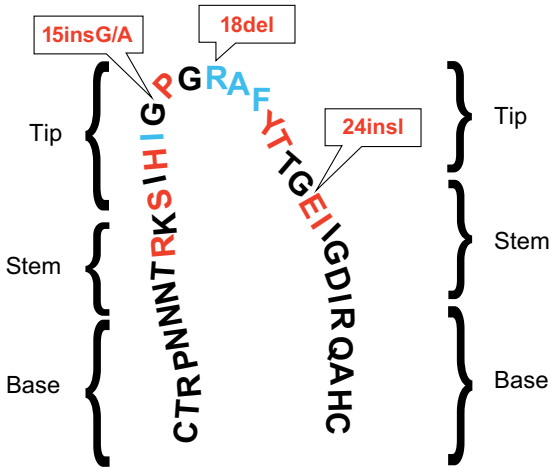

Figure 1. Changes in the V3-loop sequence identified in the Week 24 analysis mapped onto a cartoon of the V3-loop structure showing base, stem, and tip regions (Huang et al.[26]). Changes in amino acid change exclusive to resistant clones within an isolate are in red. Changes in amino acid associated with all resistant clones within an isolate are in blue. Amino acid at the residue pretreatment or not associated with resistance are in **black**. Note the sequence shown is based on HIV-1JR-FL; the insertion at codon 24 (24insl) occurred in the virus with a deletion of residue 25 pretreatment.

in this analysis were identified, and the maraviroc susceptibility of their virus at failure, together with available Week 24 clonal sequence data, was analyzed.

Optimized background susceptibility scores and adherence

A weighted susceptibility score for optimized background therapy obtained using phenotypic activity (pWOBTss) was used to estimate the number of active drugs in each treatment regimen as described previously.[24] Adherence to therapy was estimated using the presence of any maraviroc plasma concentration below the limit of quantification (5 ng/mL) observed during sparse pharmacokinetic sampling, which was performed during the first 24 weeks of therapy, as a marker of suboptimal adherence.

Results

Week 24

Genotype and reduced susceptibility to maraviroc. Fourteen of the 35 participants experiencing virologic failure with R5 virus at Week 24 had evidence of reduced phenotypic susceptibility to maraviroc (MPI <95%) and 1 had MPI 95% at failure. Pre- and on-treatment V3-loop amino acid sequences from 12 viral clones were aligned and analyzed for changes at specific residues and phenotypic susceptibility to maraviroc measured. The data are summarized in Table 1, with more detail provided in the Supplementary Table. Within individual paired sequences, changes in amino acid were recorded for sequences present in both susceptible and resistant viruses (blue) and for those present in resistant viruses alone (red).

Overall, no clear pattern was observed of changes in amino acid on treatment among the 15 participants with maraviroc-resistant CCR5 virus at failure through Week 24 (Table 1). In 3 post-treatment maraviroc-resistant viruses, a change in amino acid at position 26 (I26V) was identified; however, not all viruses with this change showed reduced susceptibility. Furthermore, in the pretreatment isolate from another participant (Pat 2), all clones had I26V and were fully susceptible to maraviroc.

Two incidents of identical changes in amino acid occurred in viruses from 2 different participants (N13H seen in Pats 10 and 11; P16A seen in Pats 4 and 9). However, these changes were not exclusively associated with resistant clones on treatment for 3 of 4 participants. Furthermore, the consensus for subtype B codon 13 is histidine, and H13 occurred as a consensus sequence among maraviroc-susceptible clones from pretreatment isolates from 6 other participants.

Seven participants had a total of 8 isolates with changes from the consensus pre-treatment virus that were exclusively observed across all resistant on-treatment clones (Table 1 – relevant changes in red font). These changes differed in position and residue, and 4 of the 8 instances involved insertions or deletions; 3 (G15insA, G15insG, G18del) were in the V3-loop tip. Tip-insertion changes were associated with the greatest reductions in MPI. When all residues with changes in amino acid associated with partial or exclusive resistance on treatment were mapped onto a diagram of the V3 loop, they were concentrated in the stem and tip of the structure, which provides the region of greatest interaction between viral gp120 and CCR5 (Figure 1).[25]

Week 48

Comparison of consensus sequence generated using the week 48 database of V3-loop screening sequences with reference consensus sequence. The Week 48 database linked V3-loop screening sequence data with Week 48 outcome data. A V3-loop consensus sequence and variants were derived and compared with a V3-loop reference consensus sequence and variants derived from a database of 391 sequences for subtype B virus based on sequences downloaded from the Los Alamos HIV Sequence Database.[23] The pattern of conserved and variable residues was similar between the databases (Table 2). The 2 consensus sequences differed at position 25 alone (aspartic acid in the MOTIVATE analysis and glutamic acid in that of the comparator reference consensus sequence; Table 2). Therefore, the MOTIVATE dataset was considered to be representative of subtype B viruses.

Retrospective assessment of V3-loop sequence variants using the week 48 database. All positions in the V3 loop with >3% prevalence of any amino acid variant from the MOTIVATE dataset consensus sequence were identified in the 704 pretreatment sequences. Of these, 52 variants were identified across 23 amino acid positions from the MOTIVATE dataset consensus (resulting in 75 variants, including the 23 consensus residues). Apart from consensus residues, amino acid variants K10R, S11G, A22T, D25E, and D29N were the most prevalent, observed at 29%, 31%, 29%, 34%, and 27% respectively (Table 2). At 8 positions, >3 different amino acid variants occurred (positions 2, 5, 13, 14, 18–20, and 25); 5 of these (positions 5, 13, 18, 20, and 25) were also highly variable in the comparator database.[23] The majority of variation was located in the stem domain of the V3-loop structure on either side of the GPG tip (positions 13, 14, and 18–20), which is where the greatest variability had been

Table 2. Week 48 database screening consensus Gp120 V3-loop amino acid sequence and variants (%) With >3% prevalence in viruses from the 704 participants analyzed (grey shading) compared with the consensus sequence from Patel et al.[23] and variants from that of 391 R5 sequences in the comparator Clade B Database (green shading).

Residue no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Consensus sequence (MOTIVATE)	**C**	T	**R**	P	N	**N**	**N**	**T**	R	K	S	I	H	I	G	**P**	**G**	R
Other variants		I (9) A (3) V (3)		L (3)	S (17) G (12) H (3)				K (3) S (3)	R (29)	G (31) R (3)	V (6)	N (16) P (14) S (12) T (7) R (5)	M (24) L (12) F (4)	A (8)			K (15) S (14) G (8) Q (5)
Consensus sequence (subtype B)	**C**	T	**R**	P	N	**N**	**N**	**T**	R	K	S	I	H	I	G	P	**G**	R
Other variants		I (11)			S (11) G (7) H (3)					R (16)	G (21)	V (3)	P (16) N (11) S (4) T (3)	M (15) L (14)	A (4)	W (5)		K (11) S (6) G (3) Q (6)
Residue no.	19	20	21	22	23	24	25	26	27	28	29	30		31	32	33	34	35
Consensus sequence (MOTIVATE)	A	F	Y	A	T	G	D	I	I	**G**	D	**I**		**R**	Q	**A**	H	**C**
Other variants	T (6) S (6) V (5)	I (9) L (7) W (4)	F (12) H (3)	T (29)	A (7)	Del (11) E (4)	E (34) Q (12) A (8) K (6) G (5) N (4) R (4)	V (10)	V (9) T (8)		N (27)				K (16) R (7)		Y (23)	
Consensus sequence (subtype B)	A	F	Y	A	T	G	E	I	I	**G**	D	**I**		**R**	Q	**A**	H	**C**
Other variants	T (6)	W (11) L (8) I (4)	F (6) H (3)	T (46)			D (30) Q (14) A (7) G (5) N (3)	V (4)	V (4) T (4)		N (13)				K (7) R (4)		Y (12)	

Table 3. Response rates for maraviroc-treated participants With 4L, 11R, and 19S mutations pretreatment.

			Weighted susceptibility score for optimized background therapy, n (%)[a]		
		Overall response	0/0.5	1.0/1.5	≥2
MOTIVATE Database	N	704	262	264	178
	Responders, n (%)	366 (52.0)	90 (34.4)	147 (55.7)	129 (72.5)
	Virologic failure, n (%)	338 (48.0)	172 (65.6)	117 (44.3)	49 (27.5)
4L variant	N	20	9	4	7
	Responders, n (%)	5 (25.0)	1 (11.1)	0 (0.0)	4 (57.1)
	Virologic failure, n (%)	15 (75.0)	8 (88.9)	4 (100)	3 (42.9)
11R variant	N	23	7	11	5
	Responders, n (%)	6 (26.0)	0 (0.0)	3 (27.3)	3 (60.0)
	Virologic failure, n (%)	17 (74.0)	7 (100)	8 (72.7)	2 (40.0)
19S variant	N	39	16	16	7
	Responders, n (%)	13 (33.0)	2 (12.5)	8 (50.0)	3 (42.9)
	Virologic failure, n (%)	26 (67.0)	14 (87.5)	8 (50.0)	4 (57.1)

[a]Weighted susceptibility scores for optimized background therapy, according to the phenotypic resistance value[24]: nucleoside reverse transcriptase inhibitor = 0.5, all other drugs = 1.[23]

observed in viruses with reduced susceptibility to maraviroc at Week 24 (Figure 1; Table 2).

Amino acid variants previously observed in individual maraviroc-resistant R5 viruses and those previously described in the 15 maraviroc-resistant viruses from participants identified at Week 24 were present in the derived consensus sequence (Table 2). Combinations of amino acid variants (11S + 26V [n = 52]; 18G + 22T [n = 12]; 19S + 26V [n = 6]; 20F + 25D + 26V [n = 23]; 20F + 21I [n = 3]), which were identified in the Week 24 clonal dataset, were also present in the 704 Screening sequences in the Week 48 population sequence database. Response rates for participants whose virus contained these combinations ranged from 42% (18G + 22T) to 67% (20F + 21I). None of these combinations were significantly associated with failure compared with the overall population using a Fisher's exact test (P value range: 0.21 to 1.00), although proportions of the population with these mutation combinations were small (range: 0.4% to 7.3%).

Association between V3-loop variants and virologic outcome. The association between V3-loop variants and virologic outcome at Week 48 was determined using the Week 48 database. The response rate for individuals with V3-loop variants was compared with the overall Week 48 response rate for the maraviroc-treated population (52%; n/N = 366/704). All the variants, including insertions, deletions, and combinations of variants, identified from the Week 24 analysis of maraviroc-resistant viruses as well as the 75 amino-acid variants with >3% prevalence previously described were included.

In univariate analysis, 3 amino acid variants (4L, 19S, and the CXCR4 tropism-associated variant 11R) were identified as potential predictors of poor response ($P < 0.05$). They were present in the database with prevalence rates of 2.8%, 5.5%, and 3.3%, respectively, and were associated with response rates of 25% (n/N = 5/20), 33% (n/N = 13/39), and 26% (n/n = 6/23), respectively, compared with a response rate of 52% among participants included in the total dataset.

Response rates were also examined in participants with viruses encoding each of these amino acids in relation to the amount of antiviral support provided by optimized background therapy (Table 3). Overall, the pWOBTss were significantly lower in participants with virologic failure; however, the presence of mutations 4L, 11R, or 19S was associated with greater proportions of virologic failure, even when controlling for the pWOBTss using the stratified Cochran–Mantel–Haenszel test ($P < 0.05$). Although numbers were small in these subgroups, it is notable that, among 262 participants with pWOBTss <1, a response rate of 34.4% at Week 48 was recorded, whereas the corresponding participants with 4L, 11R, and 19S viral variants only supported responses in 1 of 9 (11.1%), 0 of 7 (0%), and 2 of 16 (12.5%) participants, respectively. In all instances, the proportion with response was higher when the pWOBTss was ≥2, with 57.1%, 60.0%, and 42.9% for the 4L, 11R and 19S variants, respectively, compared with a response of 72.5% among the overall population of 178 participants with pWOBTss ≥2 (Table 3). Three double-mutant combinations were observed, so these participants were evaluated as having individual mutations and not in combination.

Table 4. Phenotypic tropism at failure for main database and participants with 4L, 11R, and 19S.

		Tropism at failure, n (%)		
	Participants, n	R5	CXCR4-using	BLQ/NR/NP
MOTIVATE database	Viral failures: 338	95 (28.1)	128 (37.9)	115 (34.0)
4L variant	Viral failures: 15	5 (33.3)	6 (40.0)[a]	4 (26.7)
11R variant	Viral failures: 17	2 (11.8)	11 (64.7)	4 (23.5)
19S variant	Viral failures: 26	7 (26.9)	8 (30.8)[b]	11 (42.3)

CXCR4-using: dual/mixed or X4 tropic virus; BLQ: below limit of quantification; NR: no result; NP: not phenotypable.
[a]1 and [b]2 participants also had 11R which is a marker for CXCR4-using virus.

A more expansive multivariate analysis was not possible due to the limited size of the datasets.

V3-Loop sequence and tropism. Additional analysis was performed to examine the association between the variants 4L, 11R, and 19S in relation to tropism and response. Tropism at failure was measured using the original Trofile assay, and results were compared between these 3 variants and the main dataset to determine whether emergence of CXCR4-using viruses was responsible for the poor response rate. The variant 11R was associated with CXCR4-using viruses at failure, but viruses encoding 4L or 19S were not associated with any particular tropism at failure (Table 4).

The V3-loop sequences that contained 11R at Screening were also analyzed using the Geno2Pheno algorithm to assess the genotypic tropism prediction. Of the 17 identified 11R variants in participants with virologic failure, 15 were predicted to be CXCR4-tropic, 1 as CCR5-tropic, and 1 as unassigned. Furthermore, although all 17 virologic failure participants with the 11R variant had R5 virus identified at screening, CXCR4-using virus was found using the original Trofile assay prior to exposure to maraviroc at Day 1 in 6 (35.3%). Thirteen of these 17 variants also had a tropism determination at treatment failure when the presence of CXCR4-using virus was found in 11 of the 13 variants (84.6%) using the Original Trofile assay. Six participants with 11R variants at Screening showed a virologic response (Table 3); 5 of these 6 retained an R5 tropism (Trofile) at Day 1, whereas the tropism for the other virus was nonreportable. However, when the Geno2Pheno algorithm was applied to the Screening sequences of these viruses, 4 of the 6 were predicted to be CXCR4-tropic. When the Screening samples with the 11R variant were retrospectively analyzed using the Enhanced Sensitivity Trofile Assay, 2 of the 6 responders (33.3%) and 12 of the 17 failures (70.6%) were ascribed to a CXCR4-using phenotype. The 2 responders with a CXCR4-employing phenotype had a pWOBTss of 2.

Screening V3-loop sequence and phenotypic susceptibility in participants failing with R5 virus. In all, 83 of the 704 participants experienced virologic failure with R5 virus and had a valid maraviroc phenotype result; 57 (69%) of these were phenotypically susceptible to maraviroc. The residues 4L and 19S were present in 5 (6.0%) and 7 (8.4%), respectively, of the 83 participants' viruses at Screening. Reduced susceptibility to maraviroc at failure was found at Week 48 in 3 of 5 (60%) participants with a 4L variant. Three of the 7 (43%) variants with 19S had reduced susceptibility to maraviroc at failure (2 at Week 24 and 1 at Week 48).

Clonal sequence data from Week 24 were available for all 3 participants with maraviroc-resistant virus in whom 19S was detected at Day 1 (Table 5). In all 3 participants, 19S was also detected in clones at failure and was present in both maraviroc-resistant and maraviroc-susceptible clones, indicating that this residue alone did not confer a maraviroc-resistant phenotype. Additional amino acid variants in the V3 loop were also observed in both maraviroc-resistant and maraviroc-susceptible clones with 19S at Day 1 and failure (Table 5). These additional amino acid variants in the 19S-encoding loops differed between participants and between time points.

Of the participants with the 19S variant, the viral clones in one participant (Pat 8) had 3 or 4 changes in amino acid after treatment (18R, 20F, 25D, 26V), although clonal analysis included both resistant (MPI, 61%–76%) and susceptible clones (MPI, 99%). One participant (Pat 12) had a highly diverse viral genotype pretreatment that resolved to include 11S and 26V in a relatively homogeneous genotype at failure. Key changes from consensus between Day 1 and virologic failure from Pat 13 included P13H and Y34H, both of which were also present in viruses from Pats 8 and 12 at Day 1 in maraviroc-sensitive clones (Table 5).

Adherence and maraviroc susceptibility. Sparse pharmacokinetic measurements were examined in the group of 83 participants with maraviroc susceptibility determined at virologic failure with R5 virus (maraviroc phenotypically susceptible, $n = 57$; maraviroc

Table 5. Clonal V3-loop sequences with 19S show additional mutations are associated with phenotypic maraviroc susceptibility reduction.

Pat no.	1 5 10 15 20 25 30 35 ....|....|....|....|....|....|....|	Clones, n	MPI range, %
Pat 8 Day 1	CTRPGNNTRKSIHMGPGSSIYATGAIIGDIRQAHC	9	98-100
	.....D.....	1	100
	.....E.....	2	99
Pat 8 Wk 24	.....F....DV.....	9	61-99
	.....R.F....DV.....	3	76-99
Pat 12 Day 1	CIRPNNNTRKGIHIGPGRSFYATGDIIGDIRQAHC	4	95-100
	.T.....E.....	2	95-97
	.T.....V..	1	100
	.T.....	2	100
	.....N.....	2	99-100
	.....T..	1	100
Pat 12 Wk 8	.....S.....V.....	12	21-92
Pat 13 Day 1	CTRPNNNTRKSIPVGPGSSFYATGDIIGDIRQAYC		99-100
	.....G.....H.	1	100
Pat 13 Wk 16	.....H.....H.	12	66-96

MPI: maximum percent inhibition; Pat: participant.

phenotypically resistant, n = 26). In the total CCR5 maraviroc-susceptible failure population, 21 (36.8%) of 57 participants had markers of suboptimal adherence compared with 2 (7.7%) of 26 participants who experienced failure with CCR5 maraviroc-resistant virus (Fisher's exact test; $P = 0.007$).

Discussion

Virus from participants with virologic failure and phenotypic resistance to maraviroc at Week 24 in the MOTIVATE clinical trials had a variety of amino acid changes identified in the stem and tip of the V3 loop such that a consistent pattern of resistance could not be identified in these participants. These findings further support other reports indicating the absence of a common genetic pathway to resistance for maraviroc.[14] In addition to the broad variability of changes in the V3 loop, other non–V3-loop amino acid substitutions were observed in gp120, mostly clustered in V1, V4, and V5, and less commonly in the bridging sheet and CD4-binding region, adding to the diversity and complexity of the effects of changes in amino acid.[14]

Changes observed in these maraviroc-resistant viruses among participants with virologic failure were initially taken by others to be markers of maraviroc resistance,[15–17] and the findings of significant proportions of these as naturally occurring polymorphisms raised concerns regarding the need for V3-loop genotyping prior to starting maraviroc treatment. The prevalence of these mutations and their combinations in the MOTIVATE database described here are similar to that reported by Soulie,[16] who found ~7% of R5 viruses had these V3-loop variants; however, none of these previously published analyses were supported by phenotypic susceptibility evaluations. Another analysis identified 93 viruses with these variants.[27] Fourteen of these viruses with representative variant patterns in the V3 loop were tested for phenotypic susceptibility to maraviroc, only one of which was found with phenotypic resistance. In addition, removal of the V3-loop mutations from these viruses restored susceptibility; however, adding the mutations to a different genetic background did not give rise to resistance.[27] These observations are in agreement with previous findings from initial investigations of maraviroc susceptibility in the MOTIVATE studies, whereby transferring on-treatment resistance-associated changes using site-directed mutagenesis did not always establish resistance in the virus on Day 1.[20]

Furthermore, changes identified in individual viral isolates do not necessarily confer resistance when they are present in other viruses.[12,20] It therefore appears that resistance is mediated in the context of complex

conformational changes. These changes are likely associated with additional variable networks of linked changes in gp120 and gp41 to maintain essential multifunctionality.[14] Consistent with the earlier analyses showing poor correlation between genotype and phenotypic susceptibility, the combinations of changes previously identified in individual viral isolates that showed reduced susceptibility to maraviroc were not linked to virologic outcome in this large retrospective analysis of V3-loop sequences from 704 participants treated with maraviroc. Overall, this current analysis supports the lack of association between specific V3-loop variants and maraviroc susceptibility, confirming that reliable, predictive, signature resistance-associated mutations inherently cannot be identified for maraviroc.

Of the 52 amino acid variants relative to the V3-loop consensus sequence present at 23 positions in >3% (ie, >20 of the total group of 704 participants), only the 4L, 11R, and 19S residues showed any potential association with virologic failure in univariate analysis. However, these variants were present in a small proportion of viruses, and one of these, 11R, was identified as a marker of CXCR4 use, thereby explaining its association with virologic failure. Examination of other factors that might impact response revealed that a high proportion of participants with viruses containing these residues had a low pWOBTss for their background therapy. The pWOBTss has been found to be associated with virologic outcome in multivariate analysis.[24] This was further supported by the finding that the 2 participants with pretreatment virus carrying 11R and whose infection responded to treatment had a pWOBTss of 2; therefore, they may have had sufficient antiviral activity in their background regimen to suppress the outgrowth of CXCR4-using virus. Conversely, more participants with 19S and 4L had a pWOBTss <1, indicating very little support from other drugs in the regimen. In addition, for participants infected with maraviroc-susceptible virus at treatment failure, markers of nonadherence were more common than in those whose treatment failed and were infected with maraviroc-resistant virus. Although numbers in these subgroups are small, overall, these results are consistent with the requirement for selective pressure to be maintained in order to select and preserve reduced susceptibility.[28]

Analysis of sequence data from viruses of participants with R5 virus whose infection failed maraviroc treatment showed that the 4L and 19S residues were not always associated with failure with reduced maraviroc susceptibility; therefore, they are not reliable markers of maraviroc resistance. Clonal genotypic data with phenotypic susceptibility values from the virus of 3 participants showed that 19S could be present in both sensitive and resistant clones. It is possible that additional residues within the V3 loop, or elsewhere in the gp120/gp41 complex, may contribute to the maraviroc-resistant phenotype observed with the 19S viruses. Indeed, contribution from other regions of gp120 has been indicated for maraviroc[14] and in studies with another CCR5 antagonist, vicriviroc.[29,30] The need for envelope proteins to retain essential multifunctionality (epistaticity) may partly explain diverse changes associated with reduced susceptibility to maraviroc.[14] Many changes could be associated with changes in immune response or with viral adaptations to changes in the available permissive cell population. Indeed, there is a record of a clonal virus (YU-2) adapting to low-density CCR5 on cells in vitro through a change at position 7 of the V3 loop.[31] This change was not observed in the current analyses.

In conclusion, findings from this analysis of a large number of participants with maraviroc-related treatment outcomes further support the lacking correlation between V3-loop genotype and clinical outcome, confirming that the V3-loop sequence cannot be used to predict the maraviroc susceptibility of R5 virus.

Acknowledgements

We thank the participants and investigators of the MOTIVATE 1 and 2 studies. Richard Harrigan of the British Columbia Centre of Excellence in HIV/AIDS provided population-based baseline V3-loop sequences used in the Week 48 analysis of clinical outcomes.

Data sharing

Anonymized individual participant data and study documents can be requested for further research from www.clinicalstudydatarequest.com.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: MEL, PS, JM, BJ, EVDR, CC, and MW were employed by Pfizer Inc at the time the submitted research was conducted. MEL, EVDR, and CC are now contracted to Pfizer Inc through The Research Network. CC has also received contractor fees from ViiV Healthcare. PS is currently employed by AstraZeneca. JM is currently employed by hVIVO. BJ was an employee of Pfizer at the time of the submitted work but is now an employee of Covance Inc. JS is currently employed by Cytel and is a shareholder of Pfizer Inc. LM is an employee and shareholder of Pfizer Inc. DLR has nothing to disclose. MW is currently employed by Centauri Therapeutics Ltd.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was funded by ViiV Healthcare. Editorial

assistance was provided under the direction of the authors by Sherri Damlo, ELS, MedThink SciCom, and was funded by ViiV Healthcare. The authors have authorized the submission of this manuscript by Rupali Ullal, MedThink SciCom, whose submission assistance was funded by ViiV Healthcare.

ORCID iDs

E van der Ryst https://orcid.org/0000-0002-9193-1825
C Craig https://orcid.org/0000-0003-3276-9925

Supplemental material

Supplemental material for this article is available online.

References

1. Aiamkitsumrit B, Dampier W, Antell G, et al. Bioinformatic analysis of HIV-1 entry and pathogenesis. *Curr HIV Res* 2014; 12: 132–161.
2. Wyatt R and Sodroski J. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* 1998; 280: 1884–1888.
3. Keele BF, Giorgi EE, Salazar-Gonzalez JF, et al. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A* 2008; 105: 7552–7557.
4. Archer J, Braverman MS, Taillon BE, et al. Detection of low-frequency pretherapy chemokine (CXC motif) receptor 4 (CXCR4)-using HIV-1 with ultra-deep pyrosequencing. *AIDS* 2009; 23: 1209–1218.
5. Berger EA, Murphy PM and Farber JM. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol* 1999; 17: 657–700.
6. Brumme ZL, Goodrich J, Mayer HB, et al. Molecular and clinical epidemiology of CXCR4-using HIV-1 in a large population of antiretroviral-naive individuals. *J Infect Dis* 2005; 192: 466–474.
7. Dorr P, Westby M, Dobbs S, et al. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrob Agents Chemother* 2005; 49: 4721–4732.
8. Garcia-Perez J, Rueda P, Staropoli I, et al. New insights into the mechanisms whereby low molecular weight CCR5 ligands inhibit HIV-1 infection. *J Biol Chem* 2011; 286: 4978–4990.
9. Tan Q, Zhu Y, Li J, et al. Structure of the CCR5 chemokine receptor – HIV entry inhibitor maraviroc complex. *Science* 2013; 341: 1387–1390.
10. Westby M. Resistance to CCR5 antagonists. *Curr Opin HIV AIDS* 2007; 2: 137–144.
11. Roche M, Salimi H, Duncan R, et al. A common mechanism of clinical HIV-1 resistance to the CCR5 antagonist maraviroc despite divergent resistance levels and lack of common gp120 resistance mutations. *Retrovirology* 2013; 10: 43.
12. Westby M, Smith-Burchnell C, Mori J, et al. Reduced maximal inhibition in phenotypic susceptibility assays indicates that viral strains resistant to the CCR5 antagonist maraviroc utilize inhibitor-bound receptor for entry. *J Virol* 2007; 81: 2359–2371.
13. Lazzarin A, Reynes J, Molina JM, et al. The maraviroc expanded access program – safety and efficacy data from an open-label study. *HIV Clin Trials* 2015; 16: 10–21.
14. Jiang X, Feyertag F, Meehan CJ, et al. Characterizing the diverse mutational pathways associated with R5-tropic maraviroc resistance: HIV-1 that uses the drug-bound CCR5 coreceptor. *J Virol* 2015; 89: 11457–11472.
15. Alencar CS, Nishiya AS, Ferreira S, et al. Evaluation of primary resistance to HIV entry inhibitors among Brazilian patients failing reverse transcriptase/protease inhibitors treatment reveal high prevalence of maraviroc resistance-related mutations. *AIDS Res Hum Retroviruses* 2010; 26: 1267–1271.
16. Soulie C, Malet I, Lambert-Niclot S, et al. Primary genotypic resistance of HIV-1 to CCR5 antagonists in CCR5 antagonist treatment-naive patients. *AIDS* 2008; 22: 2212–2214.
17. Vasil'ev AV, Kazennova EV and Bobkova MR. Analysis of the prevalence of CCR5 coreceptor antagonist resistance mutations among HIV-1 variants in Russia. *Voprosy Virusologii* 2011; 56: 32–37.
18. Gulick RM, Lalezari J, Goodrich J, et al. Maraviroc for previously treated patients with R5 HIV-1 infection. *N Engl J Med* 2008; 359: 1429–1441.
19. McGovern RA, Thielen A, Mo T, et al. Population-based V3 genotypic tropism assay: a retrospective analysis using screening samples from the A4001029 and MOTIVATE studies. *AIDS* 2010; 24: 2517–2525.
20. Lewis M, Mori J, Toma J, et al. Clonal analysis of HIV-1 genotype and function associated with virologic failure in treatment-experienced persons receiving maraviroc: Results from the MOTIVATE phase 3 randomized, placebo-controlled trials. *PLoS One* 2018; 13: e0204099.
21. Edgar RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinform* 2004; 5: 113.
22. LaRosa GJ, Davide JP, Weinhold K, et al. Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant. *Science* 1990; 249: 932–935.
23. Patel MB, Hoffman NG and Swanstrom R. Subtype-specific conformational differences within the V3 region of subtype B and subtype C human immunodeficiency virus type 1 Env proteins. *J Virol* 2008; 82: 903–916.
24. Schapiro JM, Boucher CA, Kuritzkes DR, et al. Baseline CD4(+) T-cell counts and weighted background susceptibility scores strongly predict response to maraviroc regimens in treatment-experienced patients. *Antivir Ther* 2011; 16: 395–404.
25. Cormier EG and Dragic T. The crown and stem of the V3 loop play distinct roles in human immunodeficiency virus type 1 envelope glycoprotein interactions with the CCR5 coreceptor. *J Virol* 2002; 76: 8953–8957.
26. Huang CC, Tang M, Zhang MY, et al. Structure of a V3-containing HIV-1 gp120 core. *Science* 2005; 310: 1025–1028.
27. Delobel P, Cazabat M, Saliou A, et al. Primary resistance of CCR5-tropic HIV-1 to maraviroc cannot be predicted

by the V3 sequence. *J Antimicrob Chemother* 2013; 68: 2506–2514.
28. Roberts NA, Craig JC and Sheldon J. Resistance and cross-resistance with saquinavir and other HIV protease inhibitors: theory and practice. *AIDS* 1998; 12: 453–460.
29. Anastassopoulou CG, Ketas TJ, Depetris RS, et al. Resistance of a human immunodeficiency virus type 1 isolate to a small molecule CCR5 inhibitor can involve sequence changes in both gp120 and gp41. *Virology* 2011; 413: 47–59.
30. McNicholas PM, Mann PA, Wojcik L, et al. Mapping and characterization of vicriviroc resistance mutations from HIV-1 isolated from treatment-experienced subjects enrolled in a phase II study (VICTOR-E1). *J Acquir Immune Defic Syndr* 2011; 56: 222–229.
31. Garg H, Lee RT, Maurer-Stroh S, et al. HIV-1 adaptation to low levels of CCR5 results in V3 and V2 loop changes that increase envelope pathogenicity, CCR5 affinity and decrease susceptibility to maraviroc. *Virology* 2016; 493: 86–99.