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Different selection patterns of resistance and cross-resistance to HIV-1 agents targeting CCR5

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Objectives: Identification of CCR5 as an antiretroviral target led to the development of several CCR5 antagonists in clinical trials and the approval of maraviroc. Evaluating the mechanism of drug resistance to CCR5 agents may have implications in the clinical development of this class of agents. We have analysed the resistance profile of two R5 HIV-1 strains [BaL and a clinical isolate (CI)] after long-term passage in cell culture in the presence of TAK-779, the first developed non-peptidic small molecule targeting CCR5.

Methods: Genotypic and phenotypic tests were used to evaluate the resistance of virus isolated from cell culture in the presence of the CCR5 inhibitor TAK-779.

Results: Mutations conferring resistance appeared in the gp120 sequence but were not confined to the V3 loop region, and both strains had a different mutation pattern. Recombination of the *env* gene of the BaL-derived resistant virus into the HIV-1 HXB2 wild-type backbone conferred resistance to TAK-779 and cross-resistance to maraviroc, with 63- and 11-fold changes in their EC_{50} (50% effective concentration), respectively, together with an apparent reduction of the maximal plateau inhibition (MPI) of TAK-779 but not of maraviroc. Conversely, the resistant CI viruses showed an ~50% reduction in MPI for both TAK-779 and maraviroc.

Conclusions: We confirm that different pathways to the generation of CCR5 drug resistance/cross-resistance may occur that strongly depend on cell culture conditions, CCR5 availability and the genetic background of the HIV strain. Our study provides complementary information to understand the complexity of resistance to CCR5 antagonists.

Keywords: entry inhibitors, maraviroc, TAK-779, virus entry

Introduction

The essential steps of HIV-1 entry in the host cell offer several potential new targets for antiviral agents.[1,2] HIV-1 enters cells using the CD4 receptor and one of two co-receptors, CCR5 and CXCR4.[3,4] TAK-779 was the first small-molecule CCR5 inhibitor described[5,6] and a relevant proof of concept that led to the development of better drug candidates. The development of other chemical structures as CCR5 antagonists has culminated with the FDA-approved CCR5 inhibitor maraviroc (Selzentry).[7]

Resistance to CCR5 agents has been described both *in vitro* and in patients failing CCR5 drug-containing antiretroviral regimens (reviewed in Moore and Kuritzkes[8]). In the majority of patients, treatment failure is accompanied by a switch in virus co-receptor use from CCR5 (R5) to CXCR4 (X4), apparently through the emergence of pre-existing X4 variants that went undetected by current diagnostic tests[9,10] and that are favourably selected under CCR5 drug pressure. In cell culture, HIV can be forced to switch co-receptor when passaged in cells expressing high levels of CXCR4 and low/undetectable levels of CCR5.[11] However, most reports suggest that when R5 viruses are passaged in the presence of CCR5 agents, resistance develops in the absence of co-receptor switch.[12–15] Two mechanisms of resistance have been described for CCR5 drugs: competitive resistance, identified by an increase in the concentration of the inhibitor required to block HIV replication by 50% (EC_{50}), and non-competitive resistance in which increasing the inhibitor concentration does not lead to complete inhibition of virus replication, the EC_{50} remains unchanged and resistance is manifested by a decrease in the maximal HIV-1 inhibition achieved by the compound [maximal plateau inhibition (MPI)].[8] HIV-1 variants made resistant to most CCR5 drugs, including AD101,[14] vicriviroc,[15–17] SCH-C and maraviroc,[12] use the non-competitive mechanism in which virus may use CCR5 in the presence of the inhibitor (co-receptor-bound inhibitor model).[8,12,15] CCR5 drug cross-resistance profiles are also complex, and not all the viruses resistant to a compound appear to be cross-resistant to a structurally related molecule. The most common genetic route to resistance involves sequence changes in the gp120 V3 region, but viruses with mutations in other regions of gp120 or, surprisingly, in gp41 have been described.[18,19]

Starting with two R5 HIV-1 strains, we have selected HIV-1 strains that are resistant to TAK-779 after nearly a year in culture and have characterized their resistance profile. Our results highlight the complexity of the development of resistance to CCR5 antagonists and suggest that multiple mechanisms of resistance may be developed depending on the virus strains and the cell culture conditions used.

Methods

Cells

The lymphoid MT-4 cell line was obtained through the MRC Centre for AIDS Reagents. Cells were cultured in RPMI 1640 (Invitrogen, Barcelona, Spain) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Innogenetics, Barcelona, Spain) and selection antibiotics when appropriate. Peripheral blood mononuclear cells (PBMCs) from five healthy donors were isolated by gradient centrifugation of buffy coat cells obtained from the Catalonia Banc de Sang i Teixits (Barcelona, Spain). PBMCs from each donor were mixed equally and resuspended at 50×10^6 PBMCs/mL in FCS containing 10% DMSO (Sigma-Aldrich, Madrid, Spain), frozen and conserved, until needed, in liquid nitrogen.

Establishment of the MT-4/CCR5+ cell line

The *CCR5* coding sequence and the puromycin resistance gene were amplified from the pBABE-puro-CCR5-expressing vector (NIH AIDS Research and Reference Reagent Program). CCR5 was amplified with primers 5′-GGAACCGGTGGAACAAGATGG-3′ and 5′-CAGCCCACTTGGATCCGT-3′. Puromycin resistance (Pur^{res}) was amplified with 5′-GCAAAACGCGTACCATGACC-3′ and 5′-TTTTCTCGAGGCATGGGGTC-3′. Primers were purchased from Sigma Genosys. The Pur^{res} PCR product was cloned into the second multiple cloning site of the pQCXIX vector (Clontech) between MluI and XhoI restriction sites. The resulting vector (pQCXIX-Pur^{res}) was used for CCR5 cloning into the first multiple cloning site flanked by the AgeI and the BamHI sites, resulting in the pQCXIX-Pur^{res}-CCR5 construct.

A total of 0.1×10^6 293GP2 cells were seeded onto 24-well plates the day before transfection. Cells were transfected with 0.6 μg of pQCXIX-Pur^{res} or pQCXIX-Pur^{res}-CCR5 together with 0.6 μg of pVSV-G (Clontech), using the CalPhos™ Mammalian Transfection Kit (Clontech). At 72 h after transfection, the supernatants containing pseudoviruses were collected and filtered (Corning 0.2 μm pore; Sigma, Madrid, Spain). 293GP2 cells from the transfection were then selected with Dulbecco's modified Eagle's medium (DMEM) containing 1 μg/mL puromycin (Sigma).

A total of 0.25×10^6 MT-4 cells were transduced with 500 μL of filtered supernatant and incubated for 72 h in a CO_2 incubator. MT-4 cells were centrifuged and medium was replaced with RPMI growth medium containing 1.5 μg/mL puromycin (Sigma). MT-4 cells were cloned by serial dilution. CCR5+ populations were detected by flow cytometry, staining with fluorescein isothiocyanate (FITC) or APC-Cy7-A-labelled anti-CCR5 (CD195) monoclonal antibodies (BD Pharmingen, Madrid, Spain). Selected MT-4/CCR5 populations were frozen in FCS (Innogenetics, Barcelona, Spain) containing 10% DMSO (Sigma, Madrid, Spain) and stored in liquid nitrogen until needed.

Viruses

The R5 laboratory-adapted HIV-1 strain BaL was obtained through the MRC AIDS Reagent Program. HIV-1 isolate (CI) from an antiretroviral-experienced patient and displaying CCR5 tropism was obtained by co-culturing PBMCs from an HIV-1-infected patient with stimulated PBMCs from healthy donors.[20] The amino acid sequences of the reverse transcriptase and protease of CI have been published elsewhere.[21]

Reagents

TAK-779 and maraviroc were obtained from the NIH AIDS Research and Reference Reagent Program. 3-Azido-3-deoxythymidine (zidovudine) was purchased from Sigma-Aldrich (Madrid, Spain) and AMD3100[22,23] was purchased from Sigma (Madrid, Spain). Enfuvirtide (Fuzeon, T-20) and C-34[24] were synthesized by the Peptide Synthesis Service of Universitat de Barcelona.

Prolonged culture of HIV-1 viruses with or without TAK-779

PBMCs were stimulated with 6 U/mL interleukin-2 (IL-2; Roche, Barcelona, Spain) and 4 mg/mL phytohaemagglutinin (PHA) for 72 h before use. Stimulated PBMCs (10^5) were infected with 20 ng of R5 virus in the presence of 1 μg/mL TAK-779, in 48-well plates and in a final volume of 700 μL. Every week viral growth was checked by evaluation of CA p24 antigen in the culture supernatant using a commercial ELISA kit (Innogenetics, Barcelona, Spain) and recovered virus was used for re-infection of fresh stimulated PBMCs as described previously.[24–27]

Sequencing of wild-type and drug-resistant strains

Cell pellets obtained from each stock were used for the analysis of the proviral sequence. Genomic DNA was extracted with the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany), and the *env* gene (nucleotides 5514–8910, relative to HXB2) was amplified with primers 5′-GATAAAGCCACCTTTGCCTAGT-3′ and 5′-TTCTAGGTCTCGAGATACTG-3′ using the Expand High Fidelity PCR System (Roche, Barcelona, Spain). Both strands were amplified with the ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences from gp120 and gp41 were collected with an Avant 3100 Genetic Analyzer, and were aligned and analysed with the Sequencher 4.2 program.

HXB2-env clones harbouring gp160 from BaL and CI

gp120 and the extracellular region of gp41 (nucleotides 5665–8486, relative to HXB2) were amplified with primers 5′-GGATTTGGCTCCATGGCTTAGGG-3′ and 5′-TGCTAAGGATCCGTTCACTAATCG-3′, which contain the NcoI and BamHI restriction sites (underlined). PCR products were cloned using pGEM-T Easy Vector Systems from Promega (Madrid, Spain) following the manufacturer's instructions. The *env* sequences from the obtained clones were sequenced and those representative for the consensus sequence of viruses were selected for cloning into the pJJ5-HXB2 backbone.[24,28] HXB2-*env* clones were nucleofected into MT-4/CCR5+ cells with the Amaxa Nucleofector (Lonza Technologies, Cologne, Germany). Viral stocks from these cultures were generated and proviral *env* sequences were confirmed.

The gp160 region of BaL and CI (nucleotides 5665–8486, relative to HXB2) were amplified with primers 5′-GGATTTGGCTCCATGGCTTAGGG-3′

Table 1. Antiviral activity of control compounds when tested against viruses from passages

Compound	EC$_{50}$ (μg/mL)[a]			Fold resistance[c]	EC$_{50}$ (μg/mL)[a]		Fold resistance[c]
	BaL parental[b]	BaL-wt	BaL-res		CI-wt	CI-res	
TAK-779	0.02±0.001	0.08±0.036	>5	>63	0.057±0.017	0.077±0.075	1
Maraviroc	0.006±0.001	0.003±0.001	0.033±0.011	11	0.004±0.001	0.003±0.001	1
AMD3100	>0.5	>0.5	>0.5	—	>0.5	>0.5	—
Enfuvirtide	0.020±0.005	0.047±0.056	0.06±0.045	1	0.024±0.001	0.041±0.007	2
C-34	ND	0.016±0.011	0.016±0.008	1	0.008±0.005	0.006±0.001	1
Zidovudine	0.004±0.001	0.008±0.003	0.013±0.012	2	1.09±1.29	>2	>1
Nevirapine	0.18±0.006	0.2±0.15	0.52±0.36	3	0.035±0.004	0.104±0.005	3

ND, not determined.
[a]EC$_{50}$, 50% effective concentration, or concentration needed to inhibit 50% HIV-induced cytopathic effect, evaluated in MT-4/CCR5+ cells with the MTT assay. Maximum concentrations tested: 5 μg/mL TAK-779; 2.5 μg/mL maraviroc; 0.5 μg/mL AMD3100; 5 μg/mL enfuvirtide; 0.25 μg/mL C-34; 1 μg/mL zidovudine; and 2 μg/mL nevirapine. Values represent the mean of two separate evaluations ±SD.
[b]Unpassaged BaL.
[c]Fold resistance, calculated as the ratio EC$_{50}$ for resistant virus/EC$_{50}$ for wild-type virus. When the EC$_{50}$ value was greater than the maximal concentration, this was used for fold resistance calculation.

and 5′-TGCTAAGGATCCGTTCACTAATCG-3′, which contain the NcoI and BamHI restriction sites (underlined). PCR products were cloned using pGEM-T Easy Vector Systems from Promega (Madrid, Spain) following the manufacturer's instructions. The *env* sequences from the obtained clones were sequenced and those representative for the consensus sequence of viruses were selected for cloning into the pJJ5-HXB2 backbone.[24,28] The recombinant clones carrying the resistant *env* (BaL-c5.6r and CI-c46.9r) and the wild-type *env* (BaL-c2.2wt and CI-c3.3wt) were nucleofected into MT-4/CCR5+ cells and infective viral stocks were generated for further characterization

Viral stocks from these cultures were generated and proviral *env* sequences were confirmed.

Titration of viral stocks and evaluation of anti-HIV-1 activity of control compounds

HIV-1 strains were titrated after 5 days acute infection, and infectivity was measured in MT-4/CCR5+ cells, exposed or not to an HIV-1 multiplicity of infection of 0.003, in the presence of various concentrations of test compound, by a tetrazolium-based colorimetric method (MTT method) as described.[20,29,30] The MT-4/MTT method has been widely used to evaluate virus drug susceptibility and to identify resistance to anti-HIV agents.[31] Inhibition values from two independent triplicate experiments were fit to a non-linear regression and MPIs were obtained with GraphPad Prism 5. Best-fit values of the MPI were compared with the extra sum-of-squares *F* test, and data of maximal inhibition points were compared using the *t*-test. MTT read-outs provide a correlation coefficient of 0.999 between the absorbance values (OD_{550}) and number of cells.[32] However, above the linear range, the read-out may provide values calculated as >100% protection and should be considered as full protection (100%) from the HIV-induced cytopathic effect.

Results

Selection of HIV-1 variants under TAK-779 pressure

Two HIV-1 R5 viruses, the laboratory-adapted BaL and one clinical isolate (CI),[11] were passaged every 6–7 days in PBMCs and in the presence (BaL-res and CI-res cultures) or absence (BaL-wt and CI-wt cultures) of 1 μg/mL TAK-779 for 285 days (41 passages). Supernatants from infected cells were collected and viruses were expanded and kept at −80°C until use. Susceptibility of HIV-1 strains to the antiretrovirals tested was not significantly affected by cell culture passage in the absence of the drug as noted for the control virus HIV-1 BaL (Table 1). No change in co-receptor use was noted after virus stocks were tested in U87/CD4+ CCR5+ or CXCR4+ cells (data not shown).

Antiviral susceptibility profiles of wt and res viruses

The anti-HIV activity of TAK-779 and maraviroc was tested for all virus strains (Table 1) and *env* recombinants were used to confirm that resistant phenotypes were induced by changes in the gp120 coding sequence (Table 2). When it was possible, differences in the mean EC$_{50}$ for each compound were calculated as the ratio between the mean EC$_{50}$ of the resistant strains relative to the mean EC$_{50}$ of its wild-type counterpart. Virus strains were considered resistant when a >4-fold difference in the EC$_{50}$ was found. Passage of virus in the presence of TAK-779 did not affect the susceptibility to the gp41 inhibitors enfuvirtide or C-34 or the reverse transcriptase inhibitors zidovudine or nevirapine. Compared with the BaL-wt and the BaL-env recombinant (c2.2wt) strains, the BaL-res and BaL-env recombinant (c5.6r) were less susceptible to TAK-779 and maraviroc as seen by the changes in the EC$_{50}$ (>63- and 74-fold less for TAK-779 and 11- and 8-fold less for maraviroc, respectively). However, the inhibition curves of both BaL-res (Figure 1a and b) and CI-res (Figure 2a and b) and their respective *env* recombinant strains differed for TAK-779 and maraviroc. When we compared wild-type MPI values (Table 3), we observed that whilst the MPI of TAK-779 could only reach 61%–73% inhibition against the BaL resistant strains and clones, respectively, no changes in MPI were observed for maraviroc. Despite the large decrease of the MPI for TAK-779 against BaL-res and BaL-c5.6r, the difference did not reach statistical significance (extra sum-of-squares *F* test), due to variance at the highest TAK-779 concentrations and because an inhibitory plateau could not be calculated for

Table 2. Antiviral activity of control compounds when tested against *env* recombinant viruses

	EC$_{50}$ (μg/mL)[a]			EC$_{50}$ (μg/mL)[a]		
Compound	BaL-c2.2wt	BaL-c5.6r	Fold resistance[b]	CI-c3.3wt	CI-c46.9r	Fold resistance[b]
TAK-779	0.005±0.005	0.369±0.238	74	3.62±1.95	ND	1
Maraviroc	0.001±0.001	0.008±0.004	8	0.012±0.001	0.011±0.004	1
AMD3100	>0.5	>0.5	—	>0.5	>0.5	—
Zidovudine	0.001±0.0007	0.002±0.001	1	0.003±1E-04	0.002±0.0001	>1

ND, could not be determined as maximum protection was ∼20% (see Figure 2).
[a]EC$_{50}$, 50% effective concentration, or concentration needed to inhibit 50% HIV-induced cytopathic effect, evaluated in MT-4/CCR5+ cells with the MTT assay. Maximum concentrations tested: 5 μg/mL TAK-779; 2.5 μg/mL maraviroc; 0.5 μg/mL AMD3100; 1 μg/mL zidovudine; and 2 μg/mL nevirapine. Values represent the mean of two separate evaluations ±SD.
[b]Fold resistance is calculated as the ratio EC$_{50}$ for resistant virus/EC$_{50}$ for wild-type virus.

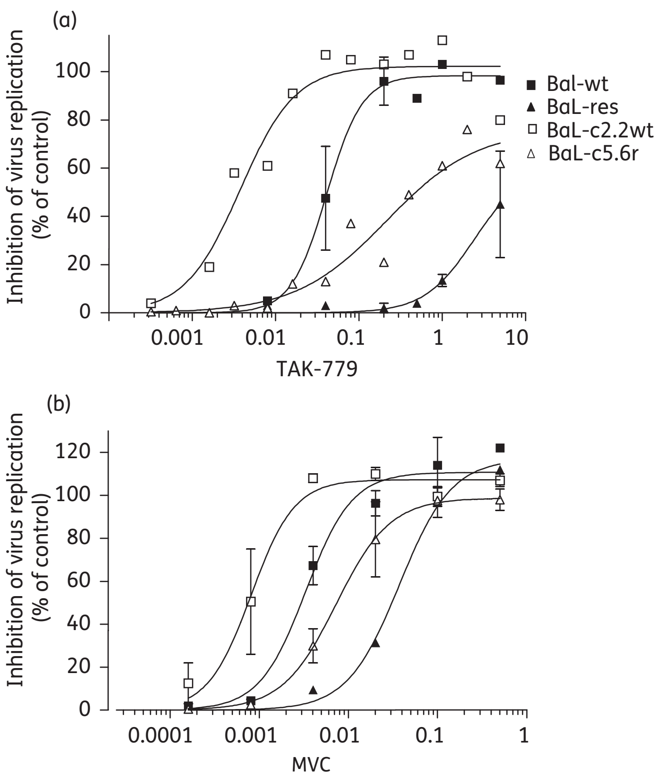

Figure 1. Inhibitory curves of TAK-779 and maraviroc (MVC) when tested against the wild-type and the TAK-779-resistant viruses derived from HIV-1 BaL. Inhibition of HIV-1 is measured as MT-4/CCR5 cell death induced after 5 days of infection. Inhibitory patterns of TAK-779 and maraviroc are similar for the viral strains obtained from passages (filled symbols) and for the *env* recombinant HIV-1 clones (open symbols). Values are from at least two independent experiments performed in triplicate ±SEM. The inhibitory curves of TAK-779 could not be extended to higher concentrations due to toxicity of the compound [50% cytotoxicity concentration (CC$_{50}$) 11.3±2.9 μg/mL].

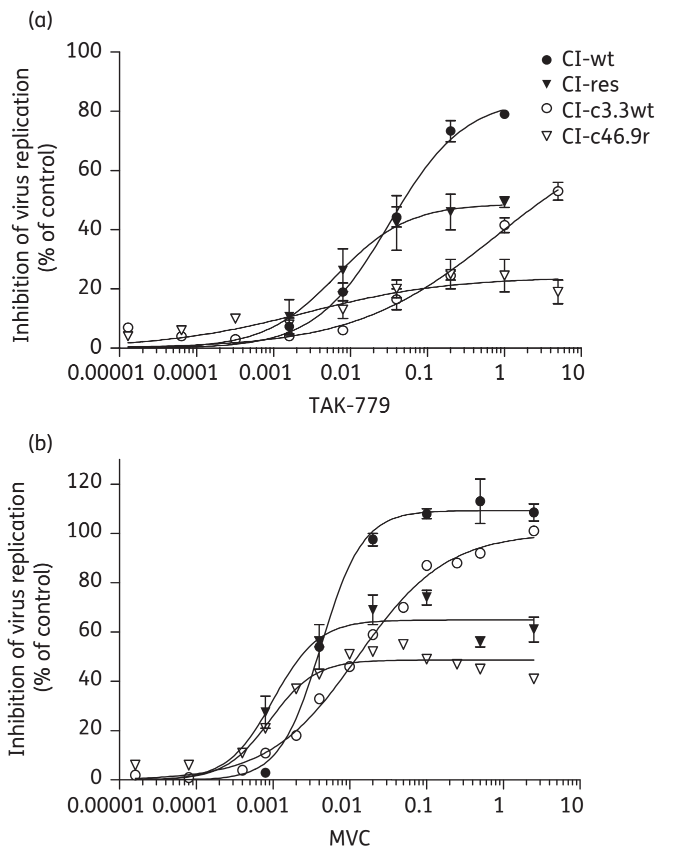

Figure 2. Inhibitory curves of TAK-779 and maraviroc (MVC) when tested against the wild-type and the TAK-779-resistant viruses derived from HIV-1 CI. Inhibition of HIV-1 is measured as MT-4/CCR5 cell death induced after 5 days of infection. Inhibitory patterns of TAK-779 and maraviroc are similar for the viral strains obtained from passages (filled symbols) and for the *env* recombinant HIV-1 clones (open symbols). Values are from at least two independent experiments performed in triplicate ±SEM. The inhibitory curves of TAK-779 could not be extended to higher concentrations due to toxicity of the compound (CC$_{50}$ 11.3±2.9 μg/mL).

BaL-res and BaL-c5.6r at subtoxic concentrations. However, *t*-test analysis of maximal inhibitory values of TAK-779 (at 1 and 5 μg/mL for BaL-wt, BaL-c2.2wt and BaL-c5.6r, and at 5 μg/mL for BaL-res) showed there to be a statistical difference between each wild-type and resistant strain [*F* ratio: $F_{(1.3)}$ 59.6 and $F_{(3.3)}$ 1.70; and *P* values of 0.017 and 0.006; for BaL-wt/res and for BaL clones, respectively]. On the other hand, despite variance at the maximal percentage inhibition of maraviroc

Table 3. Calculated MPI of TAK-779-resistant HIV-1 strains

	MPI (%±SE)	
HIV-1	TAK-779	maraviroc
BaL-wt	98.3±6.1	117.0±7.1
BaL-res	60.9±121.3	110.6±5.9
BaL-c2.2wt	102.2±5.5	107.3±5.6
BaL-c5.6r	72.6±7.9	98.8±5.5
CI-wt	84.3±4.3	109.3±2.9
CI-res	49.2±5.4	64.9±3.2
CI-c3.3wt	76.1±16.6	100.1±2.6
CI-c46.9r	24.0±3.7	48.7±1.8

against BaL (Figure 1b), there was no statistical difference between MPI of all four BaL viruses [$F_{(3.44)}$ 0.88; P value 0.46]. Conversely, a change in MPI was calculated for both TAK-779 and maraviroc, without a change in EC_{50}, when the CI-res virus was evaluated (Figure 2a and b). The EC_{50} value of TAK-779 could not be calculated against CI-c46.9r because the maximum inhibition achieved at the maximum concentration of 5 μg/mL was ~20%. The MPI for maraviroc was reduced 41% and 51% for the CI-res and the CI-c46.9r strains, respectively. MPI differences between CI viruses and clones were statistically significant for both TAK-779 [$F_{(1.18)}$ 22.22 and $F_{(1.20)}$ 99.37; P values 0.0002 and <0.0001, respectively] and maraviroc [$F_{(1.24)}$ 6.12 and $F_{(1.24)}$ 6.11; P values 0.02 and 0.02, respectively]. Changes in MPI as a mechanism of drug resistance have been previously observed for maraviroc-resistant and vicriviroc-resistant viruses.[12] However, our data suggest that the mechanism of resistance to CCR5 inhibitors may be dependent not on the CCR5 drug, but on the virus strain used.

Sequence analyses of viruses from passages

The gp160 coding sequences from the viruses obtained after passages with TAK-779 were analysed and are shown in Figure 3. Mutations detected in the resistant viruses were located in gp120, but were not confined to the V3 loop. BaL-resistant virus included mutations in V2 (S187 deletion and a mixed population of I185L and I199M), V3 (Y307N) and C4 (K431R). Two mutations were also detected in gp41, S533A downstream of the fusion peptide (FP) and before the heptad repeat (HR)-1, and R695K within the transmembrane (TM) segment of the glycoprotein (not shown). The resistant CI strain included mutations in V1 (P134S, R136K, T137N, A138V, T139_N140insNI, N145F and S148R), V2 (N170S and N199S), V3 (A318T and V325I) and V4 (N387H and D398G).

In order to understand the evolution of CCR5 drug resistance, proviral DNA from different passages available were PCR amplified and sequenced (Figure 4). Data show that virus began to accumulate mutations in the envelope coding sequence as early as passage 16 (~64 days) and as shown for CI-res, progressively accumulated mutations, finally leading to 11 mutations in the *env* coding sequence.

Discussion

The mechanism by which HIV evades the antiviral activity of CCR5 inhibitors has important implications for clinical practice. While TAK-779 is not being developed any further as a therapeutic agent, it serves as a model to understand drug escape mechanisms that may also apply to other drugs relevant for clinical use. As shown, selection of resistance to one drug may induce cross-resistance to other CCR5 inhibitors. Previous work has identified the V3 loop as a determinant of TAK-779 resistance by evaluating a library of HIV-1 mutant clones carrying recombinant V3 loop sequences.[33] Here, we show the selection of TAK-779 resistance through serial passage and evolution of virus under drug pressure. Drug resistance was associated with changes in the HIV envelope but was not confined to the V3 loop as reported for other CCR5 inhibitors.[8,34] A virus resistant to the TAK-779 analogue TAK-652 showed cross-resistance to TAK-779.[35] Although the virus contained several mutations in gp120 (C2, V3 and C4), none of them emerged in the TAK-779-resistant viruses described herein. The only mutation selected in the V3 of the BaL-res was Y307N. This N307 residue is frequently found in R5 HIV-1 but has not been related to resistance to CCR5 inhibitors.[12,33,36] Therefore, it is plausible to think that the single V3 mutation could not by itself explain drug resistance and that mutations located in other regions of gp120, such as V2 (S192del), C2 (D281N) and C3 (K431R), are determinants of the resistant phenotype as previously suggested.[2] The accumulation in time of a significant number of mutations required to generate CCR5 drug resistance suggests a relatively high genetic barrier for resistance. The influence of each specific mutation on the phenotype of the resistant virus and its effect on replicative capacity need to be determined to fully understand this issue.

As shown, the pattern of mutations may differ depending on the virus strain used in spite of the similar experimental conditions used to generate them. Importantly, the heterogeneity of the envelope backbone and the stochastic nature of virus evolution allow for an intricate pattern of drug-resistance mutations for every virus, suggesting that genotypic tests to evaluate CCR5 resistance in clinical practice may not be as relevant as those used in genotype to phenotype correlation for reverse transcriptase and protease inhibitors.

Some similarities with other CCR5-resistant viruses could be identified: the amino acid sequence AsnValThrAsn (NVTN) that results from the mutations in the V1 of CI-res coincides with a four residue insertion in V1 previously related to the resistance of a clinical isolate to AD101. The BaL-res virus contains a mutation in gp41 (S533A) that results in the same N-terminal gp41 sequence of the AD101-resistant strain.[14,37] The gp41 sequence of the AD101-resistant virus appeared not to affect drug resistance and, despite AD101 and TAK-779 not being structurally related molecules, the AD101-resistant virus was less susceptible to TAK-779. We cannot rule out the possibility that mutations in gp41 play a role in the TAK-779-resistant phenotype, as changes in gp41 have been described to be responsible for resistance to vicriviroc.[18] Furthermore, mutations A318T and V325I selected in the CI-res virus have been previously associated with the *in vitro* maraviroc resistance of another clinical isolate,[12] but these appear not to be distinct markers of CCR5 drug resistance of use in the clinical

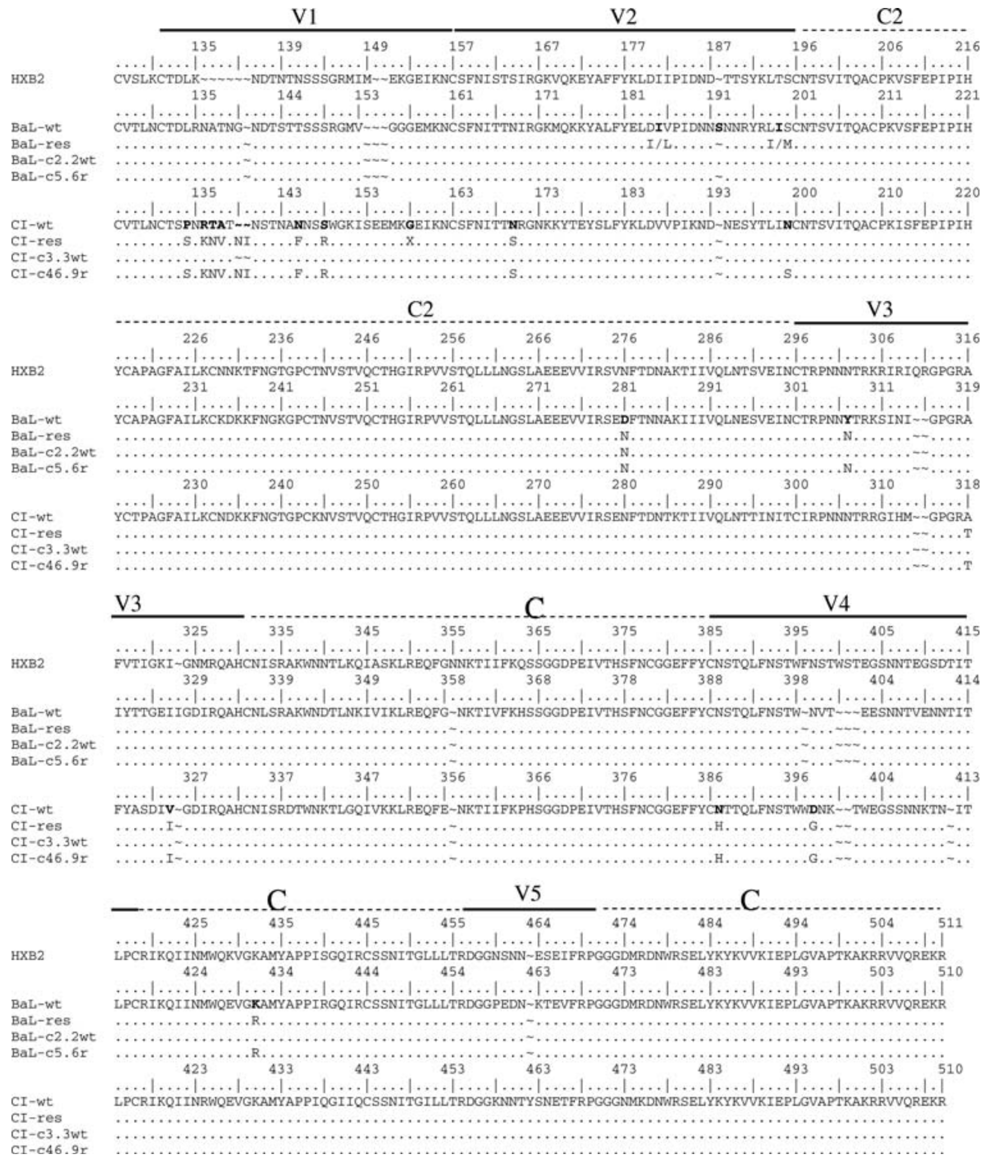

Figure 3. gp120 sequences of wild-type and resistant viruses. Sequences were aligned together with the HXB2 sequence. Numbering is displayed starting the same as that of HXB2 but is continued and referred to in the text with the viruses' own residue sequence. Viral sequences are compared with the control strain (virus passed in parallel without TAK-779). Variable loops and conserved regions are indicated. Dots indicate residues the same as those of the control strain. Tildes (~) indicate absent residues.

identification of treatment failure due to the emergence of resistant virus.

In the CI-res virus, gp120 mutations result in the loss of potential *N*-glycosylation sites (two in V1 and one in V4) and others are gained (one in V1 and another in C2). The CI-res contains the N386H mutation, which may imply losing an *N*-glycosylation site. The loss of *N*-glycosylation at this position has been associated with an enhanced exposure of the CD4-binding site.[38] It is possible that the resistance to small-molecule CCR5 inhibitors can promote a stronger association with CD4 as a compensatory mechanism.

The potency of inhibition of a CCR5 agent may differ depending on the cell type used (e.g. PBMCs versus engineered cell lines[18,39]). Our assay with MT-4/CCR5+ cells allows for the detection of different patterns (change in the MPI and/or change in the EC_{50}) of resistance to small-molecule CCR5 inhibitors. The dose–response curves of inhibition for TAK-779 and maraviroc were dependent on the viral genetic background on which resistance was developed. Changes in the MPI of TAK-779 against both resistant viruses were observed, and the inhibitory curve for both BaL-resistant viruses was also shifted to higher concentrations, increasing the EC_{50} for the compound. Conversely, the dose–response curve of inhibition of maraviroc was different for each virus (BaL or CI). Resistance was only reflected as a change in EC_{50} for the BaL strain whereas resistance by the CI-res virus was observed as a change in MPI. Due to the variability of our assay, we cannot discount undetected differences in the MPI of maraviroc against BaL viruses that

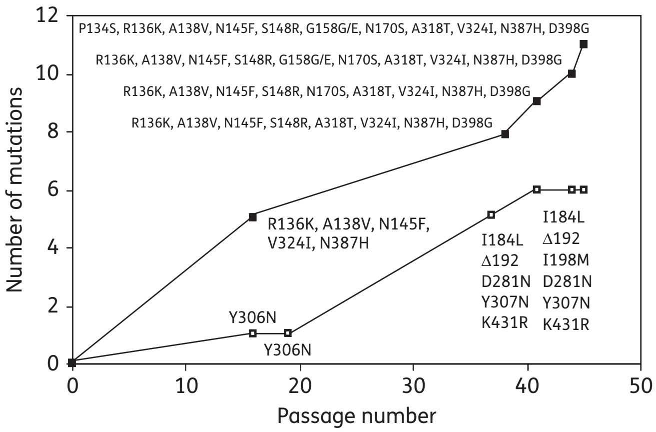

Figure 4. Emergence of mutations in the HIV-1 Env amino acid sequence in the presence of TAK-779. Cells infected with HIV-1 strain BaL (open squares) and CI (filled squares) were cultured in the presence of TAK-779. Proviral DNA was isolated and PCR amplified from the corresponding passage. Sequences from gp120 and gp41 were collected with an Avant 3100 Genetic Analyzer, and were aligned and analysed with the Sequencher 4.2 program.

may also contribute to the overall resistance to maraviroc. Nevertheless, the profile of change of maraviroc curves in BaL wt-res viruses is clearly different from that of CI wt-res viruses, revealing the different mechanisms of resistance (change in EC_{50} versus change in MPI). A model for the use of CCR5 by HIV-1 wild-type and CCR5 inhibitor-resistant variants has been proposed where two or more CCR5 forms are present on the cell surface in the presence of the CCR5 inhibitor (free/occupied forms).[18] Virus strains resistant to CCR5 inhibitors may have the capacity to use free and drug-occupied CCR5 forms. This mechanism of resistance, named non-competitive or allosteric resistance, is identified by a change in MPI. Therefore, the TAK-779-resistant strains shown herein appear to use the inhibitor-bound form of CCR5. However, the fact that no change in MPI was observed for maraviroc for the BaL-res virus suggests, despite the moderate resistance, that cross-resistance may not involve the maraviroc-bound co-receptor or that both mechanisms (competitive and non-competitive) may co-exist.

Despite CXCR4 availability, TAK-779-resistant viruses retained CCR5 use and were cross-resistant to maraviroc. We have shown that under conditions of low CCR5 availability, i.e. in SUPT-1 cells in which CCR5 is undetectable by flow cytometry but which allow for a low-level R5 virus replication, some HIV-1 strains may gain CXCR4 use. Under such conditions, HIV-1 BaL will not switch but the CI virus did and TAK-779[11] and maraviroc (data not shown) accelerated the emergence of an X4 CI strain. In clinical trials of CCR5 inhibitors, X4 viruses were detected in 35%–55% of subjects who failed therapy,[40,41] most probably due to expansion of pre-existing X4 variants that were not detected by co-receptor screening tests, suggesting that it is not CXCR4 availability but efficient block of CCR5 that leads to the emergence of the X4 phenotype. Our results coincide with these clinical observations. Only potent CCR5 blockade in a condition of already low CCR5 availability (SUPT-1 cells) allowed for CI to expand its co-receptor use. Conversely, in PBMCs, the virus could still use CCR5 even in the presence of the drug and gained the CCR5 drug resistance phenotype in the absence of co-receptor switch. Virus co-receptor use may be dependent on no other immunological factor than CCR5 availability and the intrinsic capacity of HIV to switch without a significant cost in replicative capacity.

Our results, together with studies from other groups, highlight the complexity of the development of resistance to CCR5 antagonists. TAK-779 resistance has proved to be difficult and to take a long time to generate under conditions in which resistance to other HIV inhibitors is rapidly developed. Furthermore, resistance to CCR5 antagonists was found to be dependent on several conditions such as the cells used, the availability of CCR5 or the viral strain, generating resistance through different mechanisms (allosteric, competitive, co-receptor switch), and even different complex patterns of mutations.

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Transparency declarations

We declare no conflicts of interest.

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