Resistance to CCR5 inhibitors caused by sequence changes in the fusion peptide of HIV-1 gp41

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We have investigated the mechanism of resistance of a HIV type 1 (HIV-1) R5 primary isolate, D1/85.16, to the small molecule CCR5 inhibitor, vicriviroc (VVC). Unlike other viruses resistant to this class of compound, D1/85.16 lacks sequence changes in the V3 region of the gp120 surface glycoprotein. Inspection of env sequences from D1/85.16 compared with those derived from the parental, inhibitor-sensitive virus, CC1/85, revealed a cluster of 3 conservative changes in the fusion peptide (FP) of the gp41 transmembrane glycoprotein that tracked with the resistance phenotype. Studies with engineered Env-chimeric and point-substituted viruses confirmed that these 3 FP residues were substantially responsible for VVC resistance without altering coreceptor usage, as assessed in both peripheral blood mononuclear cells and the TZM-bl cell line. VVC resistance is manifested differently in the 2 cell types, and there are assay-dependent complexities to the dose-response curves for the engineered resistant viruses. To explain them, we created a model for resistance and generated theoretical VVC inhibition curves that closely mimicked the experimental data for the resistant viruses. The basis for the model is the existence of distinct forms of CCR5, with varying affinities for small molecule CCR5 inhibitors that are presumed to be present in different proportions on different cell types, and are used selectively by resistant HIV-1 variants when ligated with an inhibitor. Together, the experimental results and theoretical model may help understand how HIV-1 uses CCR5 to enter target cells under various conditions.

The small molecule CCR5 inhibitors maraviroc (MVC) and vicriviroc (VVC) are now used to treat infection with HIV type 1 (HIV-1) (1). These and similar compounds target CCR5, a G protein-coupled receptor (GPCR) used by HIV-1 early in the course of the infection (2). HIV-1 entry is mediated by the envelope glycoprotein (Env) complex, a heterotrimer of 3 gp120 surface subunits noncovalently linked to 3 gp41 transmembrane subunits. The gp120 subunits contact first CD4, then CCR5; conformational rearrangements within the trimer drive insertion of the gp41 fusion peptide (FP) region into the host cell membrane, leading to fusion. Analysis of the behavior of resistant and sensitive clones in PBMC and the TZM-bl cell line led us to develop a model to explain cell type-dependent complexities in resistance to CCR5 inhibitors. The model predicts the existence of at least 2 forms of CCR5 with different affinities for small molecule inhibitors that are present in different relative abundances on different cell types.

Results

VVC Resistance of D1/85.16 Maps to gp41. The D1/85.16 isolate arose under VVC selection pressure (14), and uses the inhibitor-CCR5 complex as the basis for its resistance to VVC and related compounds (5). Unlike CC101.19, D1/85.16 lacked a consistent pattern of sequence changes in the gp120 V3 region, and a VVC-resistant clone, D1/85.16 cl.23, had a V3 sequence identical to VVC-sensitive CC1/85 clones (6, 14). To test whether resistance tracked with the gp120 or gp41, we swapped these subunits from D1/85.16 cl.23 (= R) with the corresponding components of the parental clone CC1/85 cl.6 (= S). Therefore, the R/S chimeric virus contains gp120 from R and gp41 from S, and conversely for the reverse chimera, S/R [for virus nomenclature, Table S1]. Both chimeric viruses were replication-competent in PBMC with titers of 10^4 to 10^5 TCID50/mL on day 7 postinfection (p24, 3–5 ng/mL). VVC resistance in these cells tracked with gp41. Thus, the R/S chimera behaved identically to the sensitive isolate and clone S, the S/R chimera to the resistant isolate and clone R (Fig. 1). The complex

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The authors declare no conflict of interest.

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Data deposition: The env sequences of the D1/85.16-derived viruses reported in this paper have been deposited in the GenBank database (accession nos. F1713453–F1713458).

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structure to the VVC-inhibition curve for S/R is analyzed further below. Similar results for both chimeric viruses were also obtained when AD101 was used.

**Sequence Changes in the FP Region of D1/85.16.** We sequenced the env genes from isolate D1/85.16 and its clone R, as well as from 4 reversion culture isolates (D1/85.16R7, D1/85.16R8, D1/85.16R10, and D1/85.16R19; Fig. S1). We sequenced clones derived from the parental CC1/85 parental virus and the AD101-resistant CC101.19 isolate (6). The only consistent pattern of changes involved the FP region of gp41. Thus, all 6 D1/85.16 isolates or clones contained 3 changes that were rare or absent from the parental CC1/85 clones: G516V, M518V, and F519I (Fig. 2). Of these, 516V and 519I were absent from all of the CC1/85 or CC101.19 sequences, whereas 518V was present in 2/7 CC1/85 clones and in all 6 CC101.19 clones. This pattern suggests 518V is a naturally occurring polymorphism that becomes enriched under selection in the FP region of gp41. Thus, all 6 D1/85.16 isolates and R/S + 3FP engineered clones were tested for VVC sensitivity in a multicycle PBMC-based replication assay measuring p24 production 7 days postinfection. The data shown are mean values derived from 7 to 15 independent experiments ± SEM.

**Site-Directed Mutagenesis of Specific Residues in the FP Region.** To test whether the G516V, M518V, and F519I changes confer VVC resistance, site-directed mutagenesis was performed on the sensitive parental clone S. This clone was chosen because it was the most closely related to the resistant clone R (14). The same 3 substitutions were also introduced into the sensitive R/S chimera to investigate their actions in a different gp120 context. The engineered clones S + 3FP and R/S + 3FP were replication-competent in PBMC; their titers were several-fold lower than the other test viruses’ ranging from $10^3$ to $10^4$ TCID$_{50}$/mL on day 7 postinfection (p24, ≈3 ng/mL). When the reverse chimera S/R and the S + 3FP and R/S + 3FP engineered clones were tested for VVC sensitivity by using PBMC, they were all resistant to high VVC concentrations (Fig. 3). As previously, the S and R/S viruses were VVC-sensitive, whereas R was fully resistant. The S/R reverse chimera and the S

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**Fig. 1.** VVC resistance maps to gp41. Clonal chimeric viruses R/S and S/R (Table S1) bearing gp120 and gp41 subunits derived either from the parental, VVC-sensitive clone S, or the VVC-resistant clone R were tested for VVC sensitivity in a multicycle PBMC-based replication assay measuring p24 production 7 days postinfection. The data shown are mean values derived from 7 to 15 independent experiments ± SEM.

**Fig. 2.** Alignment of N-terminal gp41 sequences from VVC-sensitive and VVC-resistant viruses. The first 23 amino acid residues from the parental, VVC-resistant viruses. The first 23 amino acid residues from the parental, VVC-resistant isolates or clones based on D1/85.16, and 6 clones from the AD101-resistant CC101.19 isolate. The sequences are recorded relative to that of CC1/85 cl.16 (top line), with dashes indicating amino acid sequence identity. Amino acid numbering is based on HXB2 Env, with the first residue of gp41 at position 512. The 3 conservative substitutions of hydrophobic residues, G516V, M518V, and F519I, in the FP region of resistant viruses are highlighted in bold. Among the 7 parental clones, S (GenBank accession no. AY357338) is the most closely related to R; these 2 clones, which were used for subsequent genetic studies, are boxed. The env sequences of the D1/85.16-derived viruses have been deposited in GenBank (accession nos. FJ713453–FJ713458).

**Fig. 3.** The 3 gp41 FP amino acid substitutions confer resistance to VVC. Site-directed mutagenesis was performed to introduce the 3 changes (G516V, M518V, and F519I) into the VVC-sensitive parental clone S and the R/S chimera (Table S1). The engineered mutant viruses, S + 3FP and R/S + 3FP, were then tested for VVC sensitivity in a multicycle PBMC-based replication assay measuring p24 production 7 days postinfection. For comparison, S and the VVC-resistant clone R were also tested. In the same experiments, the R/S and S/R chimeras behaved comparably with S and R, respectively, but the curves are not shown, for clarity. The depicted results are the average of 6–10 independent experiments, with the error bars indicating the SEM.
+ 3FP and R/S + 3FP mutants were also cross-resistant to AD101. Thus, the 3 FP changes are sufficient to confer VVC (and AD101) resistance on a sensitive virus, and without causing CXCR4 usage (Fig. S2). However, the same 3 FP changes do not have this effect when introduced into a different, VVC-sensitive virus, JR-FL, implying that they act only in a defined Env context (Fig. S3). Studies on the roles played by the individual changes and their combinations in conferring resistance on CC1/85 are now in progress.

However, there were intriguing aspects to the VVC dose-response curves for the resistant viruses. Thus, the replication of S/R, S + 3FP and R/S + 3FP was partially inhibited (40–60%) by low VVC concentrations (≈0.3–2 nM), but enhanced by higher concentrations (up to 5 μM; see Figs. 1 and 3). This pattern of partial, biphasic inhibition at low VVC concentrations was also observed, albeit less so, with R (Figs. 1 and 3). The replication-enhancing effect of higher VVC concentrations appeared to be more pronounced for the R/S + 3FP virus than for S + 3FP (Fig. 3). Therefore, the gp120 component derived from R, which is present in the former but not the latter virus, may contribute to the resistance phenotype, particularly at high inhibitor concentrations.

In contrast, the enhancing effects of higher VVC concentrations were similar for the reverse chimera S/R and the S + 3FP mutant (Figs. 1 and 3); therefore, this phenotypic effect of the gp41 subunit from R may be attributable to the 3 specific FP changes. A possible biological explanation for the biphasic inhibition curve is modeled below.

**Cell Type Dependence of VVC-Resistance.** Resistance to small-molecule CCR5 inhibitors varies with the cell type (4, 5, 7). When viruses that are fully resistant to a compound such as VVC and MVC in PBMC are tested in cell lines engineered to express CCR5 (e.g., U87.CD4.CCR5 or TZM-bl), they tend to be inhibited but only up to a maximum value [the maximum percent inhibition (MPI)]. The extent of inhibition is then unaffected by a further increase in inhibitor concentration (“the plateau effect”). The MPI reflects the relative ability of the virus to use the inhibitor-bound and inhibitor-free forms of CCR5 for entry; the higher the MPI value, the less efficiently a resistant virus recognizes the inhibitor-CCR5 complex (4, 5). Therefore, we tested the engineered, clonal infectious viruses for their sensitivity to VVC in TZM-bl cells, to further characterize their resistance profiles (Fig. 4).

Each virus was replication-competent in TZM-bl cells, with relative luminescence units (RLU) varying within a 10-fold range (Table 1). The parental clone S and the sensitive chimera R/S behaved similarly; each was fully inhibited by VVC (MPI >90% in both cases), with half maximum (IC50) values of 20 and 7.8 nM, respectively (Fig. 4, Table 1). The R and S/R viruses had MPI values of 93 and 94% with reduced IC50 values of 2.4 and 2.0 nM; although these MPI values are high, the upper limits of their 95% C.I. were consistently <100% (Table 1). The MPI values for the engineered resistant viruses S + 3FP and R/S + 3FP were also consistently <100% (95 and 90%, respectively), but their IC50 values were reduced even further, to 0.70 and 0.66 nM (Fig. 4, Table 1). Similar results for the S, R, and S + 3FP viruses were also obtained by using AD101.

Therefore, the engineered mutant viruses, which are strongly resistant in PBMC (Fig. 3), are ~30-fold more sensitive (as judged by IC50 values) than the parental clone to low VVC concentrations when tested in TZM-bl cells (Fig. 4, Table 1). However, these viruses do display the noncompetitive resistance indicator that is characteristic of assays based on engineered cell lines, i.e., MPI significantly <100% (4, 5, 7). Experiments using U87.CD4.CCR5 cells and pseudotyped viruses bearing Env proteins from clones S and R yielded similar findings, including the replication of a small viral fraction in the presence of high VVC concentrations and a reduced IC50 for R compared with S (Fig. S4). Also, the principal finding from both the cell line- and PBMC-based assays is that gp41 has the paramount role in resistance, principally due to the 3 specific FP changes. The gp120 context can have an additional influence, particularly at high inhibitor concentrations. Thus, the MPI values are almost identical for the S/R and S + 3FP viruses, and lower for R/S + 3FP (Table 1). The complexities of CCR5 usage that may underlie these observations are discussed below.

**Modeling the Cell Type-Dependency of Resistance.** The dose-response curves for the resistant clones depicted in Figs. 1, 3, and 4 are clearly both complex and cell type-dependent. To try to understand the underlying biology, we developed a model (Fig. S5). We postulate that 2 different subpopulations of CCR5 receptors coexist on the cell surface in proportions that vary between cell types, and that the infectivity of a viral variant is a linear function of the amount of a CCR5 form(s) that can support entry of that variant. One CCR5 subpopulation (designated CCR5-A) is assumed to bind VVC, and related inhibitors, with a low affinity, whereas the second form (CCR5-B) binds them with a higher affinity. Wild-type HIV-1 can have a preference for one of the free forms of CCR5, but cannot use either inhibitor-bound form for entry. A resistant variant can use inhibitor-CCR5-A complexes while losing the capacity to enter via the free form of CCR5-A, but retaining or increasing its ability to use the free form of CCR5-B. This version of the model is the simplest; in reality, there can be >2 subpopulations of CCR5. The mathematical formulation of the

![Fig. 4. VVC resistance is manifested differently in a cell line-based assay. The same infectious, clonal viruses studied in PBMC were tested in TZM-bl cells with a luciferase reporter gene endpoint. The VVC inhibition curves (dashed black line for the resistant virus R) asymptote to a plateau that is high, but consistently lower (upper limit of 95% C.I. <100%) for the resistant than the sensitive viruses. The data shown are the average of 3 independent experiments. Resistance-related parameters derived from these experiments are summarized in Table 1.](image-url)

**Table 1. Indicators of VVC-resistance in TZM-bl cells**

<table>
<thead>
<tr>
<th>Virus</th>
<th>MPI, % (95% C.I.)</th>
<th>IC50, nM</th>
<th>RLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC1/85 cl.6</td>
<td>95 (93–97)</td>
<td>0.70</td>
<td>4.2 × 10^4</td>
</tr>
<tr>
<td>D1/85.16 cl.23 = R</td>
<td>93 (89–96)</td>
<td>2.4</td>
<td>1.6 × 10^4</td>
</tr>
<tr>
<td>S/R</td>
<td>100 (93–110)*</td>
<td>7.8</td>
<td>6.7 × 10^4</td>
</tr>
<tr>
<td>S + 3FP</td>
<td>95 (93–97)</td>
<td>0.70</td>
<td>4.2 × 10^4</td>
</tr>
<tr>
<td>R/S + 3FP</td>
<td>90 (87–93)</td>
<td>0.66</td>
<td>1.9 × 10^4</td>
</tr>
</tbody>
</table>

Data are mean values derived from 3 independent experiments. *, P < 0.01 for R vs. S and for R vs. R/S MPI values; P > 0.05 between the MPI for R and each other clone.
Inhibition of TZM-bl cell infection by S to differ by fits the data well when the VVC affinities of CCR5-A and CCR5-B are assumed seen at the highest VVC concentrations in the different experiments. (amounts of the CCR5-A and CCR5-B modulate the different levels of inhibition in infection (gray) at the highest VVC concentration is contrasted with inhibition CCR5-B as for VVC-CCR5-A complexes. (B) An example of enhancement of S when plotted similarly. (concentrations. Datasets from other donor pools fall between these profiles the S continuous lines in the shades of the dataset each is fitted to. (A) Inhibition of S virus (black) on PBMC from different donor pools. Different relative amounts of the CCR5-A and CCR5-B modulate the different levels of inhibition seen at the highest VVC concentrations in the different experiments. (C) Inhibition of TZM-bl cell infection by S + 3FP (gray) and S (black). The model fits the data well when the VVC affinities of CCR5-A and CCR5-B are assumed to differ by >37-fold, a value that was predetermined by measuring the IC50 values for the respective viruses on TZM-bl cells. The small residual infectivity of S + 3FP at the highest VVC concentrations results from an excess of CCR5-B (high-affinity for VVC) over CCR5-A (lower affinity for VVC). This excess would be ~20-fold if the efficiency of S + 3FP viral entry were the same for free CCR5-B as for VVC-CCR5-A complexes.

Fig. 5. Fitting of model function to empirical data from PBMC and TZM-bl cell assays. The relative inhibition of virus infectivity is expressed in percentage on the y axis as a function of the VVC concentration in nM on the x axis. The black circles represent data for wild-type virus (S); data for the VVC-resistant S + 3FP mutant are shown as gray squares and triangles (error bars, SEM in each case). The theoretical curves derived from the model are shown as continuous lines in the shades of the dataset each is fitted to. (A) Inhibition of the S + 3FP (gray) and S (black) viruses in PBMC from 2 different donor pools. The resistant virus is partially or negligibly inhibited at the highest VVC concentrations. Datasets from other donor pools fall between these profiles when plotted similarly. (B) An example of enhancement of S + 3FP virus infection (gray) at the highest VVC concentration is contrasted with inhibition of S virus (black) on PBMC from different donor pools. Different relative amounts of the CCR5-A and CCR5-B modulate the different levels of inhibition seen at the highest VVC concentrations in the different experiments. (C) Inhibition of TZM-bl cell infection by S + 3FP (gray) and S (black). The model fits the data well when the VVC affinities of CCR5-A and CCR5-B are assumed to differ by >37-fold, a value that was predetermined by measuring the IC50 values for the respective viruses on TZM-bl cells. The small residual infectivity of S + 3FP at the highest VVC concentrations results from an excess of CCR5-B (high-affinity for VVC) over CCR5-A (lower affinity for VVC). This excess would be ~20-fold if the efficiency of S + 3FP viral entry were the same for free CCR5-B as for VVC-CCR5-A complexes.

The difference between the plateaus of inhibition at the highest VVC concentrations for the sensitive and resistant viruses is much smaller in the TZM-bl cell assay than with PBMC (Fig. 5C). However, as with the PBMC-based data, the experimental datasets from TZM-bl cells can be closely mimicked by the nonlinear-regression-generated curves based on the model (Fig. 5 and Fig. S6). Thus, in both the experimental data and the theoretical curves, inhibition of the VVC-resistant virus is incomplete at the plateau, but the IC50 is lower than for the wild-type virus; these 2 apparently conflicting observations are otherwise hard to reconcile with each other.

Fitting the function derived from the model to the experimental PBMC data yields values for 3 parameters: the affinities of VVC for CCR5-A and CCR5-B and w, which reflects the relative amount of the form of CCR5 that is infection-permissive when ligated by VVC; also, w can be interpreted as expressing a variable capacity of a test virus to use these VVC-CCR5 complexes (SI Materials and Methods). For the PBMC-derived data, w is close to 0 for the wild-type S virus (0.0–0.0040; Table S2); for the resistant S + 3FP virus, it varies above and below 1 between experiments on cells from different donor pools; it is <1 (0.34 ± 0.029) for the data showing partial inhibition at the plateau, it approaches 1 (1.0 ± 0.037) when there was no inhibition at the highest VVC concentrations (Fig. 5A, Table S2), but it exceeds 1 (1.8 ± 0.059) when high concentrations of VVC enhance infection (Fig. 5B, Table S2). In each of the 3 cases, the estimated dissociation constant of VVC for CCR5-A was distinct from that for CCR5-B (9.6–50 and 0.30–1.6 nM, respectively, with ratios of 7.5–170; Table S2). The modeled IC50 values for S on PBMC from 3 donor pools were in an intermediate range of 1.3–6.4 nM.

Modeling the data for S from the TZM-bl cell assay yielded a value of w close to 0 (w = 0.0095), similar to the one derived from PBMC with the same virus (w = 0.0040) (Table S2). The w value for infection of TZM-bl cells by the resistant S + 3FP virus was 7-fold higher (=0.06) than for S on those cells, but it was lower than for S + 3FP on PBMC (0.34–1.8). A dissociation constant for VVC binding to CCR5-A on TZM-bl cells could not be precisely estimated, but it must exceed 25 nM; for CCR5-B on the same cells, it could be estimated to be 0.67 ± 0.045 nM. Therefore, the corresponding ratio of >37 may fall within the range derived from the PBMC experiments (7.5–170).

Discussion

Resistance to small molecule CCR5 inhibitors, exemplified by MVC and VVC, is now well documented in vitro, and has arisen in vivo (4, 6–9, 11). Cross-resistance within the class is usually observed (5, 6, 14), but sometimes not (4). Contrary to initial assumptions, the principal resistance pathway does not involve switching to CXCR4; the virus instead acquires the ability to

model is presented in SI Materials and Methods, and the different scenarios are depicted schematically in Fig. S5. The principles of the modeling results are shown in Fig. S6.

We used nonlinear regression to fit the general function of the model (15) to experimental PBMC and TZM-bl data (Fig. 5). In this analysis, we made no assumptions about the relative proportions of the 2 CCR5 forms, or their absolute affinities for VVC, when fitting the function to the PBMC data. However, to avoid creating excessively wide C.I. when modeling the TZM-bl cell data, IC50 values were estimated for S and S + 3FP from the sigmoid dose-response plots derived experimentally by using these cells. The resulting IC50 ratio of 37 was then used as a constant affinity ratio in a 2-parameter function (SI Materials and Methods). The resulting functions were then fitted to the experimental data (Fig. 5).

As noted above (Fig. 3), the experimental VVC dose-response curves against the resistant S + 3FP mutant in PBMC have complex shapes. Inhibition is maximal at intermediate VVC concentrations, but a plateau arises at higher concentrations that varies from partial inhibition to enhanced infectivity (negative inhibition) in experiments on PBMC from different donor pools (Fig. 5 A and B). Similar data profiles, as well as intermediate forms, arise with PBMC from other donor pools. We have yet to determine whether the various curve shapes are donor-specific, and how they relate to variation in CCR5-expression levels; studies on cells from individual donors, rather than the pooled cells from 2 or 3 donors that we routinely use, will be necessary.

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recognize the inhibitor-CCR5 complex while retaining use of the free coreceptor (4, 5). Entry/replication of some resistant viruses can increase when an inhibitor is present, because they recognize the inhibitor-CCR5 complex more efficiently than free CCR5, and/or because more inhibitor-CCR5 complexes are available on the cell (5, 11). The resistant viruses replicate efficiently, and their phenotype is stable, at least in PBMC cultures, exemplified by the lack of reversion of both the CCI101.19 (13) and the D1/85.16 isolate (Fig. S1) after prolonged culture in the absence of AD101 and VVC, respectively. Therefore, the altered Env configuration enabling D1/85.16 to use the VVC-CCR5 complex must also recognize free CCR5 approximately as efficiently as Env from the parental CCI1/85 isolate. The same applies to the AD101-resistant isolate CCI101.19 and clones (5, 6).

How the Env complex adjusts to use the inhibitor-CCR5 complex is under investigation. The generally accepted model of gp120-CCR5 interactions involves the crown of the V3 region contacting the second extracellular loop (ECL2), whereas the bridging sheet and more conserved residues in the V3 stem bind the tyrosine-sulfated amino-terminal domain [N terminus (Nt)] (1, 10, 16). The available evidence suggests that resistant viruses become less dependent on, or even independent of, the V3-ECL2 interaction (8, 17). The genetic pathway typically involves accumulating multiple sequence changes in V3 that, presumably, impede its binding to ECL2 (4, 6–9, 11). However, we described a VVC-resistant clone with no V3 sequence changes (14); its phenotype was comparable with the AD101-resistant (and VVC-cross resistant) viruses derived from the same parental isolate under similar experimental conditions (6, 12). Thus, there is a second genetic pathway to the same phenotype.

Here, we show that this alternative pathway not only does not involve V3, but that the critical changes do not even lie within gp120; instead, they are located within the FP of gp41. This finding was unexpected. The 3 FP changes are collectively compatible with fusion; the resistant viruses are replication-competent, as is the corresponding, inhibitor-sensitive JR-FL mutant. However, no naturally occurring viruses in the HIV Sequence Database contain all 3 changes, although some subtype C strains have 2 (G516V + M518V or M518V + F519I). Of note is that one of the FP changes, G516V, was recently shown to confer CCR5 usage on a clonal R5 virus (18). Perhaps the coreceptor switching of that virus and the altered CCR5 interactions of the VVC-resistant CCI1/85 variants involve similar mechanisms. However, the 3 FP changes combined did not enable CCI1/85 variants to use CXCR4 in PBMC, and CXCR4 usage was not responsible for VVC resistance in TZM-bl cells (Fig. S2). We are now investigating whether any of the 3 FP changes individually (or in pairs) affect VVC sensitivity, fusion efficiency, and coreceptor usage. However, the effect of the FP changes is context-dependent. Thus, a JR-FL variant engineered to have the same FP sequence as resistant D1/85.16-derived variants remained VVC-sensitive (Fig. S3); likewise, the V3 changes that confer resistance on CCI1/85 have no such effect on JR-FL.

Although the gp120 subunit is not responsible for CCR5 inhibitor resistance, gp120 sequence changes may influence the phenotype of D1/85.16 and some mutants derived from it. We are assessing whether compensatory changes elsewhere in Env influence the stability and fitness of the FP-engineered viruses; they may subtly alter how CCR5 is recognized, particularly at high inhibitor concentrations (compare S + 3FP and R/S + 3FP in Figs. 3 and 4; see Table 1).

How FP changes alter the gp120-CCR5 interaction remains to be understood, but it seems reasonable to assume they act at a distance; there are no grounds to believe that the FP contacts the coreceptor before or during fusion. When unligated by an inhibitor, CCR5 provides a stimulus in a chain of conformational changes in Env that culminate in membrane fusion. This stimulus is abrogated for wild-type Env, but not the resistant variant if an inhibitor is bound. The V3 sequence changes may facilitate an interaction between Env and the inhibitor-CCR5 complex by changing the normal requirements for both V3 and the bridging sheet to contact different regions of CCR5 (ECL2 and the Nt). In principle, the FP substitutions may act analogously, via allosteric effects on Env conformations that functionally mimic the consequences of the V3 changes. An alternative hypothesis is that the FP substitutions lower the threshold for fusion triggering, such that bridging-sheet interactions with the CCR5 Nt now suffice to drive the Env refolding that mediates fusion. Although structural information on the native Env trimer would clarify intersubunit effects, there is now considerable information about how gp41 sequence changes affect gp120 structure and function. The first study of how Env responds to a selection pressure, a serum from a HIV-1-infected individual, involved a point substitution in gp41 (AS82T) that created a IIIb-resistant variant to neutralizing antibodies (NAbs) that specifically target CD4-binding site-associated gp120 epitopes (19, 20). Other influences of sequence changes in the gp41 ectodomain and cytoplastic tail on neutralization by gp120 ligands are now known (21–23). The VVC- and AD101-resistant D1/85.16 and CCI101.19 isolates are somewhat more NAb sensitive than their parental virus, CCI1/85; further suggesting that the FP and V3 changes affect the overall geometry of the Env complex. The greatest difference in sensitivity between D1/85.16 and CCI1/85, ~55-fold, was to NAB 2G12 against a conformational epitope comprising gp120 N-linked glycans (24). How this difference arises is yet another unknown that will probably require Env trimer structural information. Alterations in gp41, including the cytoplastic tail, can lead to CD4 independence and reduced CCR5 dependence (25), and secondary resistance substitutions affecting small molecule inhibitors of gp120-CD4 binding were also mapped to gp41 (26).

The manifestation of resistance to CCR5 inhibitors is assay-dependent; the relative efficiency with which the resistant virus uses the inhibitor-bound and inhibitor-free forms of CCR5, the viral inoculum size, and the CCR5 surface density are all relevant variables (4, 5, 7, 27). In PBMC, resistance usually appears as a rightshifts in the inhibitor dose-response curve (IC50 increase), whereas in CCR5-expressing cell lines such as TZM-bl there is a plateau effect, and resistance is defined by MPI values <100% (4, 5, 7). In general, the engineered FP mutants behaved similarly to the naturally resistant virus, but with some informative exceptions. Thus, replication of the S + 3FP and R/S + 3FP viruses in PBMC was partially inhibited (40–60%) by low VVC concentrations (~0.3–2 nM), but enhanced by higher concentrations (up to 5 μM), and there was considerable variation in the extent of resistance among donor pools (Figs. 1, 3, and 5A and B). Replication of the engineered viruses in TZM-bl cells was hypersensitive to low VVC concentrations, compared with the parental and naturally resistant clones, but there was a small yet consistent persistent fraction of viruses replicating in the presence of very high VVC concentrations (Fig. 4). The manifestation of resistance of some CXCR4-using viruses to the small molecule inhibitor AMD3100 has also been shown to involve plateau heights that vary with the cell type and among PBMC from different donors (28). The explanation may be similar to what we propose here for CCR5; thus, the derivation of analogous models could be informative.

Our model of CCR5-inhibitor resistance explains the complex curve shapes arising for the VVC-resistant clones in PBMC and TZM-bl cells. Its key feature is the existence of 2 CCR5 forms, CCR5-A and CCR5-B, which bind VVC and related inhibitors with low and high affinity (in reality, there may be >2 forms; see SI Materials and Methods and Fig. S5). The model also assumes that wild-type and resistant HIV-1 variants differentially use free and inhibitor-bound conformations of these CCR5 forms. By varying the proportions of CCR5-A and CCR5-B and their affinities for VVC, we can derive infection-inhibition curves that mimic data generated in both PBMC and TZM-bl cells. The similarities between the experimental results and the model curves support the central hypothesis that free and VVC-ligated forms of different
CCR5 subpopulations, respectively, can mediate entry of the resistant S + 3FP variant, whereas the wild-type S virus can use both free CCR5 forms to various degrees. Thus, the model explains the distinct inhibition profiles of the 2 viruses on the 2 cell types.

How reasonable are the model and its underlying assumptions? Antibody-reactive profiles show that multiple CCR5 conformations are present in proportions that vary between cell types (16). These different conformations may arise because CCR5 can be coupled intracellularly to G proteins, which affects the shape of the ligand-binding sites in the extracellular loops and Nt (29). Also, the CCR5 Nt is tyrosine-sulfated at 3 positions posttranslational, 2 of which (Tyr-10 and Tyr-14) are required for recognition by wild-type HIV-1 (30). Perhaps overexpressing CCR5 in engineered cell lines like TZM-bl and U87.CD4.CCR5 saturates the tyrosine sulfotransferases, creating partially sulfated CCR5 proteins that are antigenically and functionally distinct. The Tyr-sulfation requirements of the various resistant isolates remain to be investigated. Overall, the postulates that different forms of CCR5 exist in different proportions on different cell types and are recognized with different efficiencies by wild-type and resistant HIV-1 variants seem plausible. Quite what forms correspond to our theoretical CCR5-A and CCR5-B remains to be investigated experimentally; based on the curve shapes in Figs. 4 and 5, we argue that the CCR5-B, which has a high affinity for VVC and related inhibitors, will be overexpressed relative to CCR5-A in engineered cells like TZM-bl and, by extrapolation, U87.CD4.CCR5. Also, because CCR5-B may be used less efficiently by wild-type HIV-1 in those cells, it could perhaps be a variant that is under-sulfated on the Nt tyrosines. The relative usage of the CCR5 Nt and ECL2 does appear to alter between wild-type and resistant HIV-1 variants, the latter becoming more dependent on the Nt (8). However, why under-sulfation of the Nt would increase the affinity of CCR5 for small molecule inhibitors, as this interpretation of our model implies, remains to be understood. Perhaps intermolecular interactions involving CCR5 and/or other cellular proteins involve the Nt and indirectly modify the binding site for the small molecule inhibitors in the transmembrane helices. Overall, we hope that our experimental results, and the model they have helped generate, will drive new lines of research into how HIV-1 uses its coreceptors under different conditions, and into the general biology of GPCRs.

Materials and Methods

Viruses and Inhibitors. The study viruses are listed in Table 5 (5, 6, 14). Infectious stocks were prepared by transient transfection of 293T cells with pNL4-3/env proviral plasmids by using Lipofectamine 2000 (Invitrogen), and titrated before testing. Full-length env genes were sequenced as described (12, 14), and aligned with MacVector 10.0.2. VVC (SCH-D, SCH-417690), and AD101 (SCH-350581) (12) were provided by Julie Strizki, AMD3100 by Gary Bridger.

Construction of Chimeric NL4-3/env Proviruses. The env genes with interchanged gp120gp41 subunits from viruses S and R were constructed by using the unique MfeI sites, sequenced, then subcloned into pNL4-3 to produce chimeric infectious viruses (6). Site-directed mutagenesis was performed with QuikChange II (Stratagene), using pBlueScript KS(+) plasmids containing EcoRI/Xhol fragments that were then subcloned into pNL4-3.

Infection-Inhibition Assays. Virus sensitivity to VVC or AD101 was assessed by using TZM-bl or CD4+CCR5+ T cells. TZM-bl cells were seeded at 1 × 10⁴ per well in 96-well plates and allowed to adhere overnight; inhibitors were added for 1h before virus infection. After culture for 48h, the supernatant was removed, cells lysed, and luciferase expression quantitated as RLU by using the Bright-Glo Luciferase Assay System (Promega). Background values from mock-infected cells, which were also cultured with each VVC or AD101 dose to control for cell viability, were subtracted. A 4-parameter sigmoidal function was fitted to the inhibition data by nonlinear-regression (Prism, Graphpad). In testing theoretical models, we fit different functions as described (SI Materials and Methods). HIV-1 replication (p24 production) in U87.CD4.CCR5 and U87.CD4.CXCR4 cells was measured as described (6, 12).

For modeling CCR5 inhibitor resistance, see SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Antonia Thomas, Nicole Labutong, and Samuel Jacob for technical support; Min Lu, Reem Berro, Rogier Sanders, and Antu Dey for helpful discussions; and past contributions by Andre Marozsan, Pavel Pugach, Julie Strizki, and Shawn Kuhmann to work on VVC-resistant viruses. This work was supported by National Institutes of Health Grant R01 AI41420.
**Supporting Information**

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**SI Materials and Methods**

**Stability of a Vicriviroc (VVC)-Resistant HIV type 1 (HIV-1) Isolate.** To determine whether the VVC-resistance phenotype of the D1/85.16 isolate was stable, we returned the virus to culture in peripheral blood mononuclear cells (PBMC) for 19 passages without VVC, assessing its sensitivity to the inhibitor periodically. Each virus from the VVC-free culture, like the input D1/85.16 isolate, was completely resistant to VVC concentrations as high as 5 μM, whereas the parental CC1/85 virus was inhibited in the 1–10 nM range (Fig. S1). Thus, VVC-resistance is highly stable and there is no obvious fitness cost associated with this phenotype in PBMC cultures. In this respect, D1/85.16 resembles the AD101- and VVC-resistant CC1/1.19 isolate (1–3).

**Fusion Peptide (FP) Changes Do Not Alter Coreceptor Usage.** We reported that the uncloned, VVC-resistant D1/85.16 isolate could use CXCR4, albeit inefficiently, to replicate in some engineered cell lines, but not PBMC (4). Therefore, we tested whether the resistant clonal viruses could use CXCR4, first by using U87.CD4 cells expressing either CCR5 or CXCR4. All of the tested viruses replicated robustly in U87.CD4.CCR5 cells (day 7 p24 production, 2–11 ng/mL). In contrast, there was no detectable replication of the parental clone S and the chimeric viruses R/S (sensitive) and S/R (resistant) in U87.CD4.CXCR4 cells (p24 < 0.1 ng/mL). However, the resistant clone and the engineered resistant mutants could replicate at a low level in these cells. The p24 production levels of 1.0, 0.59, and 1.2 ng/mL for R, S + 3FP and R/S + 3FP, respectively, were 13, 7.7 and 11% of those for the same viruses in U87.CD4.CCR5 cells.

To evaluate the significance of low-level replication in U87.CD4.CXCR4 cells, we tested the viruses in PBMC from a CCR5-Δ32/Δ32 donor, and we assessed their sensitivity to the CXCR4-specific small molecule inhibitor AMD3100 in both CCR5-Δ32/Δ32 and normal donor PBMC. None of the resistant chimeric and engineered mutant viruses produced detectable amounts of p24 in PBMC from the CCR5-Δ32/Δ32 donor and their replication in normal donor PBMC was not inhibited by 1 μM AMD3100 (Fig. S2A). A dose-response curve (0.003–1,000 nM) against the same viruses confirmed that none was sensitive to AMD3100. In contrast, the reference X4 virus NL4-3, as well as the R5X4 viruses DH123 and CC2/86, replicated efficiently in the CCR5-Δ32/Δ32 PBMC and were AMD3100-sensitive, as expected (Fig. S2A).

In principle, the plateaus showing incomplete inhibition of the resistant viruses by VVC in TZM-bl cells [maximum percent inhibition (MPI) values significantly <100%] could be explained by low-level CXCR4 usage. To assess this possibility, we tested the parental clone S, the VVC-resistant clone R, the S + 3FP mutant, and the control X4 virus NL4-3, for VVC sensitivity in the presence and absence of AMD3100 (500 nM). The VVC-inhibition curves and the resulting MPI values were unaffected by AMD3100, demonstrating that CXCR4 usage did not contribute to the resistant phenotype of R or S + 3FP in TZM-bl cells (Fig. S2B). As a control, the X4 reference virus NL4-3 was inhibited by AMD3100 in the same experiments. Also, uninfected cells were cultured with AMD3100 (500 nM) in combination with each dose of VVC, to control for any effects on cell viability; the background relative luminescence units (RLU) values were unaffected by the inhibitor combinations.

Thus, although some (but not all) of the clonal, resistant viruses can replicate with low efficiency in U87.CD4.CXCR4 cells, they do not use CXCR4 in primary lymphocytes at quantifiable levels. Also, their resistance to VVC in TZM-bl cells is not due to CXCR4 use. We conclude that the various resistant clones and chimeric viruses retain the R5 phenotype of the CC1/85 parental isolate, and that VVC resistance conferred by the 3 FP changes does not involve the use of CXCR4 as an alternative entry pathway into PBMC or TZM-bl cells.

**Resistance Mutations Are Context-Dependent.** The 3 changes in the FP of D1/85.16 have similar effects to those conferred by 4 changes (K305R, H308P, A316V, and G321E) in the V3 region of CC1/1.19, the resistant CC1/85 variant generated under AD101 selection pressure (2, 3). The 4 V3 changes are context-dependent in that they did not confer resistance when introduced into JR-FL. To examine whether the 3 FP substitutions also act only in context, we introduced the G516V and F519I changes into JR-FL (this virus already has Val instead of the Met that predominates at position 518 of CC1/85). The recombinant clone, JR-FL + 3FP was confirmed to be replication-competent; its titer on day 7 postinfection ranged from 10^4 to 10^5 TCID50/mL, when p24 production was comparable with that of wild-type JR-FL (3.6 and 5.7 ng/mL, respectively). Thus, the 3 changes do not markedly affect FP function. However, in contrast to what was observed with CC1/85, the FP mutant of JR-FL remained sensitive to VVC both in PBMC (Fig. S3) and in TZM-bl cells. Although we cannot discount the possibility that more subtle changes in phenotype may be revealed by additional studies, it therefore appears that the FP changes conferring VVC resistance on CC1/85 are, like the V3 changes, context-dependent.

**VVC-Resistance in CCR5-Expressing Cell Lines Is Manifested by Reductions in both MPI and IC50 Values.** Inhibitor sensitivity tests in the TZM-bl cell line showed that VVC resistance was not only associated with incomplete inhibition plateaus as would be expected, but also with changes in IC50 values (Fig. 4, Table 1). To investigate further whether these resistance indicators were also observed in other cell lines engineered to express CCR5, we tested pseudotyped viruses bearing Env proteins from clones S and R in a single-cycle assay involving U87.CD4.CCR5 cells (Fig. S4). The Env-pseudotyped viruses were prepared and used as previously described, with the assay endpoint being luciferase reporter gene expression after infection for 48 h (5). Infections with S and R generated similar levels of luciferase expression (∼4.5 × 10^4 RLU). The parental clone S was fully inhibited by VVC (MPI >99%), with an IC50 of 5.8 nM, whereas the corresponding values for R were an MPI of 97.6% (the upper limit of 95% C.I. was consistently <100%), with a reduced IC50 of 1.2 nM. The ∼5-fold lower IC50 value for R compared with S is comparable with the ∼8-fold reduction seen using TZM-bl cells (Table 1). Thus, VVC resistance in at least 2 different CCR5-expressing cell lines is manifested both by incomplete inhibition plateaus, and by counterrative shifts toward lower IC50 values.

**Modeling the Usage of Different CCR5 Conformations by Wild-Type and Resistant HIV-1 Variants.** A model to explain the differential VVC concentration dependencies of a wild-type virus (S) and an engineered, resistant virus (S + 3FP) is depicted in Fig. S5. The model relies on simplifying assumptions. (i) That CCR5 exists on the cell surface in two forms, CCR5-A and CCR5-B (in reality, there may be more than 2); (ii) CCR5-A binds small molecule inhibitors (e.g., VVC) with a lower affinity than
CCR5, $K_{\text{diA}} > K_{\text{diB}}$; (iii) wild-type HIV-1 may use the 2 inhibitor-free forms to various degrees; (iv) CCR5-inhibitor-resistant HIV-1 variants use the inhibitor-CCR5-A complexes, but not free CCR5-A for entry, and can also use the inhibitor-free form of CCR5-B (in reality, there may be partial usage of the different CCR5 forms); and (v) the susceptibility of a target cell to infection by a particular HIV-1 variant is a function of the number of CCR5 coreceptors of the appropriate form on its surface (6–8). Pictorial representations of various infection scenarios are depicted in Fig. S5.

The relative inhibition of viral infectivity, $Q$ (%), is hypothesized to be a function of the inhibitor concentration ($C$), and is described by an equation with 3 parameters: $K_{\text{di}}$ (dissociation constant of such inhibitor binding to CCR5 that blocks infection), $K_{\text{de}}$ (dissociation constant of such inhibitor binding to CCR5 that allows infection), and $w$ (incorporating the relative amounts of CCR5-A and CCR5-B, as well as the capacity of the virus to use CCR5-A as an inhibitor-complex). Thus, the equation is general and applies to both wild-type and resistant viruses (and to any small molecule CCR5 inhibitor to which the test virus is resistant):

$$Q = (1 - (1 - (C/(K_{\text{di}} + C)) + w(C/(K_{\text{de}} + C)))) \times 100\%$$

All fitting of these functions was done by nonlinear regression (Prism, GraphPad) (8).

To illustrate the principles of how the model might predict differential inhibition of infection of PBMC and TZM-bl cells, we made assumptions about inhibitor-CCR5 affinities, the relative expression levels of CCR5-A and CCR5-B, and the abilities of the wild-type and resistant variants to use these CCR5 forms (Fig. S6). We postulated that CCR5-B has a 100-fold higher affinity for VVC than CCR5-A ($K_{\text{di}} = 0.01$ and $1 \text{ nM}$, respectively), and that the wild-type virus has an absolute preference for free CCR5-A over free CCR5-B. Also, we assumed that the resistant virus could enter cells via uncoupled CCR5-B and VVC-ligated CCR5-A with equivalent efficiencies (the wild-type virus can use neither). Keeping these assumptions constant, we modeled inhibition as a function of VVC concentration at 3 different ratios of CCR5-A and CCR5-B (set at 10:7, 2:3, and 20:1 in $A - C$ of Fig. S6, respectively). The shapes of the resulting infection-inhibition curves are reminiscent of the experimental data shown in Figs. 1, 3, and 4 in the main article. Of note is that varying the proportions of CCR5-A and CCR5-B around, but close to, 1:1 generates curves similar to those obtained experimentally using PBMC, whereas a curve similar to that from the TZM-bl cell tests is produced when CCR5-B is assumed to be in substantial excess (20:1).

One form of the mathematical model includes 3 parameters: $K_{\text{di}}, K_{\text{de}},$ the dissociation constants for such binding to forms of CCR5 that blocks or permits (even enhances) infectivity; and $w$, a multifactor parameter that reflects the relative amount of the form of CCR5 that allows infection when ligated by inhibitor and the capacity of a virus to use such complexes. However, the resolution in the plateau zone of the TZM-bl cell data is too poor to test the model of heterogeneous CCR5 affinity within the data set for each virus. To apply the model to the TZM-bl cell data, we instead proceeded in 2 steps. First, assuming that there really are 2 separable affinities (which could be averages from overlapping CCR5 populations), we estimated what the minimum ratio of $K_{\text{di}}$ to $K_{\text{diB}}$ would be. We assumed that the parallel shifts in the inhibition curves represent a minimum of such an affinity difference. This estimated ratio ($k \approx 37$) was introduced as a constant into the equation for relative inhibition, eliminating one parameter (by substituting $kK_{\text{di}}$ for $K_{\text{di}}$); thus, giving the equation:

$$Q = (1 - (1 - (C/(K_{\text{di}} + C)) + w(C/(kK_{\text{di}} + C)))) \times 100\%$$

The results of nonlinear regression fitting of the corresponding mathematical model to data from 6 infectivity-inhibition experiments using PBMC (3 with S, 3 with S + 3FP) and 2 averages of 3 experiments using TZM-bl cells (1 mean with S, 1 with S + 3FP) are listed in Table S2. In the case of the S virus on PBMC, the second term in the equation, which contains $w$, and represents entry via low-affinity complexes, always approached zero. For infection of TZM-bl cells by the S virus, $w$ did not reach 0 but was low (0.0095), a value that reflects the negligible contribution of the resistant S + 3FP virus on those cells (Table S2).

Mathematical models of infectivity can have >1 virological interpretation (8). Our model invokes affinity differences in VVC binding to distinct forms of CCR5. Thus, it is legitimate to search for alternatives to binding differences as interpretations of the mathematical model. Is the binding component of the modeling realistic? Although we have incorporated $K_{\text{di}}$ values into the models without measuring any binding, the parameter we have estimated through modeling comes close to direct measurements in the literature (9, 10). However, the parameters in the equations are fitted to inhibitory concentrations, and would coincide with dissociation constants only when half-maximal occupancy reduces infectivity by 50%. This raises the question: Could apparent $K_{\text{de}}$ reductions for inhibition of a resistant virus arise because that virus requires greater numbers of unoccupied CCR5 molecules than a wild-type virus does? Such a hypothesis could explain an aspect of the TZM-bl cell data (the shifts in mid-point inhibitory concentrations), but it would not produce the peaked curves observed in the PBMC assay. Thus, this alternative to the explanation based on CCR5 heterogeneity is not viable, overall.

Nevertheless, factors not accounted for in the modeling include possible differences in the absolute expression levels of various CCR5 forms between the cell types, as well differential inhibitory occupancies of VVC on CCR5 for sensitive and resistant virus. Such factors may contribute to the difference in the VVC concentrations that inhibit the S virus on PBMC and TZM-bl cells, and also the ranges of the apparent dissociation constants for inhibition of the S + 3FP mutant on TZM-bl cells and on PBMC from different donor pools. Although estimates of the inhibitory dissociation constant ($K_{\text{di}}$) for VVC against the wild-type S virus in the PBMC assay are variable, the values do fall between the inhibitory and the infection-mediating or infection-enhancing $K_{\text{di}}$ values for the S + 3FP mutant ($K_{\text{di}} = 1.3–6.4 \text{ nM}$ for S; $K_{\text{di}} = 0.28–1.6$ and $K_{\text{de}} = 9.6–50 \text{ nM}$ for S + 3FP). This suggests that the wild-type virus has no absolute preference between the 2 unligated forms of CCR5.

However, the inhibitory $K_{\text{di}}$ for the S virus is considerably (up to 14-fold) higher on TZM-bl cells than on PBMC. Also, the data for the S + 3FP virus suggest that CCR5-B is present at higher levels on TZM-bl cells, a factor that would lead to a lower inhibitory $K_{\text{di}}$ for S on TZM-bl cells than on PBMC, if S has no strong preference. The explanation for this apparent discrepancy may be that CCR5-B exists in qualitatively different forms (but still with a high affinity for VVC) on the 2 cell types, and is more permissive for usage by the S virus on PBMC. Also, the total CCR5 densities may be higher on TZM-bl cells, which could raise the inhibitory concentrations more for the wild-type S virus than for the resistant S + 3FP variant; it is plausible that the 2 viruses are blocked by different degrees of inhibitor occupancy on CCR5.


Fig. S1. The VVC-resistant phenotype is stable. A VVC-resistant HIV-1 isolate, D1/85.16, was cultured for 19 weeks in PBMC from 2 or 3 random blood donors, in the absence of VVC. Viruses from this VVC-free reversion culture were tested for VVC sensitivity in a PBMC-based replication assay. The sensitivity profiles of representative viruses D1/85.16R7, D1/85.16R8, D1/85.16R10, and D1/85.16R19 from weekly passages 7, 8, 10, and 19, respectively, are shown in comparison with D1/85.16 and the parental CC1/85 isolate. The amount of p24 produced in each VVC-containing culture after 7 days is shown as a percentage of that produced by the same isolate in the absence of inhibitor. The values shown are the means of at least 3 independent experiments ± SEM.
Fig. S2. VVC resistance conferred by the 3 FP changes does not involve the use of CXCR4 as an alternative entry pathway. (A) PBMC: The ability of VVC-sensitive and VVC-resistant viruses to replicate in PBMC from a CCR5 wild type donor was assessed in the presence and absence of the CXCR4-specific inhibitor AMD3100 (1 μM). The viruses were simultaneously assessed for their replication ability in PBMC from a CCR5-Δ32/Δ32 homozygous donor in the presence and absence of 100 nM AMD3100. The reference virus NL4-3 (X4) was tested with cells from both donors, DH123 (R5X4) and CC2/86 (R5X4) only with the CCR5-Δ32/Δ32 homozygous donor. HIV-1 replication was quantified by measuring p24 antigen production on day 7 postinfection. (B) TZM-bl cells: The parental clone S, the VVC-resistant clone R, and the S + 3FP mutant were tested for VVC sensitivity in the absence (gray symbols and lines) or presence (black symbols and lines) of AMD3100 (500 nM). The data shown are the average of 3–4 independent experiments, with the error bars indicating the SEM. The sensitivity to the different doses of VVC combined with AMD3100 (500 nM) was calculated from RLU values derived from virus-infected cells in the absence of any inhibitor, after subtraction of the background obtained from mock-infected cells exposed to 500 nM AMD3100.
Fig. S2. (continued).
Fig. S3. Effects of the 3 gp41 FP amino acid changes are context-dependent. The G516V and F519I changes were introduced by site-directed mutagenesis into JR-FL, a virus that already contains Val at position 518. The mutant JR-FL + 3FP virus was then tested for VVC sensitivity in comparison with wild-type JR-FL in a PBMC-based replication assay. The data shown are the average of 2 independent experiments, with the error bars indicating the SEM.
Fig. S4. VVC-resistance in CCR5-expressing cell lines is manifested by reductions in both MPI and IC₅₀ values. Env-pseudotyped viruses from clones S and R were tested for VVC sensitivity in a single-cycle infection-inhibition assay in U87.CD4.CCR5 cells, with a luciferase reporter gene endpoint. The VVC inhibition curve for R (dashed gray line) asymptotes to a plateau that is high, but consistently lower (upper limit of 95% C.I. <100%) than that for S, and it is also characterized by a reduction in IC₅₀. Background luminescence values derived from wells containing pseudovirus only were subtracted from test values. The data shown are the average of 3 independent experiments ± SEM.
A

Low-inhibitor-affinity

High-inhibitor-affinity

Conformers or sulfation variants

CCR5-A

CCR5-B

Fig. S5. Pictorial model of CCR5 heterogeneity and its consequences for inhibition of wild-type and resistant viruses by CCR5 ligands. CCR5 is postulated to exist in 2 distinct forms at the cell surface; these could be conformers (conformational isomers) or differentially tyrosine-sulfated forms. One form, CCR5-A (black), is assumed to bind small-molecule CCR5 ligands such as VVC with low affinity; the other form, CCR5-B (red), with a relatively high affinity. (A) According to the model, wild-type virus (green) can use both forms of unligated CCR5 for entry, but may have a preference between them. (B) Wild-type virus cannot enter via the VVC-complexes of either CCR5-A or CCR5-B. (C) The VVC-resistant virus (orange) has acquired the ability to use specifically the VVC-complex of CCR5-A. (D) The same adaptation confers or enhances the capacity of resistant virus also to enter via the unligated form of CCR5-B. (E) The VVC-resistant virus also differs from the wild type in that it cannot use the unligated form of CCR5-A. More complex versions of the model would also allow the mutant virus to use either unligated form of CCR5 in variable degrees. The model is general in that it would apply to any small molecule CCR5 inhibitor and any virus resistant to that inhibitor.
Fig. S5. (continued).
Fig. S5. (continued).
Mutant Env

CCR5-A

CCR5-B

Fig. S5. (continued).
Fig. S5. (continued).
Fig. S6. Principal modeling results of cell type-dependent CCR5-inhibitor resistance. In the 3 diagrams, the black curves depict inhibition of infection (percentage) of a target cell by a wild-type virus, the gray curves by a resistant variant (y axis), as a function of the VVC concentration (x axis). The dissociation constant for VVC binding to CCR5-A is postulated to be 1 nM and for binding to CCR-B, 0.01 nM. (A) The proportions of CCR5-A and CCR5-B on the target cell are fairly similar, in that there is only 30% less of CCR5-A than of CCR5-B (w = 0.70). (B) There is 50% more CCR5-A than CCR5-B on the cells (w = 1.5). Under the latter conditions, the infectivity of the resistant virus is enhanced by high concentrations of VVC, leading to negative inhibition. The 2 scenarios modeled in A and B might occur with PBMCs from different donors (see Fig. 5 A and B). (C) We assume there is a substantial excess of CCR5-B compared to CCR5-A on the target cells (w = 0.05). This scenario approximates what occurs in TZM-bl cells (see Fig. 5C).
Table S1. Nomenclature and properties of viruses used in this study

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Origin of env subunits in clones</th>
<th>Engineered FP mutations*</th>
<th>VVC phenotype</th>
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<td>N/A</td>
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<tr>
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<tr>
<td>S Parental clone</td>
<td>CC1/85 cl.6</td>
<td>CC1/85 cl.6</td>
<td>N/A</td>
</tr>
<tr>
<td>R VVC-resistant clone</td>
<td>D1/85.16 cl.23</td>
<td>D1/85.16 cl.23</td>
<td>N/A</td>
</tr>
<tr>
<td>R/S Chimera</td>
<td>D1/85.16 cl.23</td>
<td>CC1/85 cl.6</td>
<td>N/A</td>
</tr>
<tr>
<td>S/R Reverse chimera</td>
<td>CC1/85 cl.6</td>
<td>D1/85.16 cl.23</td>
<td>N/A</td>
</tr>
<tr>
<td>S + 3FP Mutant parental clone</td>
<td>CC1/85 cl.6</td>
<td>CC1/85 cl.6</td>
<td>GS16V, M518V, F519I</td>
</tr>
<tr>
<td>R/S + 3FP Mutant chimera</td>
<td>D1/85.16 cl.23</td>
<td>CC1/85 cl.6</td>
<td>GS16V, M518V, F519I</td>
</tr>
<tr>
<td>JR-FL Clone of R/S primary isolate</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>JR-FL + 3FP Heterologous mutant clone</td>
<td>N/A</td>
<td>N/A</td>
<td>GS16V, F519I*</td>
</tr>
</tbody>
</table>

Isolates designated as D1/85.16R followed by a number were derived from the PBMC culture in which D1/85.16 was cultured for a prolonged period in the absence of VVC; the number indicates the number of weekly passages (Fig. S1). N/A, not applicable. The columns list the origins of gp120 and gp41 subunits in chimeric viruses based on the reference clones of CC1/85 and D1/85.16.

* Amino acid numbering is based on HXB2 Env.
† JR-FL (GenBank accession no. U63632) already contains valine instead of the methionine that is present at position 518 in 5 of 7 CC1/85 clones (Fig. 2).
Table S2. Fitting of model to experimental PBMC and TZM-bl data

<table>
<thead>
<tr>
<th>Target cell (endpoint)</th>
<th>Virus</th>
<th>Modeling results</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$K_{da}$ nM</td>
<td>$K_{db}$ nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{da}$ nM</td>
<td></td>
</tr>
<tr>
<td>PBMC (p24)</td>
<td>S</td>
<td>2.0 ± 0.35</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>1.3 ± 0.19</td>
<td>&gt;1.3</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>6.4 ± 2.2</td>
<td>&gt;6.4</td>
</tr>
<tr>
<td></td>
<td>S + 3FP</td>
<td>0.30 ± 0.044</td>
<td>50 ± 28</td>
</tr>
<tr>
<td></td>
<td>S + 3FP</td>
<td>0.28 ± 0.055</td>
<td>9.6 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>S + 3FP</td>
<td>1.6 ± 0.41</td>
<td>12 ± 3.5</td>
</tr>
<tr>
<td>TZM-bl cells (RLU)</td>
<td>S</td>
<td>18 ± 2.8</td>
<td>&gt;18</td>
</tr>
<tr>
<td></td>
<td>S + 3FP</td>
<td>0.67 ± 0.045</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Three replicate p24 measurements for S and S + 3FP virus were obtained with PBMC from different donor pools in each experiment. The TZM-bl cell data are the means of 3 replicate RLU measurements obtained in 3 independent experiments. Values of the parameters $K_{da}$, $K_{da}$, and $w$ for PBMC data, and $K_{da}$ and $w$ for TZM-bl cell data were fit by nonlinear regression. These values were interpreted and classified as corresponding directly to, or as limiting, the theoretical values of $K_{da}$ and $K_{db}$. The parameter $w$ reflects the relative amount of the form of CCR5 that is infection-permissive when complexed by an inhibitor; $w$ can also express a variable capacity of a test virus to use such complexes. The closer the value of $R^2$ is to 1, the better the fit. The degrees of freedom for triplicate inhibition values measured at 8 concentrations were 21 for PBMC and 22 for TZM-bl. The dash in the PBMC results signifies that when $w$ approaches 0 no meaningful value of $K_{da}$ can be identified. N/A, not applicable, signifies that the $K_{da}$ parameter was absent from the function fit to the TZM-bl results.