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A pièce de resistance: how HIV-1 escapes small molecule CCR5 inhibitors

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Abstract

Purpose of review—Small molecule inhibitors targeting the CCR5 coreceptor represent a new class of drugs for treating HIV-1 infection. Maraviroc has received regulatory approvals, and vicriviroc is in phase 3 trials. Understanding how resistance to these drugs develops and is diagnosed is essential to guide clinical practice. We review what has been learned from *in vitro* resistance studies, and how this relates to what is being seen, or can be anticipated, in clinical studies.

Recent findings—The principal resistance pathway *in vitro* involves continued use of CCR5 in an inhibitor-insensitive manner; the resistant viruses recognize the inhibitor-CCR5 complex, as well as free CCR5. Switching to use the CXCR4 coreceptor is rare. The principal genetic pathway involves accumulating 2–4 sequence changes in the gp120 V3 region, but a non-V3 pathway is also known. The limited information available from clinical studies suggests that a similar escape process is followed *in vivo*. However, the most common change associated with virologic failure involves expansion of pre-existing, CXCR4-using viruses that are insensitive to CCR5 inhibitors.

Summary—HIV-1 escapes small molecule CCR5 inhibitors by continuing to use CCR5 in an inhibitor-insensitive manner, or evades them by expanding naturally insensitive, CXCR4-using variants.

Keywords

antiretroviral drugs; CCR5 inhibitors; entry inhibitors; HIV-1 resistance

Introduction

Small molecules directed at the CCR5 entry coreceptor represent a new drug class for treating HIV-1 infection [1•,2•]. The first compound (maraviroc; Selzentry; Pfizer, Inc.) has been approved for use in antiviral treatment-experienced patients [3], a second (vicriviroc, Schering-Plough Research Institute) is in phase 3 trials, and several others are in preclinical or clinical development [1•,2•]. Resistance to these inhibitors has been described *in vitro* and is now being seen in clinical studies. Here, we will review the resistance pathways and discuss their implications for clinical practice.

Mechanism of action of the small molecule CCR5 inhibitors

These compounds all act by binding within a cavity located among the membrane-spanning helices of CCR5, a G-protein coupled receptor, and thereby stabilizing the receptor in a

conformation that HIV-1 cannot recognize efficiently [4–11,12•,13–15,16•]. Normally, HIV-1 binds a coreceptor, CCR5 (R5 viruses) or CXCR4 (X4 viruses), after first interacting with CD4. These events trigger conformational changes in the gp120/gp41 envelope glycoprotein complex that drive fusion of the virus and cell membranes [17]. By preventing CCR5 binding, the small molecules abort fusion and interrupt the HIV-1 replication cycle [1•,2•]. *In vivo*, R5 viruses predominate early but X4 variants gradually emerge, usually while R5 viruses are also present, eventually becoming detectable in approximately 50% of patients infected with subtype B strains [18,19] and in an increasing proportion of subtype C infections [20]. As discussed elsewhere in this volume, because it is difficult to distinguish between mixtures of R5 and X4 viruses and variants with dual tropism for CCR5 and CXCR4, the term dual-mixed (D/M) viruses is commonly used. Many D/M viruses preferentially use CCR5 *in vitro* [21]. The appearance of D/M or X4 variants correlates with accelerated loss of CD4⁺ T cells and a greater risk of AIDS-defining illnesses [18,19]. CCR5 inhibitors are ineffective at reducing viral load in patients with detectable levels of CXCR4-using viruses, so are only recommended for treating ‘pure R5’ infections [1•,2•].

HIV-1 is notorious for becoming resistant to antiretroviral drugs [22,23], and the small molecule CCR5 inhibitors are no different in this regard. Unlike the ‘more traditional’ reverse transcriptase inhibitors and protease inhibitors, the CCR5 inhibitors have, at least in theory, the potential to drive the emergence of the more pathogenic CXCR4-using variants [1•,2•, 18]. Hence, understanding how resistance develops *in vitro* and *in vivo* helps define how CCR5 inhibitors should be used clinically, and influences the development and use of methods to diagnose the emergence of resistance during therapy.

Resistance to CCR5 inhibitors *in vitro*

The most intuitive mode of resistance to a CCR5 inhibitor would be for HIV-1 to switch coreceptors and enter cells via CXCR4 [1•,2•,18]. However, whenever a primary R5 virus is subjected to the selection pressure of a small molecule CCR5 inhibitor in primary cells [e.g., peripheral blood mononuclear cell (PBMC)], the resistant virus usually retains the R5 phenotype even when CXCR4 is available in relative abundance [12•,16•,24–27,28•]. The likeliest explanation for this counterintuitive outcome is that transitional R5 variants arising during the acquisition of CXCR4 use are less fit than the starting virus [29], and/or more sensitive to the selecting compound [30•].

Usually, primary R5 isolates are cultured in stimulated PBMC with increasing concentrations of various small molecule CCR5 inhibitors [12•,16•,24–27,28•]. In the first such study, the selecting compound was AD101, a preclinical precursor of vicriviroc; the subtype B isolate CC1/85 became fully (>20 000-fold) resistant after approximately 19 weekly passages, although a partially (~5-fold) resistant isolate emerged very early [24,25]. The fully resistant isolate was cross-resistant to other small molecule CCR5 inhibitors but remained sensitive to CCR5 MAbs [12•,24,25,31•]. Resistance was mapped to 4 amino acid changes in the gp120 V3 region: a pre-existing, partially AD101-resistant variant with the H308P polymorphism was rapidly selected for, then three *de novo* substitutions (K305R, A316V, and G321E) occurred sequentially and were necessary and sufficient for complete resistance [25]. The same CC1/85 isolate and the partially resistant H308P variant were also cultured with vicriviroc [26]. Both viruses became completely resistant, and cross-resistant to several other CCR5 small molecules, within 16 and 12 passages, respectively [26,31•]. Although resistance was mapped to *env*, V3 changes were not responsible, despite the use of the same input virus and culture procedure used to select the AD101-resistant variant, and the broad similarity between AD101 and vicriviroc [24,26]. Hence, more than one genetic pathway leads to the same resistance phenotype.

When CC1/85 and the subtype G isolate RU570 were cultured with maraviroc, complete resistance arose after about 16 and 18 passages, respectively [16•]. In contrast to the cross-resistance of the AD101- and vicriviroc-resistant viruses described above, the maraviroc-resistant CC1/85 and RU570 variants remained sensitive to SCH-C, vicriviroc, and aplaviroc [16•,32]. V3 changes accounted for the resistance of both viruses: the A316T and I323V substitutions were particularly influential on the CC1/85 phenotype, a three-residue deletion (Δ QAI at residues 315–317) on RU570 [16•]. In a study of a different vicriviroc-resistant variant of RU570, the determinants of resistance were mapped to a 200-residue stretch of gp120 spanning the C2–V5 region, but V3 changes conferred resistance only when present in certain genetic contexts [28•].

The appearance of X4 viruses in CCR5 inhibitor-selection experiments has, however, been observed. In one, X4 viruses arose in both the maraviroc-treated and control cultures, suggesting the driving force was adaptation to growth in PBMC and not inhibitor escape [16•]. In a similar study with an experimental CCR5 inhibitor, TAK-652, an X4 virus emerged only in the control culture [27]. One vicriviroc-resistant virus could use CXCR4 to replicate in engineered U87-CD4/CXCR4 cells, but not PBMC, implying a conditional recognition of CXCR4 [26]. When six R5 HIV-1 strains were passaged in a cell line engineered to express high CXCR4 and low CCR5 levels, three acquired the ability to use CXCR4, some more rapidly when TAK-779 was present [33]. How relevant this protocol is to physiological conditions is uncertain.

Together, these *in vitro* studies show that resistance to small molecule CCR5 inhibitors is not associated with a unique, or even a common, genetic signature. Although the V3 region is usually an important site of resistance mutations [16•,25,27,28•], different changes arose in different (or even the same) isolates. They are also context dependent; the 4 V3 changes that conferred AD101-resistance on CC1/85 had no effect when introduced into the V3 region of JR-FL (JPM, unpublished results). Moreover, at least one resistant variant has no V3 changes that are required for resistance [26], and tropism-influencing changes in gp41 have now been reported [34]. Adding to the complexity, cross-resistance to small molecule CCR5 inhibitors from other chemical classes may or may not arise [16•,24,26,27,31•,32]. However, as expected, the resistant viruses retain sensitivity to protease inhibitors, nucleoside reverse transcriptase inhibitors, nonnucleoside reverse transcriptase inhibitors, integrase inhibitors, the fusion inhibitor enfuvirtide, and anti-CCR5 MAbs that act by a dissimilar mechanism to small molecules [16•,26,28•,31•].

Resistance to CCR5 inhibitors *in vivo*

Little information on resistance is yet available from phase 2 and 3 clinical trials of CCR5 inhibitors, but the emergence of CXCR4-using viruses appears to be the most common virologic correlate of treatment failure. In phase 3 trials of maraviroc plus optimized background therapy (OBT) in treatment-experienced patients with only R5 viruses present at baseline (MOTIVATE 1 and 2), D/M or X4 viruses were detected in 63/115 (~55%) subjects who failed therapy, compared with only 4/89 (~5%) who failed OBT plus placebo [35,36]. In a phase 3 trial in treatment-naïve subjects (MERIT), CXCR4-using viruses were detected at virologic failure in 10/35 (31%) participants receiving maraviroc as a first-line regimen [37]. Similarly, in the ACTG A5211 phase 2b trial, D/M or X4 viruses emerged in 9/26 (35%) treatment-experienced patients at the time of virologic failure of a vicriviroc-containing regimen [38•].

In these studies, the detection of D/M and X4 viruses at treatment failure was due to expansion of pre-existing CXCR4-using variants that were not completely suppressed by the other drugs in the regimen, and that were not initially detectable using the Trofile screening test (Monogram

Inc) [35,39] (Fig. 1). However, as was found *in vitro*, the evolution of resistant R5 variants (but not new X4 variants) has sometimes been noted [35]. In the MOTIVATE trials, maraviroc resistance was detected in 15/36 (42%) patients who failed therapy with only R5 viruses detectable, as assessed using the PhenoSense entry inhibitor assay (see below) [40]; and in 2/13 (15%) subjects with maraviroc failure in the MERIT study. Vicriviroc resistance was detected in 1/29 patients who failed therapy in ACTG A5211 [41•], and in 4/26 subjects with vicriviroc failure in a study in treatment-naïve patients [42].

All the *ex vivo* maraviroc- and vicriviroc-resistant viruses had broadly similar properties to *in vitro*-selected escape mutants in the PhenoSense assay, suggesting that the underlying resistance mechanism was probably comparable in the two settings [35,41•,42]. The extent of cross-resistance among maraviroc, vicriviroc and similar inhibitors has not been reported to date.

Clonal analysis of *env* genes from the maraviroc- and vicriviroc-resistant viruses revealed that V3 sequence changes arose during therapy, but not consistently in viruses from placebo recipients who also failed therapy [35,43•,41•,42]. Site-directed mutagenesis studies of cloned *env* genes from four of the maraviroc-resistant isolates showed that the sequence changes deemed most likely to be relevant, on the basis of their prevalence, were both necessary and sufficient for resistance in two cases, sufficient but not necessary in one case, and necessary but not sufficient in the fourth [35]. Although the resistant viruses had sequence changes in the V3 loop stem, as with the resistant viruses selected *in vitro*, there was no consistent pattern; predicting *in vivo* maraviroc or vicriviroc resistance by sequence analysis was not possible [35].

Although there do appear to be similarities between how resistance arises *in vitro* and *in vivo*, the latter situation is the more complex: The HIV-1 envelope glycoproteins are under the continual selection pressure of neutralizing antibodies (NAbs) *in vivo* [44], so the humoral immune system may apply additional constraints on what sequence changes can be tolerated during escape from the pressure applied by a CCR5 inhibitor [31•]. For example, a variant that increases its exposure of the V3 region to evade an inhibitor may become sensitive to a NAb against V3. In some cases, acquisition of CCR5 inhibitor resistance *in vitro* does not appear to compromise viral replicative capacity or 'fitness'; when cultured without the selecting compound, the resistant strains do not rapidly revert to sensitivity [24,45]. In other cases, reversion of the resistant phenotype does occur, indicating that *in vitro* resistance can carry a fitness cost [32]. When vicriviroc was discontinued in a patient who had developed resistance, a vicriviroc-sensitive virus soon reappeared [41•]. It remains to be determined whether this will be generally true, and whether it represents the re-emergence of pretreatment, wild-type virus or the reversion of resistance mutations (as occurs with enfuvirtide-resistant viruses [46]).

Mechanisms of resistance

The two resistance mechanisms most relevant to CCR5 inhibitors have been termed competitive and noncompetitive (or allosteric) [12•,16•,47,48] (Fig. 2). The distinction between the two mechanisms is relevant not only to understanding how resistance arises, but also to how it is diagnosed and quantified. Competitive resistance is defined as resistance that results in a shift in the IC₅₀ of an inhibitor to a higher concentration; complete inhibition may still be achieved at a sufficient inhibitor concentration [12•,16•,47,48]. Noncompetitive resistance is saturable, such that increasing the inhibitor concentration no longer has any further effect; the EC₅₀ required to reach the saturation level is the same as the IC₅₀ for the fully sensitive virus [12•,16•,47,48].

Competitive resistance arises from a more efficient use of inhibitor-free CCR5, for example, due to an increase in the affinity of the Env complex for CCR5, or because fusion occurs more rapidly after CCR5 binding [12•]. Eventuality would allow HIV-1 to 'scavenge' low levels of inhibitor-free CCR5 [12•]. Although a partially (~5-fold) AD101-resistant isolate did appear to have the latter ability [24], this mechanism cannot account for complete resistance (>1000-fold, at saturating inhibitor concentrations); affinity or fusion rate increases on this scale seem impractical.

In noncompetitive resistance, the escape variant uses the inhibitor-bound form of CCR5 for entry as well as, but not instead of, the free coreceptor [12•,16•]. The level of residual inhibition [termed the 'plateau' or maximum percentage inhibition (MPI) value], at a saturating inhibitor concentration, reflects the efficiency with which the inhibitor-CCR5 complex is used, relative to free CCR5 [12•,16•]. This mechanism is consistent with how the small molecule CCR5 inhibitors act; that is, by locking the extracellular domains of CCR5 into a conformation that cannot be recognized by wild-type HIV-1 [4,8,12•,16•]. The resistant variants are, however, able to bind this altered CCR5 configuration, while retaining their use of the natural one [12•,16•].

Investigations of maraviroc- and AD101-resistance concluded that the resistant strains use the inhibitor-CCR5 complex for entry, and that the noncompetitive mechanism explains complete resistance to the inhibitors in PBMC replication assays [12•,16•]. Indeed, some CCR5 antagonist-resistant viruses may use inhibitor-bound CCR5 preferentially over the unbound receptor, leading to modestly increased replication of resistant viruses in the presence of inhibitor *in vitro* [12•,26,41•].

At a molecular level, the current model suggests that gp120 binds to CCR5 at two distinct sites: the 'bridging sheet' and residues at the V3 base bind to the tyrosine-sulfated N-terminus of CCR5 and the V3 crown interacts with ECL2 [14,49,50,51•]. The CCR5 inhibitors may predominantly affect the ECL2 conformation, with resistant viruses becoming more dependent on the interaction with the N-terminus [14,52,53•]. Thus Env sequence changes in the resistant variants might directly or indirectly alter the conformation of V3, such that its crown need no longer interact with CCR5 ECL2 to mediate infection. Indeed, HIV-1 or HIV-2 variants lacking the V3 crown, although replicating poorly, are resistant to small-molecule CCR5 inhibitors [52,53•].

How noncompetitive resistance to CCR5 inhibitors is detected and quantified is assay-dependent. In multicycle assays in PBMC cultures, the replication of resistant viruses is usually not impeded, and can even be enhanced, by very high inhibitor concentrations [12•,16•,26,27,28•]. Because multiple cycles of replication and other factors, such as changes in chemokine secretion over time, can affect the outcome of these assays, MPI is best determined by a single-cycle assay of HIV-1 entry using an Env-pseudotyped virus [12•] (Fig. 1). In this case the MPI varies with the cell type: An AD101-resistant virus had an MPI of ~25% in PBMC, but 80–90% in U87-CD4/CCR5 cells, a line engineered to express CD4 and CCR5 [12•] that serves as the basis of the PhenoSense and Trofile assays [40•]. The quantitative difference implies that the resistant viruses use the inhibitor-CCR5 complex for entry much less efficiently in the cell line than in primary CD4⁺ T cells [12•]. An MPI value of ~60% in the PhenoSense assay has also been reported for an *in vitro*-generated virus that has a completely maraviroc-resistant phenotype in a PBMC replication assay [16•]. MPIs <95% have correlated with at least partial maraviroc resistance in clinical trials [40]; an MPI threshold for vicriviroc resistance is not yet established. Why resistant viruses use the inhibitor-CCR5 complex less efficiently in some cells than in others remains to be determined; differences in cell-surface CCR5 density are probably relevant [28•].

The inefficient use of the inhibitor-CCR5 complex in U87-CD4/CCR5 cells may have important implications for resistance testing. Phenotypic assays will probably continue to be used to determine whether incomplete suppression of HIV-1 replication by a regimen containing a small molecule CCR5 inhibitor is due to resistance to this drug or to another component of the regimen. There are unanswered questions about phenotypic assays that rely on engineered cell lines, such as PhenoSense's U87-CD4/CCR5 cells, to assess CCR5 inhibitor resistance. If there is a large disparity between the MPI of a resistant virus in a cell line and in primary CD4⁺ T cells, what is the relevant maximum MPI value that indicates resistance in the cell line? Are these assays capable of discriminating between this cut-off value and 100% MPI? Answering these questions will ultimately help guide the development and application of these assays for routine use, which seems necessary given that no simple genetic marker of CCR5 inhibitor resistance has been observed *in vitro* or *in vivo* [26,27,28,33,37].

Conclusion

The small molecule CCR5 inhibitors represent an interesting new option for treatment of HIV-1 infection [1,2,3], and they may also play a role in prevention [54,55]. But resistance to these drugs is now being seen in the clinic and needs to be understood. The predominant pathway for the evolution of *de novo* resistance involves the continued use of CCR5 in an inhibitor-insensitive manner, but a more common route to treatment failure involves the expansion of pre-existing CXCR4-using viruses that are naturally insensitive to these inhibitors. These escape and evasion pathways will influence the clinical use of the small molecule CCR5 inhibitors.

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HIV-1 coreceptor usage assay

Adapted from Whitcomb J, et al. Antimicrob Agents Chemother. 2007;51:566-575.

Figure 1. A commonly used assay for HIV-1 coreceptor usage

Plasmids expressing a HIV-1 Env glycoprotein and an Env-deleted HIV-1 genome with a linked luciferase reporter gene are cotransfected into a producer cell line (typically 293T cells). The resulting Env-pseudotyped viruses are collected and used to infect a target cell line that expresses CD4 and either CCR5 or CXCR4 (typically U87-CD4-CCR5 or U87-CD4-CXCR4 cells). Infection is quantified by the amount of luciferase activity present in the target cells. The commercially available Trofile and PhenoSense assays from Monogram Inc are based on this method [39]. This figure is adapted from [39].

Figure 2. Competitive and noncompetitive inhibition

With competitive inhibition (left), resistance is manifested by a rightwards shift in the dose response curve (dotted line) such that the IC_{50} value for the inhibitor increases. If enough inhibitor is present, the extent of inhibition can still reach 100%. With noncompetitive inhibition (right), resistance is manifested by a reduction in the extent of inhibition (dotted line) but without a change in IC_{50} value. Increasing the inhibitor concentration beyond the point at which inhibition is maximal (the maximum percentage inhibition, MPI, value) has no additional effect. Resistance to the small molecule CCR5 inhibitors is noncompetitive, sometimes referred to as allosteric [12••,16••].