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Neutralizing antibody and anti-retroviral drug sensitivities of HIV-1 isolates resistant to small molecule CCR5 inhibitors

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Abstract

The small molecule CCR5 inhibitors are a new class of drugs for treating infection by human immunodeficiency virus type 1 (HIV-1). They act by binding to the CCR5 co-receptor and preventing its use during HIV-1-cell fusion. Escape mutants can be raised against CCR5 inhibitors *in vitro* and will arise when these drugs are used clinically. Here, we have assessed the responses of CCR5 inhibitor-resistant viruses to other anti-retroviral drugs that act by different mechanisms, and their sensitivities to neutralizing antibodies (NAbs). The rationale for the latter study is that the resistance pathway for CCR5 inhibitors involves changes in the HIV-1 envelope glycoproteins (Env), which are also targets for NAbs. The escape mutants CC101.19 and D1/85.16 were selected for resistance to AD101 and vicriviroc (VVC), respectively, from the primary R5 HIV-1 isolate CC1/85. Each escape mutant was cross resistant to other small molecule CCR5 inhibitors (aplaviroc, maraviroc, VVC, AD101 and CMPD 167), but sensitive to protein ligands of CCR5: the modified chemokine PSC-RANTES and the humanized MAb PRO 140. The resistant viruses also retained wild-type sensitivity to the nucleoside reverse transcriptase inhibitor (RTI) zidovudine, the non-nucleoside RTI nevirapine, the protease inhibitor atazanavir and other attachment and fusion inhibitors that act independently of CCR5 (BMS-806, PRO-542 and enfuvirtide). Of note is that the escape mutants were more sensitive than the parental CC1/85 isolate to a subset of neutralizing monoclonal antibodies and to some sera from HIV-1-infected people, implying that sequence changes in Env that confer resistance to CCR5 inhibitors can increase the accessibility of some NAb epitopes. The need to preserve NAb resistance may therefore be a constraint upon how escape from CCR5 inhibitors occurs *in vivo*.

Introduction

New classes of drugs to treat infection with human immunodeficiency virus type 1 (HIV-1) are still being developed, not least because of the propensity of the virus to develop resistance to the reverse transcriptase inhibitors (RTI) and protease inhibitors (PI) that have been the mainstay of therapy for the past decade (Hammer et al., 2006). The new classes include the small molecule CCR5 inhibitors; maraviroc (MVC) has now been approved for clinical use, vicriviroc (VVC) is in Phase III trials, and several others are at earlier stages in the drug-development process (Kuhmann and Hartley, 2008). These compounds act by binding to the CCR5 co-receptor and altering its conformation in a way that impedes its recognition by the

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HIV-1 gp120 glycoprotein during virus-cell fusion (Dragic et al., 2000; Kondru et al., 2007; Seibert et al., 2006; Tsamis et al., 2003; Watson et al., 2005). HIV-1 is, however, able to mutate to escape the inhibitory effects of the small molecule CCR5 inhibitors, a process that involves altering the conformation of the gp120 protein to allow its binding to the inhibitor-CCR5 complex (Pugach et al., 2007; Westby et al., 2007). The resistant viruses can also use the free CCR5 co-receptor for entry, so they are not drug-dependent (Baba et al., 2007; Marozsan et al., 2005; Trkola et al., 2002; Westby et al., 2007). The resistance phenotype is stable *in vitro*, in that the escape mutants are fit and they do not rapidly revert to sensitivity when cultured in the absence of the selecting compound (Anastassopoulou et al., 2007; Trkola et al., 2002; Westby et al., 2007). Whether this also applies *in vivo* remains to be determined, as multiple selection pressures on the HIV-1 Env glycoproteins may work together to compromise fitness under those conditions.

Details are now emerging about how resistance to the small molecule CCR5 inhibitors arises at a molecular level. The natural interaction between gp120 and CCR5 appears to involve two principal points of contact; the V3 region and the bridging sheet of gp120 bind to the second extracellular loop (ECL-2) and the tyrosine-sulfated N-terminus (Nt) of CCR5, respectively (Cormier and Dragic, 2002; Huang et al., 2007). In the escape mutants, the sequence changes in gp120 may disrupt the former interaction, rendering the virus much more dependent on the binding of the bridging sheet to the CCR5 Nt (our unpublished results). Genetically, this is usually achieved by the introduction of sequence changes within V3 (Baba et al., 2007; Kuhmann et al., 2004; Ogert et al., 2008; Westby et al., 2007). However, at least one VVC-resistant clone has no V3 sequence changes, which implies the existence of alternative genetic pathways to the same phenotype (Marozsan et al., 2005).

All the above observations were made using escape mutants that were generated in cell culture, but early clinical studies of the small molecule CCR5 inhibitors suggest that resistant viruses generated *in vivo* have broadly similar properties (Mori et al., 2007; Strizki et al., 2006). We have therefore used two different CCR5 inhibitor-resistant viruses to address two questions of relevance to the clinical use of these new drugs: Do the changes in gp120 that confer resistance to CCR5 inhibitors affect how the virus is neutralized by antibodies that target the viral envelope gp120/gp41 glycoprotein complex? Are the resistant viruses still sensitive to inhibitors with different mechanisms of action, including PIs and RTIs and other fusion/entry inhibitors that target different steps in the fusion process? The former sub-study is particularly relevant to understanding how CCR5 inhibitor resistance might evolve *in vivo*, because most recipients will have plasma antibodies against the HIV-1 envelope glycoproteins. To enable it to persist in the face of humoral immunity, HIV-1 has evolved multiple natural defensive mechanisms that shield the vulnerable regions of its Env complex from antibody binding, including the CCR5 binding site (Burton et al., 2004; Labrijn et al., 2003). Thus, the V3 region of most primary viruses is not well exposed for antibody binding (Hartley et al., 2005). The question therefore arises as to whether the sequence changes in the CCR5 inhibitor-escape mutants disturb any of the antibody-resistance mechanisms possessed by wild-type viruses such as their parental strain. If so, then there will be additional constraints on how CCR5 inhibitor-resistant viruses arise *in vivo*; the escape mutants must not only acquire resistance to the drug, but also retain their natural resistance to NAbs.

Our observations are that the escape mutants are moderately more sensitive than the parental CC1/85 isolate to some sera from HIV-1-infected people and also to a subset of neutralizing monoclonal antibodies (MAbs). These findings imply that acquisition of resistance to CCR5 inhibitors can affect how the Env complex resists the action of NAbs, which may be relevant to how resistance to these drugs develops *in vivo*. We also show that the small molecule CCR5 inhibitor-resistant viruses are generally cross resistant within this drug class but retain wild-type sensitivity to RTIs, PIs and various attachment and entry inhibitors that act independently

of CCR5. However, as reported previously, the escape mutants remain sensitive to protein ligands of CCR5 such as the modified chemokine PSC-RANTES and anti-CCR5 MAbs that act by different mechanisms (Ji et al., 2007; Pugach et al., 2007; Westby et al., 2007).

Results

Responses of the CCR5 inhibitor-resistant isolates to NABs

To determine whether the AD101 and VVC escape mutants CC101.19 and D1/85.16 had altered responses to antibodies against the HIV-1 envelope glycoproteins, we tested their sensitivity to four broadly reactive NABs, b12, 2G12, 2F5 and 4E10, in a PBMC-based multi-cycle replication assay, using the CC1/85 parental isolate and the CCcon.19 passage control isolate as reference standards (Fig.1, Table 1). To reduce the variation commonly observed with primary cells, we pooled PBMCs from two different donors for each experiment (Ketas et al., 2007).

We have previously reported that prolonged adaptation of the CC1/85 isolate to PBMC culture renders it more sensitive to inhibitors of the gp120-CD4 interaction, probably because of a selection pressure for increased CD4 affinity that promotes target cell infection (Pugach et al., 2004). This finding was confirmed in the present experiment: CCcon.19 was markedly (15-fold) more sensitive than CC1/85 to b12, a MAb to the CD4-binding site (CD4bs) on gp120. The two CCR5-inhibitor escape mutants had intermediate sensitivities to b12, with ~4-fold reductions in IC_{50} compared to CC1/85. These modest effects could arise from either *in vitro* passage during the resistance selection process, and/or any additional effects of becoming CCR5 inhibitor resistant.

The VVC-resistant isolate D1/85.16 was substantially more sensitive to NAb 2G12 against a glycan-dependent gp120 epitope, with a 50-fold decrease in the IC_{50} value compared to CC1/85. However, the AD101-resistant and passage control isolates had unchanged sensitivities to 2G12. The increase in the 2G12 sensitivity of D1/85.16 is therefore a consequence of the non-V3 sequence changes that arise as the virus becomes VVC resistant, but may not be obligatorily linked to resistance.

The 2F5 and 4E10 NABs recognize epitopes in the membrane-proximal external region (MPER) of gp41 (Zwick et al., 2001). The D1/85.16 isolate was moderately (~6-fold) more sensitive to 2F5 than the parental isolate, whereas 2F5 did not detectably inhibit CC101.19. Both CCR5 inhibitor-resistant viruses were >5-fold more sensitive than the parental and passage control isolates to 4E10 (IC_{50} ~10 μ g/ml); the magnitude of the sensitivity increase is hard to judge because neither CC1/85 nor CCcon.19 was sensitive to this MAb (IC_{50} >50 μ g/ml). Nonetheless, CCR5 inhibitor resistance does appear to be associated with a modestly increased sensitivity to MPER-targeted MAbs, with the exception of 2F5 against CC101.19.

We also assessed whether the resistant viruses had altered sensitivities to three V3 MAbs (447-52D, F425 and 39F) and to MAb 17b to a CD4i epitope that overlaps the CCR5 binding site. These studies were inconclusive; none of the control or resistant viruses was sensitive to any of the tested MAbs at concentrations up to 50 μ g/ml (data not shown).

Binding assays using monomeric gp120 do not predict changes in NAB sensitivity of the CCR5 inhibitor-resistant isolates

Changes in neutralization sensitivity to a gp120-targeted MAb could arise because of alterations in the structure of its epitope at the level of the gp120 subunit, or because of changes in how the epitope is presented on the functional Env trimer (Moore et al., 1995). To assess which mechanism applied to the CCR5 inhibitor resistant isolates, we measured the binding of various MAbs and CD4-IgG2 to gp120 proteins present in detergent-inactivated preparations

of the control and resistant viruses. Titration curves were generated, with half-maximal MAb binding concentrations calculated and presented (Table 2).

The binding of b12 and CD4-IgG2 to each of the gp120 proteins was comparable (± 2 -fold, the precision limit of this type of assay) (Table 2). Hence, the ~ 4 -fold increased b12 neutralization sensitivity of the resistant isolates may arise because of alterations in the exposure of the CD4bs and its associated epitopes on the Env trimer. Although we have no direct evidence for this supposition, a similar, but more dramatic, effect was previously observed with CCcon.19. This passage control virus was 100-fold more sensitive to neutralization by soluble CD4 without any increase in soluble CD4 binding to monomeric gp120 (Pugach et al., 2004).

There was only a modest (<3 -fold) increase in the binding of 2G12 to D1/85.16 gp120 compared to CC1/85 gp120 (Table 2), which is unlikely to be sufficient to account for the ~ 50 -fold increase in the relative neutralization sensitivity of D1/85.16 (Fig.1, Table 1). Structural changes in the presentation of the 2G12 epitope on the Env trimer are probably relevant, although again we have no direct evidence that this is the case.

All three V3 MAbs bound strongly to gp120 from CC1/85 (Table 2). Their inability to neutralize this virus therefore arises because their epitopes are poorly exposed on the Env trimer, as is commonly observed with V3 MAbs (Moore et al., 1995). Since the V3 sequence of D1/85.16 is identical to that of CC1/85, there was, as expected, no difference in the binding of the three V3 MAbs to these two gp120s (Table 2). CC101.19, in contrast to D1/85.16, has 4 sequence changes in V3, which account for its CCR5 inhibitor resistance (Kuhmann et al., 2004). There was no detectable binding of MAb 39F to CC101.19 gp120, presumably because the V3 sequence changes destroy its epitope. However, both 447-52D and F425 did still bind CC101.19 gp120 (Table 2). The failure of these MAbs to neutralize CC101.19, and the failure of all three to neutralize D1/85.16, suggests that the alteration in how the resistant viruses recognize CCR5 does not involve a gross change in how the V3 region is exposed to antibodies on the native trimer.

Sensitivities of the CCR5 inhibitor-resistant isolates to neutralizing sera

To further characterize the neutralization phenotype of the resistant viruses, we assessed their sensitivities to sera from 15 different HIV-1-infected individuals, and to a preparation of HIVIg, again using a PBMC-based replication assay. Our general experience with this type of assay is that 50% neutralization titer differences of < 3 -fold are not meaningful, because of experimental variation. Applying this *ad hoc* cut-off, a subset of sera neutralized the CCR5 inhibitor-resistant viruses more potently than the CC1/85 parental and CCcon.19 passage control viruses. In general, D1/85.16 was the most neutralization sensitive isolate, albeit not profoundly so. More specifically, 6/16 sera neutralized D1/85.16 at higher than expected titers, whereas 2/16 sera did so against CC101.19 (Table 3). However, the increases were generally modest in magnitude (<10 -fold).

The CCR5 inhibitor-resistant isolates are cross-resistant to other small molecule CCR5 inhibitors but sensitive to inhibitors that act elsewhere in the HIV-1 replication cycle

Both the CC101.19 and D1/85.16 isolates were highly resistant to five different small molecule inhibitors: AD101, VVC, MVC, aplaviroc and CMPD-167 (Fig.2). This cross-resistance does not extend, however, to inhibitors that target CCR5 via a different mechanism, as we have previously reported that CC101.19 and D1/85.16 retain wild-type sensitivity to chemokines and MAbs that bind to CCR5 and inhibit HIV-1 replication (Pugach et al., 2007; Trkola et al., 2002).

To assess whether resistance to small molecule CCR5 inhibitors affects the response to other anti-retroviral drugs that act at other stages of the HIV-1 replication cycle, we used BMS-806, PRO-542 (CD4-IgG2) and enfuvirtide (T-20) as representatives of attachment/fusion inhibitors, zidovudine (AZT) and nevirapine as RTIs and atazanavir as a PI. Neither CC101.19 nor D1/85.16 differed by ± 2 -fold from CC1/85 and CCcon19 in their sensitivities to any of these inhibitors (Fig.2). The CCcon19 passage control virus was modestly (10-fold) more sensitive than the others to PRO-542 and, conversely, 4-fold less sensitive to BMS-806 (Fig. 2). These altered sensitivities to inhibitors of gp120-CD4 binding are consistent with the increase in CD4 affinity that occurred during the prolonged *in vitro* passage of this virus (Pugach et al., 2004).

Discussion

One goal of this study was to assess whether the clinical use of CCR5 inhibitors to treat HIV-1 infection might drive the virus down a resistance pathway that altered its sensitivity to plasma NABs. Both CCR5 inhibitors and NABs affect envelope glycoprotein functions, and both cause resistance mutations to emerge in these proteins. Hence the potential for reinforcing (or interfering) actions clearly exists (Moore and Doms, 2003). A particularly interesting scenario is that the principal resistance pathway for a CCR5 inhibitor might render HIV-1 more sensitive to neutralization by disrupting some of the natural defenses against NAb binding that have evolved in its Env protein complex. Another, related possibility is that the sequence changes producing a resistant virus also create neo-epitopes that drive the production of new NABs to which the virus is sensitive, thereby creating a new selection pressure. We have not yet addressed the latter eventuality; doing so will require testing whether any resistant viruses that emerge *in vivo* have altered sensitivities to longitudinal, autologous serum samples. We have, however, used two different small molecule CCR5 inhibitor-resistant viruses that were generated *in vitro*, to assess whether the acquisition of resistance is associated with altered sensitivity to MABs and sera from infected individuals. The outcome of these experiments is that there is indeed such a linkage, albeit not a dramatic one.

Our two resistant variants were both derived from the same R5 primary isolate, CC1/85, using two different CCR5 inhibitors, AD101 and VVC. Although the phenotypes of the two escape mutants are generally very similar, two different genetic pathways to resistance were followed. The AD101-resistant variant, CC101.19, has 4 sequence changes in V3 that appear to disrupt the binding interaction between this region of gp120 and ECL-2 of CCR5, rendering the escape mutant more dependent on the binding of the gp120 bridging sheet to the CCR5 N-terminus (our unpublished results). In contrast, the VVC-resistant virus D1/85.16 has no sequence changes in V3, and probably relies on other sequence changes to create a broadly similar rearrangement of the components of its CCR5 binding site (we are still in the process of determining which particular Env changes in D1/85.16 are responsible for resistance) (Marozsan et al., 2005). For both viruses, the eventual outcome of the genetic changes is the ability to use the inhibitor-CCR5 complex as well as free CCR5 for entry (Marozsan et al., 2005; Trkola et al., 2002).

The two resistant variants, CC101.19 and D1/85.16, were each moderately more sensitive to the CD4bs-associated MAb b12, but to a lesser extent than the passage control isolate CCcon. 19. This virus adapted to extended growth in PBMC culture by increasing its affinity for CD4 (Pugach et al., 2004), an outcome that also modestly affects its inhibition by PRO 542 (CD4-IgG2) and BMS-806 (Fig.2) In contrast, both escape mutants had unchanged sensitivities to CD4-IgG2 or BMS-806, so the principal driving force behind their slightly greatly susceptibilities to b12 is probably not related to changes in CD4 binding. However, sequence changes that arose during either the *in vitro* adaptation process or the development of CCR5 inhibitor resistance could be involved.

The D1/85.16 variant, but not CC101.19, was markedly (~50-fold) more sensitive to 2G12, a MAb that binds a discontinuous epitope comprising mannose moieties from at least three N-linked glycans (Sanders et al., 2002; Scanlan et al., 2002). There are no sequence changes in D1/85.16 that directly affect the known glycan contributors to the 2G12 epitope, but there is a G354P change in the C3 region adjacent to residue F353 that is known to influence 2G12 binding, albeit in the genetic context of HIV-1 JR-FL (Scanlan et al., 2002). Because 2G12 did not have a significantly higher affinity for gp120 derived from D1/85.16, the most likely explanation of the increased 2G12 sensitivity is an alteration in the conformation or accessibility of the 2G12 epitope on the functional Env trimer. Whether, and if so how, this is linked to CCR5 inhibitor resistance remains to be determined. However, because of the unique nature of the 2G12 MAb and its epitope, it seems unlikely that the increased sensitivity of a CCR5 inhibitor-resistant virus to 2G12 will have much relevance to what may arise during the clinical use of these drugs.

There were also modest increases in the sensitivity of the resistant viruses to the MPER-directed MAbs 4E10 and, in the case of D1/85.16, also to 2F5. The only sequence change in close proximity to the continuous epitopes for these MAbs was E662A in D1/85.16 (a change not present in CC101.19). This is a common polymorphism in the first position of the 2F5 epitope, E/ALDKWAS, that was also present in one clone from the CC1/85 parental isolate. It could account for the modest (~6-fold) increase in the 2F5 neutralization sensitivity of D1/85.16, particularly as 2F5 did not neutralize CC101.19 detectably. There is no obvious sequence-based explanation for why either escape mutant is moderately (>5-fold) more sensitive to 4E10, as is often the case (Binley et al., 2004). A reduction in the rate of fusion can increase sensitivity to neutralization by 2F5 and 4E10, by increasing the time available for these MAbs to bind to gp41 (Reeves et al., 2005). Hence we are now investigating if resistance to CCR5 inhibitors is associated with alterations in fusion rates.

Given how we believe the resistant viruses acquire their ability to use the CCR5-inhibitor complex, by becoming more dependent on the bridging sheet region for a binding interaction with the CCR5 N-terminus, perhaps the most interesting possibility for an alteration in their neutralization sensitivity would involve an increase in V3 exposure on the native Env trimer. *In vivo*, such an occurrence could render the escape mutant more sensitive to existing V3-directed antibodies and/or create a new, and perhaps more immunogenic, V3 configuration for the generation of NAbs specific to the escape mutant. The V3 region is normally poorly exposed on R5 primary viruses (Hartley et al., 2005; Moore et al., 1995); accordingly, none of the V3-directed MAbs we tested could significantly neutralize the parental CC1/85 virus, even when their epitope was present on the gp120 monomer. We found that neither escape mutant had acquired any sensitivity to the available V3 MAbs. The 4 sequence changes within V3 destroyed the epitope for MAb 39F on CC101.19, but the epitopes for two other MAbs, 447-52D and F425, were unaltered, and this was true of all three V3 MAbs on D1/85.16. The preservation of resistance to these V3 MAbs on the resistant viruses therefore implies that the V3-dependent and V3-independent changes that create CCR5 inhibitor resistance do not necessarily alter how V3 is exposed to antibodies on the functional Env trimer.

Some modest and sporadic increases in sensitivity of D1/85.16, but not CC101.19, to sera from HIV-1-infected people were noted. These changes, of course, reflect heterologous neutralization via unknown specificities, but they are generally consistent with the observations made using MAbs to known epitopes. A more relevant pattern of data will emerge as and when *in vivo*-generated escape mutants are tested using heterologous plasma. Nonetheless, the existing results do suggest that CCR5 inhibitor-resistant viruses are likely to be somewhat more sensitive to neutralization than their parental viruses, implying that the humoral immune system could indeed exert additional selection pressures during the escape process (Moore and Doms, 2003).

We also confirmed earlier reports that the acquisition of resistance to small molecule CCR5 inhibitors does not cause HIV-1 to become resistant to drugs with different mechanisms of action, such as RTIs, PIs and attachment/fusion inhibitors that act independently of CCR5 (Marozsan et al., 2005; Westby et al., 2007). Hence resistant variants that arise *in vivo* can still be treated with other drug classes. Moreover, the escape mutants were still sensitive to MABs and chemokines targeting regions of CCR5 that are spatially distinct from the binding site for the small molecule CCR5 inhibitors, as has been reported previously (Dragic et al., 2000; Pugach et al., 2007; Trkola et al., 2002; Tsamis et al., 2003). In principle, therefore, small molecule CCR5 inhibitors could therefore be usefully combined with CCR5 MABs that are now being evaluated clinically, particularly as the two categories of CCR5 inhibitor act synergistically to inhibit HIV-1 entry (Murga et al., 2006).

Methods

Antibodies and reagents

Sera from HIV-1-infected individuals were provided by Dr. Robert Doms (University of Pennsylvania, Philadelphia, PA) and Dr. Steven Wolinsky (Chicago MACS, Northwestern University, Chicago, IL). AZT, Nevirapine, Atazanvir, MAB 447-52D, MAB F425 and HIVIG were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. CD4-IgG2 (PRO-542), APL and T-20 were donated by Dr. William Olson (Progenics Pharmaceuticals Inc, Tarrytown, NY). MABs 2G12, 2F5, 4E10 were gifts from Dr. Hermann Katinger (Polymun Scientific Inc, Vienna, Austria), MAB b12 from Dr. Dennis Burton (Scripps Research Institute, La Jolla, CA) and MAB 39F from Dr. James Robinson (Tulane University, New Orleans, LA). VVC and AD101 were provided by Dr. Julie Strizki (Schering-Plough Research Institute, Kenilworth, NJ), CMPD 167 was from Dr. Marty Springer (Merck Research Labs, Rahway, NJ), BMS-378806 from Richard Colonna (Bristol Myers Squibb, Wallingford, CT) and MVC from Dr. Chris Hitchcock (Pfizer, Sandwich, UK).

Gp120 binding assays

Stocks of infectious HIV-1 were inactivated with 1% Nonidet-P40 (NP-40) detergent and used as a source of gp120 proteins for determination of antibody binding by ELISA. The gp120s were captured onto plastic plates coated with the sheep polyclonal antibody D7324 to the C-terminus of gp120, then the binding of MABs or CD4-IgG2 was detected as described elsewhere (Moore and Sodroski, 1996; Trkola et al., 1995). The background OD₄₉₀ signal (no gp120) was subtracted from the signal derived using gp120 at each input MAB concentration.

Virus production

Generation of HIV-1 isolates was described previously (Marozsan et al., 2005; Trkola et al., 2002). Clonal, replication-competent, chimeric NL4-3/*env* viruses were prepared by transfecting 15 µg of the full-length proviral plasmid into 293T cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

HIV-1 infection of primary CD4⁺ T cells

Mitogen-activated, CD8⁺ T cell-depleted PBMC were prepared from leukopacks pooled from the blood of 2 healthy volunteers. The pooled fractions were treated with the "RosetteSep CD8⁺ depletion cocktail" (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions, before Ficoll density gradient separation of leukocytes to deplete CD8⁺ T cells from the PBMC by rosette formation (Trkola et al., 2002). The cells were split into two cultures that were stimulated for three days with 5 µg/ml of phytohemagglutinin (PHA; Sigma) or surface-immobilized anti-CD3 MAB (clone OKT3). All PBMC cultures were

maintained in PBMC culture medium (RPMI 1640; Invitrogen) with 10% FBS, 1X PenStrep and 100 U/ml interleukin-2 (IL-2; ARRRP, donated by Hoffmann-La Roche, Inc.).

The activated PBMC from the two stimulation cultures were then pooled at a 1:1 ratio and seeded (2×10^5 per well) into a 96-well culture plate. The cells were then treated with inhibitors for 1h at 37°C before addition of 50 TCID₅₀ of a HIV-1 isolate. Production of the viral p24 antigen after 7–10 days of culture was quantified using an in-house ELISA (Trkola et al., 1995). In each assay, each data point was derived from triplicate wells. The amount of p24 produced was corrected by subtracting the residual p24 remaining from the inoculum virus. HIV-1 replication in the presence of inhibitors is expressed as a percentage of what occurred in their absence.

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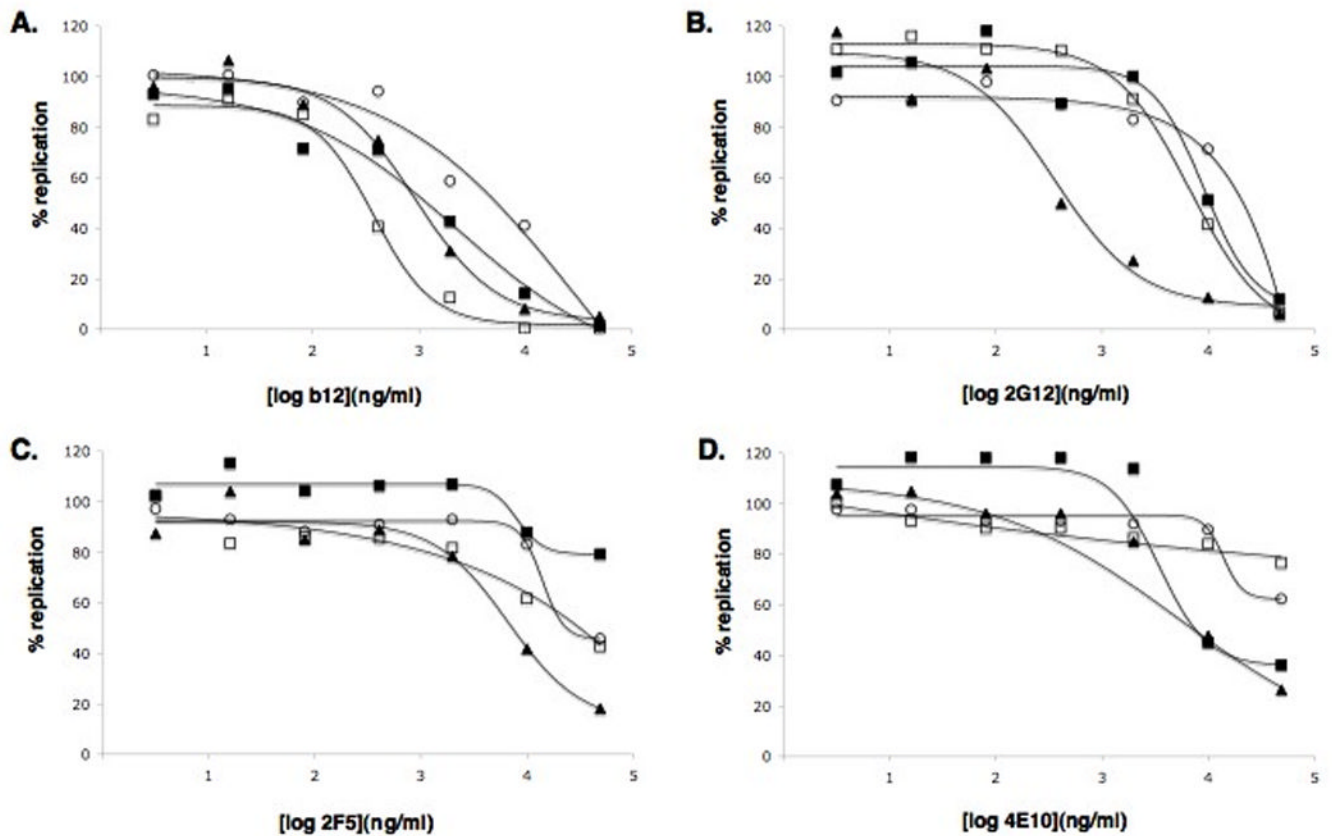


Figure 1. Inhibition of CCR5-inhibitor-resistant and control isolates by MABs
 HIV-1 isolates CC1/85 (open circles), CC101.19 (filled squares), D1/85.16 (filled triangles) and CCcon.19 (open squares) were tested for their sensitivity in primary CD4⁺ T cells to A) b12; B) 2F5; C) 2G12; D) 4E10. The extent of virus replication is presented as a percentage of p24 antigen production in the control culture (defined as 100%). The data presented represent average values derived from three independent experiments. Error bars representing standard errors of the mean have been omitted for clarity, but are typically $\pm 10\%$ of the mean value. Curve fitting was performed with nonlinear regression analysis using Prism software.

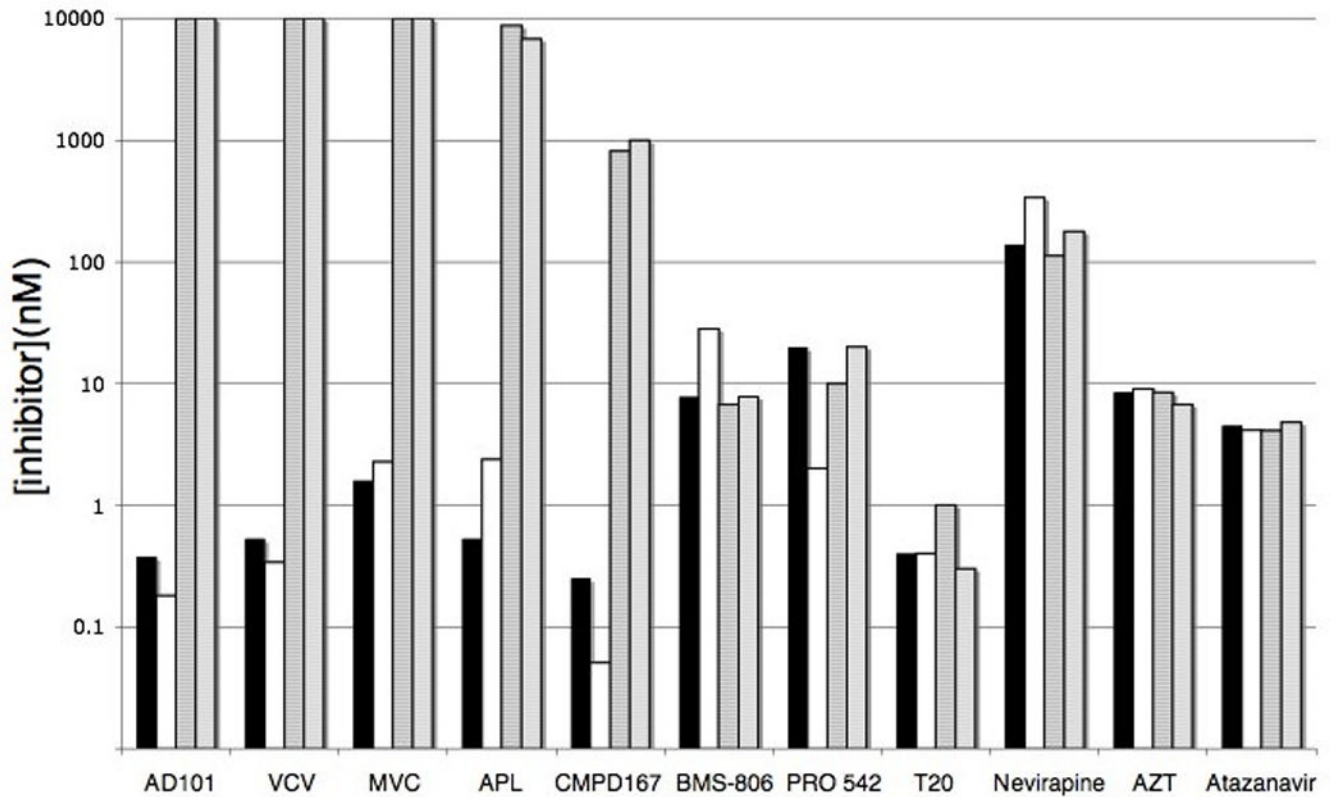


Figure 2. Sensitivity of CCR5-inhibitor-resistant and control isolates to inhibitors of HIV-1 replication

HIV-1 isolates CC1/85 (filled bars), CCcon19 (open bars), CC101.19 (hatched bars) and D1/85.16 (gray bars) were tested in primary CD4⁺ T cells for their sensitivity to the various inhibitors listed on the x-axis. The inhibitor concentrations required to inhibit replication by 50% are shown. The data presented represent average values derived from two independent experiments.

Table 1

Neutralization of the parental and CCR5 inhibitor-resistant viruses by MAbs

	CC1/85	CCcon.19	CC101.19	D1/85.16
b12	4.5	0.3	1.3	1.1
2G12	23	9.0	11	0.4
2F5	44	23	>50	7.7
4E10	>50	>50	9.3	5.2

The values recorded are the MAb concentrations ($\mu\text{g/ml}$) that reduce the replication of each test virus by 50% in a PBMC-based assay. The values highlighted in bold differ by > 5-fold for the resistant viruses compared to the parental and passage control isolates. The data presented represent average values derived from three independent experiments.

Table 2

MAB binding to gp120s from the parental and CCR5 inhibitor-resistant viruses

	CC1/85	CC101.19	D1/85.16
CD4-IgG2	0.08	0.12	0.06
2G12	0.19	0.24	0.09
b12	0.22	0.20	0.17
447-52D	0.29	0.37	0.30
F425	0.01	0.02	0.01
39F	0.01	N.A.	0.01

The values recorded are the MAb or CD4-IgG2 concentrations ($\mu\text{g/ml}$) that give 50% binding to gp120 proteins that were derived from the corresponding viruses by detergent treatment. N.A. = no detectable binding. The data presented represent average values of two independent wells at each antibody concentration and are the results from a representative experiment.

Table 3

Neutralization of CCR5 inhibitor and control isolates by sera from HIV-1-infected individuals

	CC1/85	CCcon.19	CC101.19	D1/85.16
HIVIG	270	185	285	600
20316	70	60	85	270
20422	120	110	215	455
20507	30	70	20	115
20451	320	200	160	530
20777	240	55	60	285
20207	800	2640	1900	2730
20394	1245	1870	8000	2550
20608	3800	2400	11800	3660
20479	1900	1630	3630	2240
20555	1730	3200	680	3130
20699	3725	1900	1815	3150
170	55	N.D.	150	340
214	75	N.D.	185	850
371	20	N.D.	40	110
494	80	N.D.	130	270

The values listed are the reciprocal serum dilutions that reduce virus production by 50% for each serum/virus combination. The sera designated 20XXX were obtained from the MACS cohort via Dr. Steven Wolinsky, the other four sera were provided by Dr. Robert Doms. The values highlighted in bold for the two VVC-resistant isolates CC101.19 and D1/85.16 differ by >3-fold from the mean values for the parental and passage control isolates CC1/85 and CCcon.19 (or, for the last 4 listed sera, from the value for CC1/85). N.D. = Not Done, due to a shortage of these sera. The data presented represent average values derived from two or three independent experiments, depending on the availability of the sera.