CC chemokine receptor 5 (CCR5) is a G-protein-coupled receptor for the chemokines CCL3, -4, and -5 and a coreceptor for entry of R5-tropic strains of human immunodeficiency virus type 1 (HIV-1) into CD4+ T-cells. We investigated the mechanisms whereby nonpeptidic, low molecular weight CCR5 ligands block HIV-1 entry and infection. Displacement binding assays and dissociation kinetics demonstrated that two of these molecules, i.e. TAK779 and maraviroc (MVC), inhibit CCL3 and the HIV-1 envelope glycoprotein gp120 binding to CCR5 by a noncompetitive and allosteric mechanism, supporting the view that they bind to regions of CCR5 distinct from the gp120- and CCL3-binding sites. We observed that TAK779 and MVC are full and weak inverse agonists for CCR5, respectively, indicating that they stabilize distinct CCR5 conformations with impaired abilities to activate G-proteins. Dissociation of [125I]CCL3 from CCR5 was accelerated by TAK779, to a lesser extent by MVC, and by GTP analogs, suggesting that inverse agonism contributes to allosteric inhibition of the chemokine binding to CCR5. TAK779 and MVC also promote dissociation of [35S]gp120 from CCR5 with an efficiency that correlates with their ability to act as inverse agonists. Displacement experiments revealed that affinities of MVC and TAK779 for the [35S]gp120-binding receptors are in the same range (IC50 ~6.4 versus 22 nM), although we found that MVC is 100-fold more potent than TAK779 for inhibiting HIV infection. This suggests that allosteric CCR5 inhibitors not only act by blocking gp120 binding but also alter distinct steps of CCR5 usage in the course of HIV infection.

HIV-1 entry into target cells requires sequential interactions of the surface subunit (gp120) of the viral envelope glycoprotein with cell surface CD4 and a G-protein-coupled receptor (GPCR) acting as a coreceptor, namely either CC chemokine receptor 5 (CCR5) or CXC chemokine receptor 4 (CXCR4) (1–4). The role of CCR5 as a HIV co-receptor was originally suggested after the identification that the CCR5 chemokines macrophage inflammatory protein 1α (MIP1α)/CCL3, MIP1β/CCL4, and regulated upon activation, normal T-cell expressed and secreted (RANTES)/CCL5 act as HIV-suppressive factors (5, 6). CCR5-using viruses (R5 viruses) are predominantly transmitted and persist throughout infection, whereas variants that use CXCR4 (X4 or R5/X4 viruses) emerge late in infection in a number of infected individuals (7). Additionally, individuals carrying the CCR5 Δ32 allele, which results in reduced receptor expression at the cell surface, resist infection without any obvious harmful effects on health (8). In view of these data, much research effort has been devoted to develop CCR5 ligands with antiviral properties, including chemically modified chemokines (9, 10), monoclonal antibodies (11, 12), and in more recent years a new class of nonpeptidic, low molecular weight compounds with potential for oral availability. Members of this latter class of molecules include vicriviroc (SCH-D, SCH 417690), which is currently under late phase clinical trials (13), and maraviroc (UK-427857, marketed as Selzentry® or Celsentry® (Pfizer) and hereafter referred as MVC), which recently received approval for use in the treatment of patients harboring only R5 viruses (14–16).

Nonpeptidic small CCR5 ligands prevent gp120 from binding to CCR5 and inhibit HIV entry, but the molecular mechanisms by which they act as antiviral molecules remain incompletely solved. These entry inhibitors lack agonist activity, and as such do not promote CCR5 internalization, and antagonize signaling and endocytosis of the receptor mediated by chemokines (17–21). Mutational studies and molecular modeling have proposed that these molecules do not interact with the regions of CCR5 involved in the binding of gp120 and chemokines, particularly the N-terminal domain and the second extracellular loop (ECL2) (22, 23), but lodge in a hydrophobic cavity located between the transmembrane domains of the receptor (24–28), thus suggesting that these molecules mediate their inhibitory effects on gp120 binding and chemokine functions by an allosteric mechanism, i.e. by inducing conformational changes of CCR5. Further supporting the allosteric nature of these molecules are data showing that they can inhibit their inhibitory effects on gp120 binding and chemokine functions by an allosteric mechanism, i.e. by inducing conformational changes of CCR5. Further supporting the allosteric nature of these molecules are data showing that they can inhibit triphosphate; Gpp(NH)p, 5′-guanylyl imidodiphosphate; PBMC, peripheral blood mononuclear cell.
duce differential alterations of distinct receptor functions (18, 19, 21). In other words, members of this class of molecules prevent some receptor functions but not others. This has clearly been highlighted for aplaviroc, which permits simultaneous binding of CCL5 to CCR5 and CCL5-mediated chemotaxis and receptor endocytosis (18, 21), but fully suppresses CCL5-mediated intracellular calcium release. In contrast to CCL5, aplaviroc blocks the binding of other chemokines to CCR5, thus indicating that this inhibitor is probe-dependent, which is considered a characteristic feature of allosteric modulators (19, 21). In line with this, small molecule CCR5 inhibitors have been found to alter differently the binding of different monoclonal antibodies to the receptor (18).

As compared with chemokines, allosteric interactions between gp120 and the small molecule CCR5 inhibitors have not been formally demonstrated. Also, data remain scarce on which CCR5 conformations, to which gp120 would not bind, are induced by these inhibitors. We and others have previously demonstrated that some of these molecules behave as inverse agonists for CCR5, thus suggesting that they stabilize CCR5 in inactive conformations that are uncoupled from G-proteins (29, 30), but whether or not all members of this class of molecules share this property is not well documented. In fact, viruses showing resistance to some inhibitors retain susceptibility to others (31), thus suggesting that, although all of these molecules locate in the same binding pocket, their effects on CCR5 conformation differ to some extent. Inverse agonism might take part in inhibitor-mediated blocking of chemokine binding to CCR5, as suggested by a recent work showing that coupling to G-proteins is necessary for CCR5 to bind CCL4 with high affinity (32). To what extent inverse agonism at CCR5 could also alter gp120 binding and/or other entry-associated events and thus contribute to antiviral activity is not known. However, gp120-mediated intracellular signaling through CCR5 has been reported (33–35), thus opening the possibility that G-proteins coupled to CCR5 may play a role in gp120 binding to the receptor and more generally in HIV infection.

In this study, we have investigated the mechanisms whereby TAK779, which was the first small molecule CCR5 inhibitor to be described (17), and MVC exert their antiviral activity. Radioligand dissociation and displacement binding assays demonstrated that the inhibitors produce an allosteric inhibition of gp120 and chemokine binding to CCR5. Interestingly, those inhibitors showed inverse agonism, whose relative efficacy correlated with their capacity to dissociate gp120 from CCR5 but not with their antiviral potency, thus making it likely that allosteric blocking of HIV-1 infection by small molecule CCR5 inhibitors does not rely only on their ability to decrease affinity of gp120 for CCR5.

**EXPERIMENTAL PROCEDURES**

**Materials, Cells, and cDNA Constructs**—The human recombinant chemokine [125I]MIP1α/CCL3 (specific activity 2200 Ci/mmoll) was purchased from PerkinElmer Life Sciences. MIP1β/CCL4 was provided by Dr. F. Baleux (Institut Pasteur, Paris, France). Recombinant soluble human CD4 (sCD4) and TAK779 were obtained from the AIDS Research and Reference Reagent Program catalog of the National Institutes of Health (Bethesda). MVC was kindly provided by Pfizer (Sandwich, UK). Twenty mm stock solutions of TAK779 and MVC were prepared in DMSO and stored at −80 °C before use. [35S]GTPγS was purchased from Amer-sham Biosciences. Gpp(NH)p was obtained from Sigma. The anti-CCR5 mAb 45531 was obtained from R&D Systems (Minneapolis, MN).

Human peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of healthy blood donors and were purified by centrifugation through a Ficoll-Hypaque density gradient (Pharmacia Corp., Peapack, NJ). PBMCs were maintained for 2 days in RPMI 1640 medium containing IL-2 (300 IU/ml) and phytohemagglutinin (5 μg/ml), and then for additional 5 days in the presence of IL-2 alone. HEK 293T or U87 cells expressing CCR5 and/or CD4 were cultured in DMEM (Invitrogen) supplemented with 10% FCS, 2 mM l-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. The WT-CCR5- and R126N-CCR5-expressing HEK 293T cells used in this study were described previously (30). Transient expression of CD4 in CCR5-expressing HEK 293T cells was performed using the calcium phosphate-DNA coprecipitation method of transfection.

**Production of Soluble Monomeric [35S]gp120**—The gp120 coding sequence from the R5-tropic, HIV-1 primary strain Bx08 was subcloned from plasmid p133.3 (36) into the Semliki forest virus-derived expression vector pSFV2 (37). Recombinant defective Semliki forest virus particles (SFVgp120Bx08) were produced as described previously (38). Soluble gp120 was produced by infection of baby hamster kidney cells by SFVgp120Bx08. At 6 h post-infection, culture medium (BHK-21 Glasgow’s modified Eagle’s medium with 5% FCS, 1% penicillin/streptomycin, 20 mM HEPES, and 10% tryptose phosphate broth) was replaced by 0% FCS methionine/cysteine-free DMEM (ICN Biochemicals) prior to addition of 200 μCi/ml [35S]cysteine and -methionine (EasyTag Express 35S, PerkinElmer Life Sciences). Synthesis of soluble, monomeric 35S-labeled gp120 was continued up to 24 h post-infection. The culture medium was then clarified and concentrated using a VivaSpin 20 column (30-kDa molecular mass cutoff, Sartorius) prior to dialysis against PBS. [35S]gp120 concentration was determined by Western blot analysis using gp140MN/Lai as a standard and the mouse anti-gp120 (V3) mAb K24 (38).

**Membrane Preparation**—Crude membranes from parental, WT-, or R126N-CCR5-expressing HEK 293T cells were prepared as described previously (30). Briefly, cells were incubated for 30 min at 4 °C in buffer A (15 mM Tris–HCl, pH 7.5, 2 mM MgCl2, 0.3 mM EDTA, and 1 mM EGTA), disrupted using a Dounce homogenizer (Kontes), and centrifuged at 500 × g for 5 min at 4 °C. Postnuclear supernatants were ultracentrifuged at 40,000 × g for 30 min at 4 °C. Membrane pellets were washed once in buffer A, before being resuspended in buffer B (75 mM Tris–HCl, pH 7.5, 12.5 mM MgCl2, 0.3 mM EDTA, 1 mM EGTA, 250 mM sucrose). Membrane aliquots at a protein concentration of 1 mg/ml were stored at −80 °C until use.

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Radioligand Binding Assays—Equilibrium saturation binding of \([^{35}S]\text{CCL}3\) to membranes from CCR5-expressing HEK 293T cells was performed in Minisorp tubes (Nunc, Rochester, NY). Samples containing 1 \(\mu\)g of membrane proteins and increasing concentrations of \([^{125}I]\text{CCL}3\) were incubated for 90 min at room temperature in a 0.1-ml final volume of binding buffer (50 \(\text{mM}\) HEPEs, pH 7.4, 1 \(\text{mM}\) CaCl$_2$, 5 \(\text{mM}\) MgCl$_2$, 0.5% BSA). Nonspecific binding was measured in the presence of 10 \(\mu\)M of either MVC or TAK779 or using membranes from parental HEK 293T cells. Unbound \([^{125}I]\text{CCL}3\) was removed by filtering membranes through GF/B filters presoaked in 1% BSA and washing them with 12 ml of binding buffer. Filters were counted in a gamma counter (multi-crystal LB 2111 gamma counter, Berthold Technologies). For saturation binding experiments of \([^{35}S]\text{gp}120\), 20 \(\mu\)g of membrane proteins per tube were used in binding buffer containing 1% BSA, increasing concentrations of \([^{35}S]\text{gp}120\), and a saturating concentration of sCD4 (200 \(\text{nM}\)). Membranes were filtered through GF/B filters and washed with 12 ml of washing buffer (50 \(\text{mM}\) HEPEs, pH 7.4, 1 \(\text{mM}\) CaCl$_2$, 5 \(\text{mM}\) MgCl$_2$, 500 \(\text{mM}\) NaCl). Filters were incubated for 48 h in Optiphase scintillation liquid (PerkinElmer Life Sciences) and then counted in a Wallac 1450 MicroBeta TriLux\textsuperscript® (PerkinElmer Life Sciences). Binding of either \([^{125}I]\text{CCL}3\) or \([^{35}S]\text{gp}120\) to intact CCR5-expressing HEK 293T cells was conducted as follows. Cells (3 \(\times\) 10$^6$ or 1 \(\times\) 10$^6$, respectively) were incubated for 90 min at room temperature in 0.1 ml of assay buffer (50 \(\text{mM}\) HEPEs, pH 7.4, 5 \(\text{mM}\) MgCl$_2$, 1 \(\text{mM}\) CaCl$_2$, 0.1% Na$_2$HPO$_4$) containing increasing concentrations of the radioligands. Binding of \([^{35}S]\text{gp}120\) was performed in the presence of 200 \(\text{nM}\) sCD4. Nonspecific binding was determined with 10 \(\mu\)M MVC. To remove unbound radioactivity, cells were pelleted at 16,000 \(\times g\) for 10 s and washed once. Bound radioactivity was measured in the LB 2111 gamma counter or the Wallac 1450 MicroBeta TriLux\textsuperscript®. For displacement experiments of radioligand binding, membranes were incubated with either \([^{125}I]\text{CCL}3\) or \([^{35}S]\text{gp}120\) (in the presence of sCD4, unless noted) at the indicated concentrations and increasing concentrations of unlabeled gp120-sCD4 complexes, CCL4, TAK779, or MVC, in the presence or in the absence of 10 or 100 \(\mu\)M Gpp(NH)p. Experiments were carried out in either 96-well basic FlatPlates or Minisorp tubes, with similar results. Incubations were run for 2 (CCL4 and gp120) or 4 h (TAK779 and MVC) at room temperature. Analysis of the data was made using the Prism software (GraphPad Software Inc., San Diego).
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FIGURE 1. $^{[125]I}$CCL3 and $^{[35]S}$gp120 show different binding properties to CCR5. a, equilibrium saturation binding of $^{[125]I}$CCL3 to CCR5. Total binding of increasing concentrations of $^{[125]I}$CCL3 to membranes from CCR5-expressing HEK 293T cells was determined at room temperature for 90 min in assay buffer (see “Experimental Procedures” for details). Specific binding of the chemokine to CCR5 was obtained by subtracting from total binding the nonspecific binding, which was measured using membranes from nontransduced HEK 293T cells. Data were fitted to a one-site binding model. A representative experiment out of four performed in duplicate is shown. Inset, Scatchard transformation of the binding data. b and F refer to specifically bound and free $^{[125]I}$CCL3, respectively. b, equilibrium saturation binding of $^{[35]S}$gp120 from the Bx08 HIV-1 strain was carried out as in a in the presence of 200 nM soluble CD4. Nonspecific binding varied from 20 to 50% of total binding over the range of the $^{[35]S}$gp120 concentrations used. Data were fitted with the one-site model. The inset represents the Scatchard analysis of the binding data. A representative experiment out of four is shown. c, competition of 0.08 nM $^{[125]I}$CCL3 binding to CCR5-expressing membranes by increasing concentrations of unlabeled gp120-sCD4 complexes. Results were normalized for nonspecific binding (0%), which represented 14% of total binding, and specific binding in the absence of competitor (100%) and were fitted according to a one-site competitive binding model. d, specific binding of increasing concentrations of $^{[125]I}$CCL3 or $^{[35]S}$gp120 (inset) to CCR5-expressing HEK 293T cells is shown. Experiments with $^{[35]S}$gp120 were performed in the presence of 200 nM soluble CD4. Nonspecific binding was determined in the presence of MVC at 10 μM. Data were fitted to a one-site binding model. Representative experiments out of three performed in duplicate are shown. e, the panel shows the effects of Gpp(NH)p, TAK779 (10 μM), or MVC (10 μM) on the total binding of either 10 nM $^{[35]S}$gp120 (in the presence of 50 nM sCD4) or 0.2 nM $^{[125]I}$CCL3 to CCR5-expressing membranes. Gpp(NH)p was used at a final concentration of 10 μM (for $^{[125]I}$CCL3) or 100 μM ($^{[35]S}$gp120). Hatched bars represent the specific binding of either 10 nM $^{[35]S}$gp120 (+ 30 nM sCD4) (left) or 0.08 nM $^{[125]I}$CCL3 (right) to intact CCR5-expressing HEK 293T cells incubated (light bars) or not (dark bars) overnight with pertussis toxin (PTX, 100 ng/ml). Values represent means ± S.E. of triplicate determinations from representative experiments. **, p < 0.01; ***, p < 0.001 as compared with untreated controls in unpaired two-tailed Student’s t test. f, competition of 10 nM $^{[35]S}$gp120 binding to CCR5-expressing membranes by increasing concentrations of CCL4 in the absence (filled circles) or in the presence (open circles) of 100 μM Gpp(NH)p. Binding was performed in the presence of 50 nM sCD4. Results were normalized for nonspecific binding (0%), which represented 30% of total binding, and specific binding in the absence of CCL4 (100%). The data in the presence of Gpp(NH)p were best fitted according to a two-site competitive binding model (F value = 17.4, p = 0.001). Fitting of the data in the presence of Gpp(NH)p was performed according to a one-site competitive binding model (dotted line) and was not further improved by the two-site model. A representative experiment out of two independent experiments run in duplicate is shown.

RESULTS

$^{[125]I}$MIP-1α/CCL3 and $^{[35]S}$gp120 Show Different Binding Properties to CCR5: the Influence of G-protein Coupling—CCR5 was stably expressed in HEK 293T cells using a lentivirus-based strategy, as reported previously (30, 42). Receptor expression level was first assessed by means of equilibrium saturation binding experiments of either the chemokine $^{[125]I}$MIP-1α/CCL3 (Fig. 1a) or the $^{[35]S}$gp120 from the R5-tropic HIV-1 primary strain Bx08 (Fig. 1b) to crude membranes from the CCR5-expressing HEK 293T cells. Specific binding of the chemokine could be described as a hyperbolic function, from which we determined an equilibrium dissociation constant ($K_d$) value of 0.41 ± 0.07 nM and a maximum number of binding sites ($B_{max}$) of 6.7 ± 0.2 pmol/mg of protein. Scatchard transformation of the binding data were a straight line (Fig. 1a, inset), thus further confirming that $^{[125]I}$CCL3 binds to a single class of receptors. Binding experiments of $^{[35]S}$gp120 conducted in the presence of an excess of soluble CD4 (sCD4, 200 μM) also revealed a single class of saturable binding sites displaying a $K_d$ value of 9.9 ± 1.2 nM, in agreement with the reported affinities of other R5-tropic envelopes for CCR5 (43, 44). The maximum number of binding sites for $^{[35]S}$gp120 ($B_{max}$ = 1.4 ± 0.7 pmol/mg of protein) was consistently lower than that for $^{[125]I}$CCL3, thus suggesting that gp120 does not interact with some of the $^{[125]I}$CCL3 binding receptors. To directly investigate this possibility, we then measured displacement of $^{[125]I}$CCL3 binding to CCR5 by increasing concentrations of unlabeled gp120-sCD4 complexes. The results in Fig. 1c reveal that gp120 displaced binding of all $^{[125]I}$CCL3 molecules, however, with a
Kᵢ value of 1 order of magnitude higher than its Kᵦ value deduced from the saturation binding assays (Kᵦ = 103 ± 18 nM). This indicates that crude HEK 293T membranes contain [125I]CCL3 binding receptors, which are of low affinity for gp120. Interestingly, saturation binding experiments that were repeated in intact cells demonstrated that both radioligands bind to a comparable number of receptors (Bₘax = 220,274 ± 16,494 versus 177,507 ± 25,546 receptors/cell for [125I]CCL3 and [35S]gp120, respectively, Fig. 1d), suggesting that these low affinity receptors for gp120 represent intracellular receptors that are not yet mature. Importantly, [125I]CCL3 and [35S]gp120 bind CCR5 on intact cells with Kᵦ values similar to those found when using crude membranes (Kᵦ = 0.9 ± 0.3 and 8.4 ± 1.1 nM for [125I]CCL3 and [35S]gp120, respectively, Fig. 1d). This finding strongly suggests that for each of the two radioligands, high affinity binding either to membranes or to intact cells is mediated by a same fraction of receptors.

Formation of active receptor conformational states with high affinity for agonists is recognized to be promoted by receptor coupling to nucleotide-free G-proteins (45, 46). Therefore, we repeated the binding experiments with crude membranes in the presence of Gpp(NH)p, a nonhydrolysable GTP analog that binds to α-subunits of activated G-proteins and then uncouples receptors permanently from G-proteins. As shown in Fig. 1e, Gpp(NH)p strongly decreased [125I]CCL3 binding to CCR5, thus confirming previous results showing that G-protein coupling is needed for high affinity binding of chemokines to the receptor (32). In striking contrast, [35S]gp120 binding was unaffected by Gpp(NH)p, thus indicating that the viral envelope binds to CCR5 independently of the receptor coupling to G-proteins. We obtained similar results in intact cells that were pretreated overnight with pertussis toxin at 100 ng/ml (Fig. 1e). To further confirm these findings, we performed competitive binding experiments in which binding of [35S]gp120 to CCR5-expressing membranes was measured in the presence of increasing concentrations of the unlabeled CCR5 agonist MIP18/CCL4 (Fig. 1f). Displacement of [35S]gp120 binding by CCL4 gave rise to a biphasic curve, thus indicating the presence of two distinct classes of [35S]gp120 binding receptors with high (Kᵦ = 1.2 ± 0.6 nM) and low (Kᵦ = 224 ± 120 nM) affinities for the chemokine.

Receptors of high affinity for CCL4 represented 38 ± 5% of total gp120-binding sites. Addition of Gpp(NH)p resulted in a monophasic curve representing the low affinity state (Kᵦ = 86 ± 11 nM). Altogether, these results indicate that high affinity binding of chemokines requires CCR5 coupling to G-proteins, whereas gp120 most probably binds equally well to both G-protein-uncoupled and -coupled receptors and to this regard behaves as a neutral antagonist for CCR5.

**FIGURE 2. TAK779 and MVC Are Inverse Agonists for CCR5 That Stabilize Different Receptor Conformations**—Using a [35S]GTPγS binding-based assay, we found that stimulation of CCR5 by CCL4 led to a dose-dependent activation of G-proteins (Fig. 2a). As we demonstrated previously (30), basal [35S]GTPγS binding to CCR5-expressing membranes was also increased, compared with that to membranes from parental cells (data not shown) or cells expressing the R126N-CCR5 mutant receptor, which is defective in its ability to activate G-proteins (Fig. 2, a and b) (data not shown). This indicates that a fraction of CCR5 converts spontaneously from inactive to active conformations and constitutively activates G-proteins even in the absence of agonist. Exposure of CCR5-expressing membranes to TAK779 resulted in a dose-dependent decrease in basal [35S]GTPγS binding (Fig. 2b). At high concentrations of TAK779, constitutive activity of CCR5 was no longer apparent, thus indicating that TAK779 is a full inverse agonist for CCR5. MVC also reduced basal [35S]GTPγS binding to CCR5-expressing membranes, but less efficiently than TAK779. Indeed, even at the highest concentrations, MVC only weakly inhibits constitutive activity of CCR5, thus indicating that MVC acts as a partial inverse agonist for CCR5. Together, the results suggest that TAK779 and MVC stabilize distinct conformations of CCR5, which exhibit different abilities to activate G-proteins. In particular, TAK779 would be much more effective than MVC at stabilizing the G-protein-uncoupled inactive states of CCR5.

We therefore hypothesized that TAK779, and perhaps to a lesser extent MVC, might display increased affinity for G-protein-uncoupled receptors. To directly address this issue, we performed competition experiments between [125I]CCL3 and increasing concentrations of either MVC or TAK779, in
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FIGURE 3. TAK779 and MVC accelerate dissociation of \([^{125}\text{I}]\text{CCL3}\) from CCR5. a, CCR5-expressing membranes (13 \(\mu\)g of proteins) from HEK 293T cells were incubated with 0.3 nM \([^{125}\text{I}]\text{CCL3}\) in 1.3 ml of binding buffer for 1 h at room temperature. Membranes were then centrifuged to remove unbound \([^{125}\text{I}]\text{CCL3}\). Fifteen percent of the amount of \([^{125}\text{I}]\text{CCL3}\) added initially remained attached to the membrane pellet. Equal aliquots of membranes (1 \(\mu\)g of proteins) were then resuspended in either 0.1 ml (i.e. 6-fold dilution of \([^{125}\text{I}]\text{CCL3}\) of buffer alone (open circles) or containing 250 nM of the anti-CCR5 mAb 45531 (filled diamonds) or in 2.5 ml (i.e. 150-fold or infinite dilution of \([^{125}\text{I}]\text{CCL3}\) of buffer alone (open squares) or containing 1 \(\mu\)g of either MVC (filled squares) or TAK779 (filled circles). Results are expressed as the ratio between the amount of \([^{125}\text{I}]\text{CCL3}\) bound to membranes at the indicated dissociation time (\(B_t\)) and that bound to membranes at time 0 (\(B_0\)) and are plotted on a logarithmic scale over the dissociation time (in hours). Data points represent means \(\pm\) S.E. of four determinations from two independent experiments out of at least three. Results were best fitted as a two-phase exponential decay model (\(F = 82, 151, 58, \) and 72 for dissociation in 2.5 ml of buffer alone or in the presence of the 45531 mAb, MVC, or TAK779, respectively; \(p < 0.001\)). b, effect of Gpp(NH)p on the dissociation rate of \([^{125}\text{I}]\text{CCL3}\) from CCR5. Dissociation of the chemokine was initiated by dilution in a 150-fold volume of buffer in the absence (filled circles) or in the presence (open circles) of 50 \(\mu\)M Gpp(NH)p. A representative experiment out of two is shown.

the presence or in the absence of Gpp(NH)p (Fig. 2c). We found that MVC and \([^{125}\text{I}]\text{CCL3}\) competed for binding to a single class of binding sites. The IC\(_{50}\) value for half-maximal inhibition of the chemokine binding was 0.9 \(\pm\) 0.4 nm. This value is in the same range as the \(K_p\) value of MVC determined in saturation binding assays with tritiated MVC (our data not shown and Ref. 47), in agreement with a previous work (21).

In the presence of Gpp(NH)p, the IC\(_{50}\) value for MVC decreased 3-fold (IC\(_{50}\) = 0.3 \(\pm\) 0.1 nm), although the difference did not reach statistical significance (\(p = 0.28\) in unpaired, two-tailed Student’s \(t\) test). This suggests that CCR5 uncoupling from G-proteins increases only slightly the MVC binding to the receptor. Regarding the experiments with TAK779, the results were best described by a model involving two classes of \([^{125}\text{I}]\text{CCL3}\)-binding sites displaying high (IC\(_{50}\) = 2 \(\pm\) 1 pm) and low (IC\(_{50}\) = 19 \(\pm\) 7 nm) affinities for the inhibitor. It should be noted that the IC\(_{50}\) value for the low affinity receptors is in the same range as the equilibrium dissociation constant for TAK779-CCR5 interactions reported previously (21). Addition of Gpp(NH)p substantially enhanced the ability of TAK779 to compete for the binding of \([^{125}\text{I}]\text{CCL3}\). Indeed, the nucleotide increased the proportion of receptors that are of high affinity for TAK779 from 25.1 \(\pm\) 2.7 to 68.6 \(\pm\) 5.1% of total \([^{125}\text{I}]\text{CCL3}\)-binding sites (\(p = 0.0035\)), thus suggesting that these receptors represent G-protein-uncoupled receptors. At the same time, Gpp(NH)p improved somewhat the binding of TAK779 to low affinity receptors (IC\(_{50}\) = 8.5 \(\pm\) 4 nm).

Collectively, these results are consistent with the idea that the full inverse agonist TAK779 binds preferentially to, and thus stabilizes, G-protein-uncoupled inactive conformations of CCR5. In contrast, binding of MVC seems to be less sensitive to receptor coupling to G-proteins, thus explaining why this molecule behaves as a weak inverse agonist for CCR5.

TAK779 and MVC Enhance Dissociation of \([^{125}\text{I}]\text{CCL3}\) from CCR5—We next investigated the molecular mechanisms by which TAK779 and MVC inhibit \([^{125}\text{I}]\text{CCL3}\) binding to CCR5-expressing membranes. In particular, we asked whether inverse agonism of each of these molecules contributes to binding inhibition of the chemokine. Allosteric compounds have been characterized by their ability to change the dissociation rate of orthosteric radioligands from receptors (48). For those experiments, dissociation could be initiated either by extensive (also termed “infinite”) dilution of the radioligand or by addition of an excess of unlabeled competitive ligands, such that minimal reassociation of the dissociated radioligand occurs. We studied the effects of TAK779 and MVC on the rate of dissociation of \([^{125}\text{I}]\text{CCL3}\) from CCR5 by the method of infinite dilution (Fig. 3). We first allowed \([^{125}\text{I}]\text{CCL3}\) to bind to CCR5-expressing membranes at equilibrium, then washed the membranes to remove unbound radioligand, and initiated \([^{125}\text{I}]\text{CCL3}\) dissociation by a 150-fold dilution of the chemokine in dissociation buffer alone or containing an excess concentration of unlabeled ligands.

Then, the amount of \([^{125}\text{I}]\text{CCL3}\) that remained bound to CCR5 was counted at different time points (Fig. 3a). We found that infinite dilution of \([^{125}\text{I}]\text{CCL3}\) accelerated dissociation of the chemokine from CCR5, as compared with that measured after dilution in a smaller volume of buffer in which dissociated \([^{125}\text{I}]\text{CCL3}\) could reassociate to free CCR5. Addition in the dissociation buffer of an excess concentration of a competitive ligand such as the anti-CCR5 mAb 45531 that binds to ECL2 of CCR5 did not change the dissociation rate of \([^{125}\text{I}]\text{CCL3}\), thus indicating that the conditions of infinite dilution was fulfilled and that the maximal dissociation rate of the chemokine was achieved under these conditions.

We found that dissociation of \([^{125}\text{I}]\text{CCL3}\) was best described by a two-phase exponential decay function, thus revealing that a proportion of the chemokine was dissociated rapidly (\(k_{\text{off fast}} = 2.8 \pm 0.1 \text{ h}^{-1}\)), whereas the rest was dissociated with a slower rate (\(k_{\text{off slow}} = 0.23 \pm 0.02 \text{ h}^{-1}\)). The ratio \(k_{\text{off slow}}/k_D\) yielded a \(k_{\text{on}}\) value of 1.6 \(\times\) 10\(^5\) M\(^{-1}\) s\(^{-1}\), which is within the range of on-rate constant values for peptide-receptor interactions (49). Addition of Gpp(NH)p in the dissociation buffer enhanced the dissociation rate of \([^{125}\text{I}]\text{CCL3}\) from CCR5 (Fig. 3b), as mentioned previously (32), suggesting that fast dissociating \([^{125}\text{I}]\text{CCL3}\) represents the fraction of chemokines that dissociated from G-protein-uncoupled receptors, which are in an agonist low affinity state. Accordingly, addi-
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Using [125I]CCL3 as a radioactive tracer, we found that the IC50 values are not statistically changed within the S.E. of at least three independent experiments. A one-way analysis of variation showed that the IC50 values are not statistically changed with increases of the [35S]gp120 concentration.

TAK779 or MVC (Fig. 4)

Figure 4. TAK779 and MVC displace [35S]gp120 from binding to CCR5 by a noncompetitive mechanism. a, inhibition of 10 nM [35S]gp120 binding to CCR5-expressing membranes from HEK 293T cells by increasing concentrations of either TAK779 (circles) or MVC (squares), in the absence (filled symbols, solid lines) or in the presence (open symbols, dotted lines) of 100 μM Gpp(NH)p. Binding was performed in the presence of 50 nM sCD4. Results were normalized for nonspecific (0%) and specific binding in the absence of inhibitor (100%). Experiments were fitted to a one-site competitive binding model. A representative experiment out of three independent experiments performed in duplicate is shown. b, and c, displacement of [35S]gp120 binding by TAK779 (b) and MVC (c) for various concentrations of [35S]gp120-sCD4 complexes (see panels for concentrations). Experiments were carried out as in a, d, relationship between the observed IC50 values for TAK779 (circles) or MVC (squares) displacement of [35S]gp120 binding and initial concentration of [35S]gp120-sCD4 complexes. Results represent means ± S.E. of at least three independent experiments. A one-way analysis of variance showed that the IC50 values are not statistically changed with increases of the [35S]gp120 concentration.

TAK779 and MVC Inhibit gp120 Binding to CCR5 by a Noncompetitive Mechanism—We next addressed the issue of the mechanisms by which TAK779 and MVC inhibit HIV entry into cells. For that purpose, we first performed competition experiments in which binding of [35S]gp120 was measured in the presence of increasing concentrations of either TAK779 or MVC (Fig. 4a). In contrast to what we observed using [125I]CCL3 as a radioactive tracer, we found that TAK779 and gp120 competed for binding to a single class of receptors, whose apparent affinity (IC50 = 22 ± 9 nM) for the inhibitor was not modified by Gpp(NH)p. This further confirms that gp120-bound receptors are primarily uncoupled from G-proteins. The same pattern was observed for the binding of MVC. The IC50 values obtained with MVC were slightly lower than those of TAK779 (IC50 = 6.4 ± 3 nM), thus suggesting that MVC binds more efficiently to gp120-binding receptors than TAK779.

We next performed competition experiments with TAK779 or MVC using increasing concentrations of [35S]gp120-sCD4 complexes from 5 to 50 nM (Fig. 4, b and c). Indeed, it has been reported that the relationship between the observed IC50 value and the concentration of radioactive tracer in displacement experiments can inform whether the inhibition of binding is competitive (i.e. displaceable) or noncompetitive (21). The IC50 value is expected to increase linearly with the radioligand concentration for competitive inhibition, whereas an unchanged IC50 value may indicate that noncompetitive inhibition occurs and, if so, is an estimate of the equilibrium dissociation constant value of the inhibitor-receptor complex (21). We found that the IC50 values do not change with increases of the concentration of radioactive gp120 (Fig. 4d), thus indicating that TAK779 and MVC inhibit gp120 binding by means of a noncompetitive mechanism.

TAK779 and MVC Enhance Dissociation of [35S]gp120 from CCR5—To assess the hypothesis that gp120 from the Bx08 HIV-1 strain and the entry inhibitors TAK779 and MVC bind to distinct and nonoverlapping sites on CCR5, we carried out dissociation experiments using [35S]gp120 as a radioactive tracer. CCR5-expressing membranes were incubated with 10 nM [35S]gp120 in the presence of sCD4 (20 nM), washed, and then resuspended in buffer with or without added molecules. The supplemental Fig. S1 shows that addition of sCD4 at 20 nM in the dissociation buffer did not modify the dissociation rate of [35S]gp120, thus suggesting that dissociation of the interaction between sCD4 and [35S]gp120 is not rate-limiting for [35S]gp120 dissociation from CCR5. Accordingly, the subsequent dissociation kinetics were determined in the absence of sCD4. In contrast to what we observed for [125I]CCL3 dissociation, Gpp(NH)p did not change the dissociation rate of [35S]gp120, thus further supporting the conclusion that binding of gp120 to CCR5 is independent of G-proteins (supplemental Fig. S2). Dissociation of [35S]gp120 that was measured in the presence of excess unlabeled gp120 and sCD4 was monophasic (i.e. dissociation fitted closely to a straight line when represented on a logarithmic scale, see Fig. 5a), thus indicating that the protein dissociates from a homogeneous population of receptors at its maximal unchanging rate (koff = 0.46 ± 0.06 h−1). Roughly similar results were obtained when dissociation was initiated by a 750-fold dilution of [35S]gp120 (koff = 0.49 ± 0.06 h−1, see Fig. 5a). The deduced rate of association of the [35S]gp120-sCD4 complex with CCR5 was 1 order of magnitude lower than that of [125I]CCL3 (koff = 1.3 × 103 M−1s−1), thus suggesting that conformational rearrangements in gp120 need to be overcome upon binding to the coreceptor.

Dissociation experiments were then repeated following a 80-fold dilution of [35S]gp120 in buffer alone or containing concentrations ranging from 0.3 to 100 μM of either TAK779 (Fig. 5b) or MVC (Fig. 5c). In buffer alone, [35S]gp120 dissociated from CCR5 more slowly than in the presence of excess unlabeled gp120 (i.e. bold dotted line in Fig. 5, a–c), which indicates that some of the dissociated [35S]gp120 molecules...
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Enhanced \(^{35}S\)gp120 dissociation from CCR5 by TAK779 and MVC—We directly compared the two inhibitors in their ability to prevent HIV-1 entry and replication. For that purpose, NL4-3-based viruses containing the envelope glycoprotein of the HIV-1 isolate Bx08 and Renilla luciferase as a reporter gene were used to infect CD4- and CCR5-expressing U87 or HEK 293T cells or activated PBMCs in the presence of serial dilutions of either TAK779 or MVC (Fig. 6, a–c, and Table 1). To quantify virus replication, target cells were lysed and luciferase activity was measured 48 h post-infection. We found that MVC was consistently more than 2 orders of magnitude more potent than TAK779 for inhibiting infection of all of three cell types used. Similar results were observed using the envelope glycoprotein of the R5-tropic HIV-1 strain JR-CSF instead of that of the Bx08 strain (Fig. 6, d–f, and Table 1), thus making it unlikely that the differential ability of MVC and TAK779 to inhibit infection depends merely on the envelope glycoprotein used. Half-maximal inhibitory concentration (IC\(_{50}\)) values for MVC were in the nanomolar range in U87- and PBMC-based assays, in accordance with an earlier work (14), but dramatically increased in the experiments with the CD4- and CCR5-expressing HEK 293T cells. A similar trend has been observed for TAK779 as well, which may be due to the fact that HEK 293T cells express substantially higher amounts of CCR5, as compared with U87 cells and PBMCs (see Table 1). Indeed, previous reports highlighted that increasing the CCR5 expression level at the cell surface decreases the antiviral potency of small molecule CCR5 inhibitors (51, 52). High amounts of CCR5 in HEK 293T cells might suggest that these cells contain more receptors than G-proteins, as compared with the other cell types. However, as shown here (Fig. 2e), TAK779 and to a lesser extent MVC bind preferentially to G-protein-uncoupled receptors, thus making it unlikely that the occurrence of "spare" receptors explains why infection of HEK 293T cells is less sensitive to entry inhibitors. Nevertheless, the two-log difference in the IC\(_{50}\) values between MVC and TAK779 is apparent in each of the cells used and is thus unlikely to be dependent upon receptor expression levels.
The 100-fold weaker antiviral activity of TAK779, as compared with MVC, cannot be explained by our results showing that both inhibitors have affinities for the gp120-binding receptors that are in the same range (Fig. 4) and contrasts with the increased ability of TAK779 to promote gp120 dissociation from CCR5 (Fig. 5). This strongly suggests that the antiviral activity of these small molecule CCR5 inhibitors might not rely only on their ability to interfere with gp120 binding to the coreceptor.

**DISCUSSION**

**TAK779 and MVC Are Inverse Agonists for CCR5, which Stabilize Distinct Receptor Conformations**—CCR5 like other GPCRs can convert spontaneously from inactive to active conformations (30, 53, 54). We found that basal activity of CCR5 was reversed to different extents by TAK779 and MVC, thus identifying both molecules as inverse agonists for the receptor. These molecules are thought to bind to a conserved hydrophobic cavity formed by the upper half of the transmembrane helices (24–26), some of which contain amino acids previously found to be important structural determinants for activation of the receptor (55, 56). So, direct or indirect interactions with these residues could explain how these, and others (29, 57), small molecule CCR5 inhibitors prevent the receptor from spontaneous activation. The full inverse agonist activity of TAK779 we documented in this study is likely to rely on its ability to bind preferentially to and to stabilize G-protein-uncoupled, inactive conformations of CCR5.
This assumption is in particular supported by our finding that disrupting CCR5 coupling to G-proteins by Gpp(NH)p improves the efficiency with which TAK779 competes with the chemokine [\(^{[35]S}\)CCL3 for binding to the receptor (Fig. 2c). Preferential binding of TAK779 to inactive receptors is also consistent with the recent observation that inverse agonists act by preventing formation of active conformations rather than by destabilizing preformed receptor-G-protein complexes (46). In contrast to TAK779-occupied receptors, those that have bound the partial inverse agonist MVC maintain constitutive coupling to G-proteins to some extent, thus indicating that TAK779 and MVC stabilize different conformations of CCR5. This may explain why some viruses that are resistant to MVC retain susceptibility to TAK779 (31, 58). Furthermore, given that CCR5 exists in multiple conformations displaying distinct functional properties (23, 59), our results raise the possibility that these conformations differ in their capacity to bind TAK779 or MVC.

The stabilization of different CCR5 conformations by TAK779 and MVC can be explained by specific binding modes. Although both molecules locate in the transmembrane domains of the receptor, they occupy overlapping but distinct regions of the transmembrane cavity and interact with different sets of residues (24–26). For example, whereas replacement of Tyr-37 by alanine has been described to impair TAK779 binding to CCR5 (24), we observed that this mutation actually preserves MVC binding. Interestingly, replacement of Tyr-37 by alanine also suppresses chemokine binding to CCR5, but only moderately affects that of gp120 (26), thus suggesting that Tyr-37 is mandatory for maintaining CCR5 in a high affinity conformation for agonists. It is tempting to speculate that CCR5 coupling to G-proteins, which provides high affinity binding of chemokines (Fig. 1e) (32) and is disrupted by TAK779 binding (Fig. 2b), might play a role in this process. As another example, in contrast to TAK779, MVC binding to CCR5 involves the receptor helix 5. The replacement of Ile-198 by an alanine results in a 100-fold decrease in the binding affinity of MVC for CCR5, although it has little effect on that of TAK779 (25, 26). Of note, although the I198A substitution preserves binding of both chemokines and gp120 (25, 26), it dramatically impairs HIV-1 infection (26). These observations are in keeping with the 100-fold higher antiviral potency shown by MVC, as compared with TAK779 (Fig. 6), even though both inhibitors compete for [\(^{[35]S}\)gp120 binding to CCR5 with nearly similar potencies (Fig. 4). This strongly suggests that inhibition of HIV-1 infection by MVC or by the I198A substitution involves a mechanism other than inhibition of gp120 binding to CCR5.

**TAK779 and MVC Are Noncompetitive and Allosteric Inhibitors of CCL3 and gp120 Binding to CCR5**—On the basis of our results showing that the IC\(_{50}\) values for displacement of [\(^{[35]S}\)gp120 binding by TAK779 or MVC do not vary with the radioligand concentration (Fig. 4), we provided evidence that both inhibitors act as noncompetitive antagonists of the glycoprotein binding to CCR5. Thus, these results indicate that the fraction of receptors occupied by either TAK779 or MVC does not change over the range of the [\(^{[35]S}\)gp120 concentrations used. This can be explained by the fact that TAK779 and MVC dissociate from CCR5 with substantially slower rates (\(k_{\text{off}} = 0.013\) and 0.0036 h\(^{-1}\), respectively (21)) compared with [\(^{[35]S}\)gp120 as calculated in this study (\(k_{\text{off}} = 0.49\) h\(^{-1}\)). Similar conclusions were previously drawn using [\(^{[125]I}\)CCL3 as a radioligand (21). The dissociation kinetics of [\(^{[125]I}\)CCL3 and [\(^{[35]S}\)gp120 from CCR5 measured in the presence of TAK779 or MVC are consistent with both small molecule CCR5 ligands inhibiting binding of the radioligands to CCR5 through allosteric mechanisms (Figs. 3 and 5). Indeed, in contrast to orthosteric ligands, an excess concentration of either of the two inhibitors accelerated the [\(^{[125]I}\)CCL3 and [\(^{[35]S}\)gp120 dissociation rates, thus suggesting that TAK779 and MVC can bind to [\(^{35}S\)]gp120-occupied CCR5, and then change the receptor conformation in such a way that the glycoprotein is released from the receptor. These results thus imply that both inhibitors bind to regions of CCR5 separate from the gp120- and CCL3-binding sites. Alternatively, but not exclusively, it cannot be excluded that allosteric interactions between the entry inhibitors and gp120 or CCL3 might also transmit across CCR5 oligomers. Indeed, recent data support the notion that GPCRs, including CCR5, form homo- and heterooligomers and that negative binding cooperativity can occur between the associated receptors (32, 60). Overall, these results clarify why resistance to CCR5 inhibitors was found to result in incomplete inhibition of infection at saturating inhibitor concentration, i.e. the maximum percent inhibition plateaus at a value lower than 100%, whereas resistance to competitive inhibitors is manifested, instead, by increased IC\(_{50}\) values for the inhibitors (61). The decrease in the maximum percent inhibition values is suggestive of viruses having acquired the ability to use inhibitor-bound receptors in addition to the free receptors (31, 52), which is consistent with our present results showing that gp120 and members of this class of molecules bind to separate domains of CCR5.

**Mechanisms of Allosteric Inhibition of CCL3 and gp120 Binding to CCR5 by TAK779 and MVC**—The ability of TAK779 and MVC to promote dissociation of [\(^{[125]I}\)CCL3 and [\(^{[35]S}\)gp120 from CCR5 correlated with that of their inverse agonist activity. The dissociation rate of [\(^{[125]I}\)CCL3 also increased in the presence of Gpp(NH)p (Fig. 3b), suggesting that CCR5 uncoupling from G-proteins is a mechanism by which TAK779 and MVC allosterically interfere with the binding of the chemokine to the receptor. In contrast to CCL3, however, dissociation of [\(^{[35]S}\)gp120 did not depend on the receptor coupling to G-proteins, indicating that the ability whereby TAK779 and MVC promote dissociation of [\(^{[35]S}\)gp120 from CCR5 does not stem from their inverse agonist activity. Among the mechanisms involved, it could be envisaged that TAK779 and MVC might act on gp120 binding by changing the conformation of ECL2. In support of this assumption, we have observed that both inhibitors interfere with the binding of the anti-CCR5 mAb 45531 to the distal half of ECL2 (but not with the binding of the anti-CCR5 mAbs 2D7 and CTC5 to the proximal half of ECL2 and the receptor N-terminal tail, respectively). These findings are in

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\(^4\) J. Garcia-Perez, P. Rueda, E. Kellenberger, J. Alcami, F. Arenzana-Seisdedos, and B. Lagane, unpublished data.
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fact fully consistent with recent structural data on related CCR5 inhibitors indicating that their binding to the receptor disrupts interhelix interactions and interactions between helices and ECL2 (27).

gp120 Is a Neutral Antagonist for CCR5—Lack of effect of Gpp(NH)p on dissociation of [35S]gp120 from CCR5 is consistent with our result in Fig. 1 showing that, in contrast to CCL3, high affinity binding of gp120 to CCR5 does not depend on the receptor coupling to G-proteins. Altogether, these results identify the viral glycoprotein as a neutral antagonist for CCR5. It should be noted that this conclusion does not formally rule out the possibility that gp120 may induce signaling events when interacting with CCR5, as documented by previous studies (62). Indeed, like for other GPCRs (63), it is reported that CCR5 signaling includes G-protein-independent signaling pathways, including those of the JAK/STAT and β-arrestin proteins (64), which might be activated in response to gp120. Consistent with this assumption, studies have emphasized that R5-tropic gp120-induced intracellular signals through CCR5 are not sensitive to pertussis toxin, thus indicating that they are not dependent upon Gαi/0 proteins (33–35). It could be hypothesized that gp120 might act as a “biased” agonist activating some signaling pathways but not others, as it has been reported for other GPCR ligands (65).

Antiviral Activity of TAK779 and MVC Does Not Correlate with Their Ability to Interfere with gp120 Binding to CCR5—Although CCR5 coupling to G-proteins and interaction with gp120 are two processes that were demonstrated here not to be dependent upon each other, the magnitude with which these processes are altered by both inhibitors varies in the same way. Indeed, it could be deduced from our results that gp120 from the Bx08 HIV-1 strain is dissociated from TAK779-bound CCR5 and MVC-bound CCR5 15-fold (k off[TAK779] = 7.2 ± 0.8 h⁻¹) and 5-fold (k off[MVC] = 2.4 ± 0.4 h⁻¹) more rapidly, respectively, than from the free receptor (k off = 0.49 ± 0.06 h⁻¹). The fact that gp120 is detached from CCR5 more readily in the presence of TAK779 than in the presence of MVC might be accounted for by gp120 having a lower affinity for TAK779- than for MVC-bound CCR5. It should be noted, however, that our results from the competition experiments in Fig. 4 do not follow the same trend, as TAK779 is almost 3-fold less potent than MVC for competing for binding of [35S]gp120 to CCR5 (IC₅₀ = 22 ± 9 and 6.4 ± 3 nm for TAK779 and MVC, respectively). These discrepancies can be explained by the fact that MVC exhibits a more prolonged receptor occupancy than TAK779 (k off = 0.0036 versus 0.013 h⁻¹ (21)). As explained above, the IC₅₀ values for both inhibitors that have been deduced from the [35S]gp120 displacement binding experiments can be considered as estimates of their equilibrium dissociation constants for the receptor. So, the data from these experiments indicate that the affinities of MVC and TAK779 for the gp120 binding receptors are in almost the same range of magnitude. Yet, we have observed that TAK779 is more than 100-fold less potent than MVC at inhibiting HIV entry and replication (Fig. 6), thus strongly suggesting that the antiviral potency of both molecules does not rely only on their capacity to interfere with gp120 binding to the coreceptor. Our results are in keeping with previous data showing that anti-CCR5 mAbs that are poor inhibitors of gp120 binding to CCR5 inhibit very efficiently viral entry, and conversely (23, 66). This is consistent with the view that gp120-coreceptor interactions and HIV entry and replication require different structural determinants and/or conformations of CCR5. Remarkably, a previous study has shown that the anti-CCR5 mAb PA14 and small molecule CCR5 inhibitors synergize in blocking HIV-1 entry, thus suggesting that these two types of molecules act at different stages of CCR5 usage (11). Similarly, we propose that TAK779 and MVC, which were shown here to inhibit gp120 binding with roughly similar potencies, affect differentially distinct steps of CCR5 usage in the course of HIV-1 infection. This assumption is fully consistent with the previous observations that small molecule CCR5 inhibitors, as allosteric molecules, can alter differentially distinct functions of the receptor (18, 19, 21). Hence, MVC might be more efficient than TAK779 at inhibiting events that precede gp120 binding, including the interactions between CD4 and CCR5 that have been suggested to play a key role in HIV entry into target cells (67). In this regard, we previously demonstrated that TAK779 impairs CCR5-CD4 interactions (68), thereby suggesting that this process, which might result from conformational changes of CCR5, contributes to the antiviral activity of this molecule. TAK779 and MVC might also act differentially at stages following gp120 attachment to CCR5, for example during recruitment of the coreceptor within a fusion complex or triggering of gp120/CCR5 conformational changes that are required for fusion, as was speculated regarding the mechanism of action of PA14 (11). Finally, signaling downstream of CCR5, which has been speculated to influence post-entry events of the virus life cycle (62), might also be differentially affected by TAK779 and MVC. We believe that targeting these different stages of CCR5 usage might provide clues for improving the development of small molecule CCR5 inhibitors and their therapeutic usage.

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