0026-895X/09/7503-490–501$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2009 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 75:490–501, 2009

Vol. 75, No. 3
52555/3439226
Printed in U.S.A.

The Relative Activity of "Function Sparing" HIV-1 Entry Inhibitors on Viral Entry and CCR5 Internalization: Is Allosteric Functional Selectivity a Valuable Therapeutic Property?

Vanessa M. Muniz-Medina, Stacey Jones, Jodi M. Maglich, Cristin Galardi, Robert E. Hollingsworth, Wieslaw M. Kazmierski, Robert G. Ferris, Mark P. Edelstein, Karen E. Chiswell, and Terry P. Kenakin

Infectious Diseases Discovery Performance Unit (V.M.M.-M., S.J., J.M.M., C.G., R.E.H., W.M.K., R.G.F., M.P.E.), Drug Development Sciences (K.E.C.), and Molecular Discovery Research (T.P.K), GlaxoSmithKline Research and Development, Research Triangle Park, North Carolina

Received October 8, 2008; accepted November 24, 2008

ABSTRACT

Six allosteric HIV-1 entry inhibitor modulators of the chemokine (C-C motif) receptor 5 (CCR5) receptor are compared for their potency as inhibitors of HIV-1 entry [infection of human osteosarcoma (HOS) cells and peripheral blood mononuclear cells (PBMC)] and antagonists of chemokine (C-C motif) ligand 3-like 1 [CCL3L1]-mediated internalization of CCR5. This latter activity has been identified as a beneficial action of CCL3L1 in prolonging survival after HIV-1 infection (*Science* **307:**1434–1440, 2005). The allosteric nature of these modulators was further confirmed with the finding of a 58-fold (HOS cells) and 282-fold (PBMC) difference in relative potency for blockade of CCL3L1-mediated internalization versus HIV-1 entry. For the CCR5 modulators, statistically significant differences in this ratio were found for maraviroc, vicriviroc, aplaviroc, Sch-C, TAK652, and TAK779. For instance, although TAK652 is 13-fold more potent as an HIV-1 inhibitor (over blockade of CCL3L1-mediated CCR5 internalization), this ratio of potency is reversed for Sch-C (22-fold more potent for CCR5-mediated internalization over HIV-1 entry). Quantitative analyses of the insurmountable antagonism of CCR5 internalization by these ligands suggest that all of them reduce the efficacy of CCL3L1 for CCR5 internalization. The relatively small magnitude of dextral displacement accompanying the depression of maximal responses for aplaviroc, maraviroc and vicriviroc suggests that these modulators have minimal effects on CCL3L1 affinity, although possible receptor reserve effects obscure complete interpretation of this effect. These data are discussed in terms of the possible benefits of sparing natural CCR5 chemokine function in HIV-1 entry inhibition treatment for AIDS involving allosteric inhibitors.

HIV-1 has been shown to use the chemokine C receptor CCR5 to gain entry into cells to cause infection (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996); therefore, efforts have centered on the development of CCR5 HIV entry inhibitors for potential treatment of AIDS and AIDS prevention. Several molecules have been described previously (Kazmierski et al., 2003, 2005, 2007; Schols, 2006); presently, one of them, maraviroc, has been approved for therapeutic use (Fätkenheuer et al., 2005). Another CCR5 entry inhibitor, aplaviroc (Demarest et al., 2004; Maeda et

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.108.052555.

ABBREVIATIONS: CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C motif) receptor; Sch-C, (*Z*)-(4-bromophenyl){1′-[(2,4-dimethyl-1-oxido-3-pyridinyl)carbonyl]-4′-methyl-1,4′-bipiperidin-4-yl}methanone *O*-ethyloxime; maraviroc (UK 427,857), 4,4-difluoro-*N*-((1*S*)-3-{(3-*endo*)-3-[3-methyl-5-(1-methylethyl)-4*H*-1,2,4-triazol-4-yl]-8-azabicyclo[3.2.1]oct-8-yl}-1-phenylpropyl)cyclohexanecarboxamide; TAK779, *N*,*N*-dimethyl-*N*-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5*H*-benzocyclohepten-8-yl]carbonyl]amino]benzyl]tetrahydro-2*H*-pyran-4-aminium chloride; TAK652, (*S*)-8-[4-(2-butoxyethoxy)phenyl]-1-isobutyl-*N*-(4-{[(1-propyl-1*H*-imidazol-5-yl)methyl]sulfinyl}phenyl)-1,2,3,4-tetrahydro-1-benzazocine-5-carboxamide monomethanesulfonate; vicriviroc (Sch-D), 4,6-dimethyl-5-{[4-methyl-4-((3*S*)-3-methyl-4-{(1*R*)-2-(methyloxy)-1-[4-(trifluoromethyl)phenyl]ethyl}-1-piperazinyl)-1-piperidinyl]carbonyl}pyrimidine; aplaviroc 873140, 4-{[4-({(3*R*)-1-butyl-3-[(*R*)-cyclohexyl(hydroxy)methyl]-2,5-dioxo-1,4,9-triazaspiro[5.5]undec-9-yl}methyl)phenyl]oxy}benzoic acid hydrochloride; MIP-1α (CCL3), macrophage inflammatory protein 1-α; MIP-1β (CCL4), macrophage inflammatory protein 1-β; RANTES (CCL5), regulated on activation normal T-cell expressed and secreted; HOS, human osteosarcoma; CCL3L1 (LD78b), APLAADTPTACCFSYTSRQIPQNFIADYFETSSQCSKPSVIFLTKRGRQVCADPSEEWVQKYVSDLELSA (MIP-1α, LD78-β isoform, human); Org 27569–0, 5-chloro-3-ethyl-1*H*-indole-2-carboxylic acid [2-(4-piperidin-1-yl-phenyl)-ethyl]-amide; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood lymphocytes; DMEM, Dulbecco's modified Eagle's media; FCS, fetal calf serum; PHA, phytohemagglutinin; PBS, phosphate-buffered saline.

al., 2004), has been shown to block the receptor in an allosteric manner (Maeda et al., 2004, Watson et al., 2005). Thus, although aplaviroc blocks the binding of the radioactive chemokine ^{125}I-CCL3, it does not affect the affinity for the radioactive chemokine ^{125}I-CCL5 (Maeda et al., 2004; Watson et al., 2005); such probe dependence is a characteristic of allosteric modulators (Jakubík et al., 1997; Christopoulos and Kenakin, 2002) and absolutely precludes a mechanism of orthosteric binding to the chemokine binding pocket.

Binding studies have shown prohibitive mutual binding kinetics between aplaviroc and the CCR5 entry inhibitors TAK779, Sch-C, maraviroc, and vicriviroc (Watson et al., 2005). These and other data support the idea that this new generation of HIV-1 entry inhibitors also are allosteric modulators of the CCR5 receptor (Dragic et al., 2000; Castonguay et al., 2003; Tsamis et al., 2003; Nishikawa et al., 2005; Maeda et al., 2006; Seibert et al., 2006; Westby et al., 2007; Kondru et al., 2008). In light of the fact that allosteric mechanisms are permissive with respect to allowing some receptor functions to be differentially altered more than others (Kenakin, 2005), this study explores the relative effects of CCR5 HIV entry inhibitors on HIV entry and chemokine-induced CCR5 receptor internalization. This latter mechanism has been suggested to be of value in protecting cells from HIV infection in the presence of chemokines. Because it has been reported that the chemokine CCL3L1 is associated with protection from progression to AIDS in patients infected with HIV (Gonzalez et al., 2005), the relative potency of six CCR5 entry inhibitors on HIV entry and CCL3L1-induced CCR5 internalization was studied. We show that there is a wide range of *relative* activity of these modulators on these two effects. These data are discussed in terms of general statements regarding the therapeutic use of allosteric modulators in AIDS treatment and also the potential for added efficacy for HIV-1 entry blockade through preservation of natural chemokine-induced internalization of CCR5.

Materials and Methods

Cells. Human osteosarcoma (HOS) cells expressing hCCR5 and hCD4 (Deng et al., 1996) were acquired from the NIH AIDS Research and Reference Reagent Program (Germantown, MD) and engineered to express an HIV-1 LTR-Luciferase reporter (Jenkinson et al., 2003). The cells were maintained in Dulbecco's modified Eagle's media (DMEM) containing 10% fetal calf serum (FCS), 1× nonessential amino acids, 400 μg/ml G418, 1 μg/ml puromycin, 40 μg/ml mycophenolic acid, 250 μg/ml xanthine, and 13.5 μg/ml hypoxanthine to maintain a selection pressure for cells expressing HIV-1 LTR-luciferase, hCCR5 and hCD4, respectively. Normal donor peripheral blood mononuclear cells (PBMCs) were isolated from random buffy coats (35–40 ml of elutriated whole blood in anticoagulant from HIV-negative donors) received from the American Red Cross, Southeast Division. PBMCs were isolated by density gradient centrifugation over lymphocyte separation medium (Mediatech, Herndon, VA) and stimulated in 150 ml of RPMI 1640 medium containing 20% FCS, 10% interleukin-2 (Zeptometrix, Buffalo, NY), 10 μg/ml gentamicin, and 5 μg/ml phytohemagglutinin (PHA; Sigma, St. Louis, MO) for 24 to 48 h.

Virus Strains. High-titer CCR5-tropic HIV-1 Ba-L was purchased from Advanced Biotechnologies (Columbia, MD) and expanded in PHA-stimulated PBMCs for 2 weeks with a refeeding of the culture after the first week. At the end of the 2-week period, the supernatants were clear-spun to remove cell debris, separated into 0.5-ml aliquots, and frozen at −80°C. An aliquot of the PBMC-expanded stock Ba-L was titrated into assay medium and assayed under standard conditions with test compounds not present. Viral input was determined by selecting a dilution that gave a signal within the linear range of the assay and a signal-to-background ratio of 75 to 100. Virus was diluted in assay medium before use.

HOS HIV Replication Cell Assay. HOS cells were lifted from tissue culture flasks by standard trypsinization techniques, resuspended in DMEM + 2% FCS to a density of 1.2×10^5 cells/ml. Fifty microliters of cells were dispensed into black-walled, clear-bottomed 96-well tissue culture-treated plates and placed in a humidified incubator at 37°C, 5% CO_2 overnight. The following day, 50 μl of test compounds, serially diluted in medium, were added to the plates containing the HOS cells, and the plates were returned to the incubator for 1 h. A separate aliquot of diluted compound was added to an equal volume of diluted HIV-1 Ba-L, and 100 μl of this mixture was then added to the HOS/compound mixture. Infection proceeded in a humidified incubator at 37°C, 5% CO_2 for 4 days. After this incubation period, supernatants were reduced to a volume of 50 μl, and an equal volume of reconstituted Steady-Glo reagent (Promega, Madison, WI) was added to each well. The plates were read in a TopCount luminometer (PerkinElmer Life and Analytical Sciences, Waltham, MA) at 1 s per well.

PBMC HIV Replication Cell Assay. HIV-1 Ba-L replication in PBMCs was quantitated by measuring reverse transcriptase activity present in the supernatant as described previously (Hazen et al., 2007). PHA-stimulated PBMCs were pelleted at 260*g* for 15 min, washed once with sterile phosphate-buffered saline, pelleted as before, resuspended to a density of 4×10^6 cells/ml in RPMI 1640 medium (containing 20% FCS, 10% interleukin-2, and 50 μg/ml gentamicin), and 100 μl were distributed to 96-well tissue culture plates. An equal volume of test compounds, serially diluted in medium, was added, and the plates were placed in a humidified incubator at 37°C, 5% CO_2 for 1 h. A separate aliquot of diluted compound was added to HIV-1 Ba-L, and 100 μl of this mixture was then added to the PBMC/compound mixture. Infection proceeded in a humidified incubator at 37°C, 5% CO_2 for 7 days. Fifty microliters of cell-free culture supernatant was transferred to a new 96-well plate. Ten microliters of RT extraction buffer (500 mM KCl, 50 mM dithiothreitol, and 0.5% Nonidet P-40) was added and mixed, followed by 40 μl of RT assay buffer (1.25 mM EGTA, 125 mM Tris/HCl, 12.5 mM $MgCl_2$, 68 Ci/mmol [methyl-^{3}H]deoxy-TTP, 0.62 O.D. units/ml poly(rA)-poly(dT)12–18). After mixing, room temperature reaction proceeded in a humidified incubator at 37°C, 5% CO_2 for 2 h. Radioactivity was captured on 96-well plates (Unifilter DE-81; Whatman, Clifton, NJ) using a Univac vacuum manifold (Whatman). Wells were washed a total of three times: the first wash was 5% Na_2HPO_4, followed by distilled water, and, finally, 95% ethanol. The plates were read in a TopCount luminometer (PerkinElmer Life and Analytical Sciences) at 10 s/well.

Internalization of CCR5. U373-MAGI-CCR5-E cells were generated by Dr. Michael Emerman and obtained through the National Institutes of Health AIDS Research and Reagent Program. Culture media was DMEM/Ham's F12 with 10% fetal bovine serum, and 1% GlutaMAX (Invitrogen, Carlsbad, CA). Cells were plated in 96-well view plates (PerkinElmer Life and Analytical Sciences) and harvested from the culture flask using Cell Dissociation Solution from Sigma. They were then plated at 17×10^3 cells/well in 96-well view plates (PerkinElmer Life and Analytical Sciences) and incubated for ≈15 h at 37°C, 5% CO_2.

Cell Treatment. Cells were washed twice with 50 μl/well PBS, pH 7.2. Test compounds were prepared as 1:5 serial dilutions in 100:1 dimethyl sulfoxide then diluted 1:100 in minimal essential medium-α + 1% FCS. Cells were incubated with 100 μl of test compound for 35 min at 37°C, 5% CO_2. CCL3L1 (20 nM) in α-minimal essential medium + 1% FCS was then added to the cells/compounds, giving a final CCL3L1 of 10 nM (~IC_{80}). The cells were incubated for 35 min at 37°C, 5% CO_2. Cells then were washed twice with PBS, fixed with 3.7% paraformaldehyde, and incubated at room tempera-

ture inside a chemical hood for 30 min. The cells were then washed twice with 50 μl/well wash buffer (0.5% Tween 20 in PBS, pH 7.2).

Cell membrane was stained with 4 ng/ml wheat germ agglutinin (reconstituted in physiological buffer solution, pH 7.4)-Alexa Fluor 488 from Invitrogen for 30 min in the dark. Then cells were washed twice with 50 μl/well wash buffer, and 50 μl/well 3.7% paraformaldehyde was added. This mixture was incubated for 5 min in a chemical hood. The cells were then washed twice with 50 μl/well wash buffer before blocking with 50 μl 1% BSA and 1% normal goat serum (Sigma) in PBS, pH 7.2 (block buffer), for 30 min, then permeabilized with PBS, pH 7.2, and 0.2% Triton-X (Sigma T8787) for 15 min in the dark.

The CTC8 (R&D Systems, Minneapolis, MN) mouse anti-human CCR5 primary antibody was reconstituted in PBS, pH 7.2, + 0.5% BSA to a concentration of 500 μg/ml. The Primary Antibody Solution was prepared by mixing block buffer with CTC8 to a 3 μg/ml final concentration. The permeabilization buffer from the previous step was aspirated, and 50 μl/well of primary antibody solution was added. Plates were then incubated at room temperature (in the dark) for 40 min. After the incubation, cells were washed twice with 50 μl/well wash buffer. Fifty microliters of secondary goat anti-mouse Alexa Fluor 568 F′(ab) (Invitrogen), 6 μg/ml in 2 μM Hoeschst 33342 (Lonza Walkersville, Walkersville, MD) in blocking buffers was added, and the plate was incubated at room temperature and protected from light for 40 min. After the incubation, the cells were washed three times with 50 to 100 μl/well wash buffer, then stored in 100 μl/well PBS buffer, pH 7.2. The plates were sealed with TopSeal-A (PerkinElmer Life and Analytical Sciences), and the bottom of the plates were wiped with 70% ethanol to remove any possible water marks. The plates were read using the Cellomics ArrayScan II instrument (Thermo Fisher Scientific Cellular Imaging, Pittsburgh, PA); data were analyzed with the GPCR BioApplication algorithm (Thermo Fisher Scientific Cellular Imaging).

Compounds. All compounds in this work [maraviroc (Perros et al., 2001), aplaviroc (Mitsuya et al., 2002), vicriviroc (Baroudy et al., 2000a,b), Sch-C (Baroudy et al., 2000a,b), TAK779 (Baba et al., 1999), and TAK652 (Shiraishi et al., 2003; Baba et al., 2005)] were synthesized as described in the literature.

Statistical Analysis. For the HOS and PBMC internalization assays, analysis was performed on the pIC_{50} scale using one-way analysis of variance. Mean and standard deviation were reported for each modulator and assay. S.E.M. was calculated using the analysis of variance estimate. The ratio of IC_{50} values for internalization versus HIV entry inhibition in HOS cells and PBL ("selectivity") was estimated as 10 to the power of the difference between the mean pIC_{50} for HIV-1 entry and mean pIC_{50} for internalization. 95% confidence limits were calculated using critical values from a t distribution. The selectivity of different modulators was compared on the log-scale using a t test. Degrees of freedom for the t distribution were approximated using Satterthwaite's method (Steel et al., 1997). The correlation between pIC_{50} values for HIV entry inhibition in HOS cells and PBL was calculated by fitting a total least-squares regression model using maximum likelihood. Observed potency (calc. pK_b) of CCR5 modulators was regressed against log concentration of CCL3L1 used in the internalization experiment. A linear regression model with a separate intercept and slope for each modulator was fit using ordinary least squares. For the HOS, PBMC, and U373 assays, normalized response data from duplicate titrations were fit using nonlinear least-squares to an unconstrained four parameter logistic equation, defined as follows:

$$y = E_{min} + \frac{E_{max} - E_{min}}{1 + (x/K)^{n_H}} \quad (1)$$

where x is compound concentration, y is normalized response data, E_{max} is upper bound of response, E_{min} is lower bound of response, n_H is the Hill coefficient, and K is the concentration giving a normalized response halfway between E_{min} and E_{max}.

Raw data from the PBMC assay were normalized according to the following equation:

$$\frac{\log_{10}(\text{raw}) - \log_{10}(P_C)}{\log_{10}(N_C) - \log_{10}(P_C)} \quad (2)$$

where P_C is the geometric mean of positive controls (mock-infected wells) on each assay plate, and N_C is the geometric mean of negative controls (untreated but infected wells) on each assay plate. IC_{50} values were then derived from the following back calculation using parameter estimates from eq. 1.

$$IC_{50} = K\left(\frac{E_{max} - Y.50}{Y.50 - E_{min}}\right)^{1/n_H} \quad (3)$$

where $Y.50 = \log_{10}[(P_C + N_C)/2]$ and n_H is the Hill coefficient. The molar concentration producing 50% maximal entry inhibition (pIC_{50}) was calculated as $-\log_{10}(\text{molar } IC_{50})$.

Raw data from the antiviral assays and U373 internalization assay were normalized by expressing raw values as a percentage of the arithmetic mean of the no-compound controls on each assay plate. IC_{50} values were estimated using the K parameter estimate from eq. 1. pIC_{50} was calculated as $-\log_{10}(\text{molar } IC_{50})$.

Results

HIV Entry Inhibition. The effects of six CCR5 modulators on HIV entry into HOS cells and human PBMCs were quantified by means of measuring the $-\log(IC_{50})$. As shown in Table 1, potencies of these ligands ranged from 0.4 (TAK652) to 12.6 nM (TAK779) in the HOS assay, the order of potency being TAK652 > maraviroc > aplaviroc > vicriviroc > Sch-C > TAK779. Likewise, in the PBMC assay, potencies ranged from 0.38 (TAK652) to 100 nM (TAK779), the order of potency being TAK652 > aplaviroc > maraviroc > vircriviroc > Sch-C > TAK779. The correlation between anti-HIV activity in HOS and PBMC is shown in Fig. 1 ($r = 0.68$). The correlation of 0.68 and the graph show that HOS and PBMC seem to be related; however, the fitted line in Fig. 1 is quite different from the line of identity (expected if the two assays gave identical pIC_{50} values). For the less potent compounds, HOS tends to give higher pIC_{50} than PBMC, but for compounds that are more potent, the two assays give quite similar pIC_{50} values. The interpretation of this with respect to differences between results based on PBL versus HOS is not clear.

CCR5 Internalization. The activation of seven transmembrane receptors, such as CCR5, has been shown to cause

TABLE 1
Antiviral effects (pIC_{50}) of CCR5 modulators
HOS S.E.M. values calculated based on a pooled estimate of residual standard deviation = 0.2999. PBMC S.E.M. values calculated based on a pooled estimate of residual standard deviation = 0.5850.

Compound	N	Mean	S.D.	S.E.M.
HOS				
Aplaviroc	270	8.79	0.277	0.018
TAK652	5	9.39	0.184	0.13
Maraviroc	33	8.98	0.231	0.05
TAK779	77	7.9	0.444	0.062
Vicriviroc	47	8.67	0.207	0.042
Sch-C	159	8.17	0.318	0.023
PBMC				
Aplaviroc	306	8.8	0.566	0.033
TAK652	19	9.42	0.519	0.134
Maraviroc	108	8.72	0.558	0.056
TAK779	36	7.01	0.544	0.098
Vicriviroc	42	8.19	0.637	0.09
Sch-C	65	7.51	0.711	0.073

their internalization into the cell, and this is a common observation for CCR5 receptor activation by a number of chemokines (Amara et al., 1997; Mack et al., 1998). As shown in Fig. 2A, the chemokine CCL3L1 produces concentration-dependent internalization of CCR5 (pEC_{50} = 8.7) that is prevented by maraviroc (Fig. 2B). Specifically, addition of CCL3L1 (at a concentration producing 80% maximal internalization) to cells pre-equilibrated with a range of concentrations of maraviroc produces a curve for antagonism of CCL3L1-induced internalization. The antagonism is then quantified through the midpoint of this antagonistic curve in the form of a pIC_{50} (see Fig. 2B).

It was important to determine the system dependence of the estimate of antagonism, because conclusions about molecular mechanisms can better be made with equilibrium dissociation constants for antagonist-receptor complexes (pK_B). For a surmountable competitive antagonism, a correction factor needs to be applied to the pIC_{50} to convert these to pK_I values; this is not the case for insurmountable (noncompetitive) antagonism (Kenakin et al., 2006). Therefore, data to assess the mode of action of the CCR5 modulators was obtained by determining concentration-response curves for internalization to CCL3L1 in the absence and presence of a range of concentrations of modulator. As can be seen in Fig. 3, aplaviroc produces an insurmountable antagonism of the internalization effects of CCL3L1 with little displacement of the CCL3L1 curve location parameter (maximal displacement, 2.6-fold; see Table 2). Similar patterns of inhibition were observed for TAK779 (Fig. 4A), maraviroc (Fig. 4B), TAK652 (Fig. 4C), and vicriviroc (Fig. 4D). Table 3 shows a summary of the data for inhibition of CCL3L1-induced CCR5 internalization. It can be seen from Table 3 that the modulators range in potency from 6.6 (TAK779) to 0.76 nM (maraviroc). The relatively small dextral displacement of the

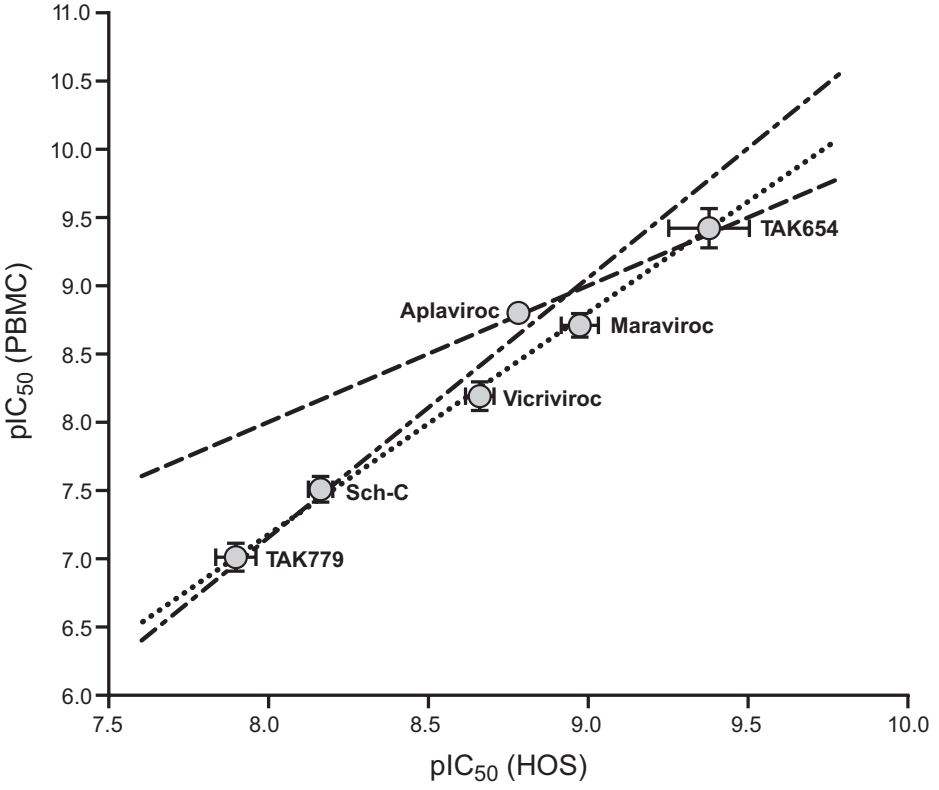

Fig. 1. Relationship between mean pIC_{50} values for inhibition of HIV entry in HOS cells (abscissae) and PBMC (ordinates). Bars represent S.E.M. Dashed line represents line of identity (equal pIC_{50} values in each assay); dotted line is the least-squares regression line fitted to the means; alternate dash-dotted line is the structural measurement error model fitted to replicates.

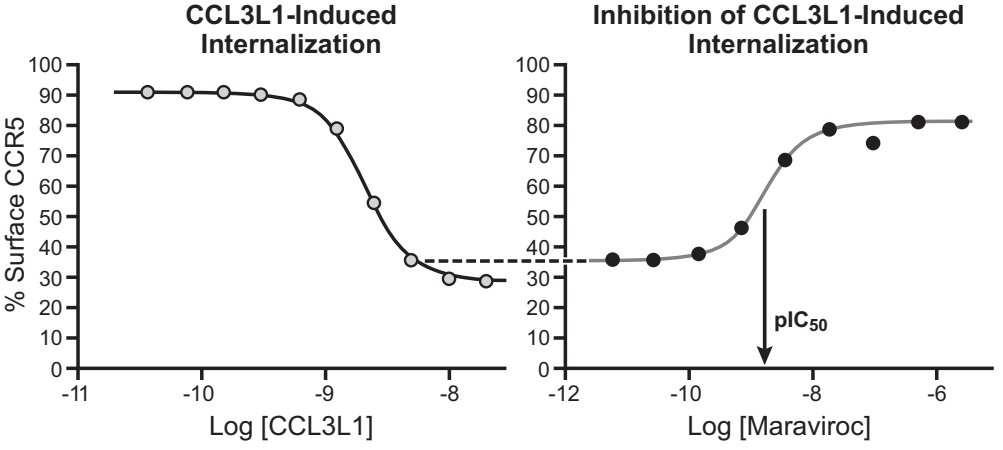

Fig. 2. Internalization of CCR5 by CCL3L1 (A) and blockade of the effect by maraviroc (B). A, surface CCR5 receptors expressed as a percentage of the initial surface density of CCR5. B, in the presence of maraviroc, an EC_{80} concentration of CCL3L1 was added to the medium; maraviroc prevented the effects of CCL3L1. The IC_{50} is the molar concentration of maraviroc that produces 50% of the maximal effect to maraviroc.

curves with depressed maxima is given in Table 2. From these data it can be seen that virtually no displacement was observed for aplaviroc, maraviroc, and vicriviroc, whereas a 7-fold maximal displacement was observed for TAK779 and TAK652. The low level of displacement indicates that pIC_{50} values are within 3- to 4-fold range.

Relative Inhibition of HIV and Internalization. The curves for inhibition of HIV entry and CCL3L1-induced CCR5 inhibition were compared to yield a ratio of potency for the modulators on these respective processes. Figure 5 shows the percentage inhibition curves for inhibition of HIV entry into HOS cells versus inhibition of internalization for TAK779 and TAK652. It can be seen that although TAK779 is more potent as an inhibitor of internalization than HIV (in this example, pIC_{50} internalization = 8.64 versus pIC_{50} HOS = 7.68), this order of relative potency is reversed for TAK652. Specifically, TAK652 is more potent as an inhibitor of HOS than internalization (in this example, pIC_{50} internalization = 8.5 versus pIC_{50} HOS = 9.42). This profile can be presented as a single curve by expressing the internalization curve as a function of the pIC_{50} concentration required to block HIV entry. Specifically, the abscissal axis for TAK779 for the internalization curve can be converted to a ratio of concentrations required for internalization versus HIV entry inhibition ([CCL3L1/IC_{50} HOS]) to yield a curve that expresses the relative activity of the modulator on internalization as a function of activity in the HOS. Fig. 5, top, shows this conversion for TAK779 and TAK652. From this figure it can be seen that the concentration required to produce 50% inhibition of internalization for TAK779 is 0.09 times the concentration required to block HIV entry into HOS cells (location parameter of the relative activity curve is centered on 0.09). This indicates that TAK779 is 11 times more active as a blocker of internalization than HIV entry. In contrast, the location parameter of the relative activity curve for TAK652 is centered on 9. This indicates that the concentration of TAK652 required to block internalization is 9 times greater than the concentration required to block HIV entry; i.e., TAK652 is 9 times more active as a blocker of HIV entry than CCL3L1-mediated CCR5 internalization.

Figure 6 shows the curves for internalization for all of the modulators expressed as a fraction of the concentration required to block HIV entry into PBMC. It can be seen from this figure that there is considerable texture in the relative effects of these modulators on internalization versus HIV entry. The data describing the specific activity of these modulators on internalization versus HOS cell and PBMC HIV entry is given in Table 4. The selectivities for these modulators on internalization versus HIV entry is shown graphically in Fig. 7. It can be seen from this figure that the modulators fall into loose categories with respect to internalization versus HIV entry. Specifically, for the HOS, compounds cluster into groups comprising TAK652 > (aplaviroc, vicriviroc, maraviroc, TAK779 > SCh-C). For the PBMC, the clusters are slightly different: TAK652 > (aplaviroc, vicriviroc, maraviroc) > (TAK779, Sch-C). To compare the modulators with one another with respect to internalization versus HIV-entry selectivity, t tests were carried out; the summary of these comparisons is shown in Fig. 8. Where modulators do not share a common letter, the differences in their selectivity are statistically different ($P \leq 0.01$).

Molecular Mechanism of Internalization Blockade. These entry inhibitors have been shown to be allosteric with respect to binding of HIV and chemokines to CCR5. Under these circumstances, their binding is permissive (Kenakin, 2005) in that there are protein species consisting of simultaneously bound CCL3L1 and modulator and also HIV and modulator. Such systems allow the cobinding ligand (either CCL3L1 or HIV) to influence the effect of the modulator. The most simple model to describe such an allosteric system combines the Ehlert model for allosteric binding (Ehlert, 1988) and the operational model for GPCR function (Black and Leff, 1983); it dictates that the fractional quantity of receptor species bound to either HIV or CCL3L1 in the presence of the modulator is described by the following equation (Kenakin, 2005; Price et al., 2005; Kenakin et al., 2006)

$$R = \frac{[A]/K_A\tau(1 + \alpha\beta[B]/K_B)E_{max}}{[A]K_A(1 + \alpha[B]/K_B + \tau(1 + \alpha\beta[B]/K_B)) + [B]/K_B + 1} \quad (4)$$

where R (response) denotes either CCL3L1-induced internalization or HIV entry. The equilibrium dissociation constants for the cobinding ligand (denoted [A]) and the modulator

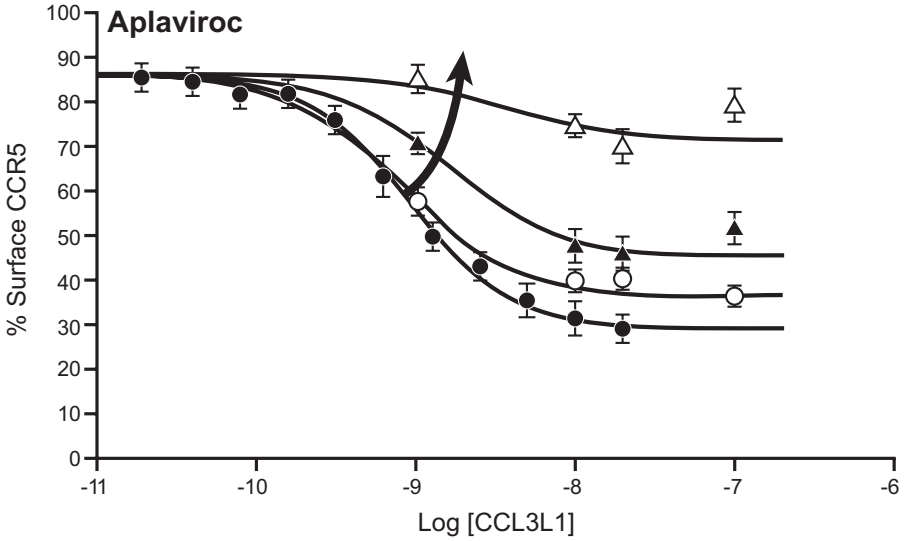

Fig. 3. Concentration-response curves for CCL3L1-mediated internalization of CCR5 in the absence (●) and presence of 0.128 (○), 0.64 (▲), and 3.2 nM (△) aplaviroc. Arrow shows direction of change for concentration response curves with increasing concentration of aplaviroc. For all datapoints, n = 2 or 3 with error representing range or S.E.M.

(denoted [B]) are K_A and K_B, respectively. τ represents the pharmacological efficacy coupling of the receptor for fusion or separately for internalization, α is the change in the affinity of the cobinding ligand (either HIV or CCL3L1) when the modulator is bound, and β is the change in efficacy of the cobinding ligand with modulator bound [note that in Kenakin (2005) and Kenakin et al. (2006), the efficacy term was denoted ζ; this has been changed to β to be consistent with Price et al., 2005]. It is important to note that, in keeping with the known probe dependence of allosteric modulators (Christopoulos and Kenakin, 2002), the values for α and β for HIV may (and most likely will) be different from those for CCL3L1.

Equation 4 can be used to deduce selective effects of modulators on affinity (α) and efficacy (β) of CCL3L1 for CCR5. The change in the location parameter of CCL3L1 curves in the presence of modulator (expressed as a dose ratio of EC_{50} values where D_R = [EC_{50} in presence of modulator]/[EC_{50} in absence of modulator]) is given by:

$$D_R = \frac{([B]/K_B + 1)(1 + \tau)}{(1 + \alpha[B]/K_B + \tau(1 + \alpha\beta[B]/K_B))} \quad (5)$$

Because these modulators completely suppress CCL3L1-induced internalization, $\beta = 0$, reducing the relation to:

$$D_R = \frac{([B]/K_B + 1)(1 + \tau)}{(1 + \tau + \alpha[B]/K_B)} \quad (6)$$

It can be seen from eq. 6 that if the modulator reduces the affinity of CCL3L1 for CCR5 ($\alpha < 1$), then DR will necessarily be > 1; i.e., CCL3L1 curves will shift to the right with increasing concentrations of modulators. However, if the modulator has no effect on affinity ($\alpha = 1$) or potentiates CCL3L1 ($\alpha > 1$), then DR values will be low (little shift to the right) for systems of low receptor reserve (low τ values). Dextral displacement still can be observed, however, in sys-

TABLE 2
Displacement of CCL3L1 curves in the presence of modulators
Ratio indicates EC_{50} (presence of modulator)/EC_{50} (no modulator).

Concentration	EC_{50}	Ratio
	nM	
Aplaviroc		
0 nM	0.84	1.0
0.128 nM	0.8	0.95
0.64 nM	1.24	1.48
3.2 nM	2.2	2.62
Maraviroc		
0 nM	0.95	1.0
0.128 nM	1.0	1.05
0.64 nM	1.05	1.11
3.2 nM	1.10	1.16
TAK652		
0 nM	0.91	1.0
0.128 nM	2	2.20
0.64 nM	4.1	4.51
3.2 nM	7	7.69
TAK779		
0 nM	0.91	1
0.128 nM	1.0	1.10
0.64 nM	2.10	2.31
3.2 nM	6.40	7.03
Vicriviroc		
0 nM	0.65	1
0.128 nM	0.7	1.08
0.64 nM	0.67	1.03
3.2 nM		

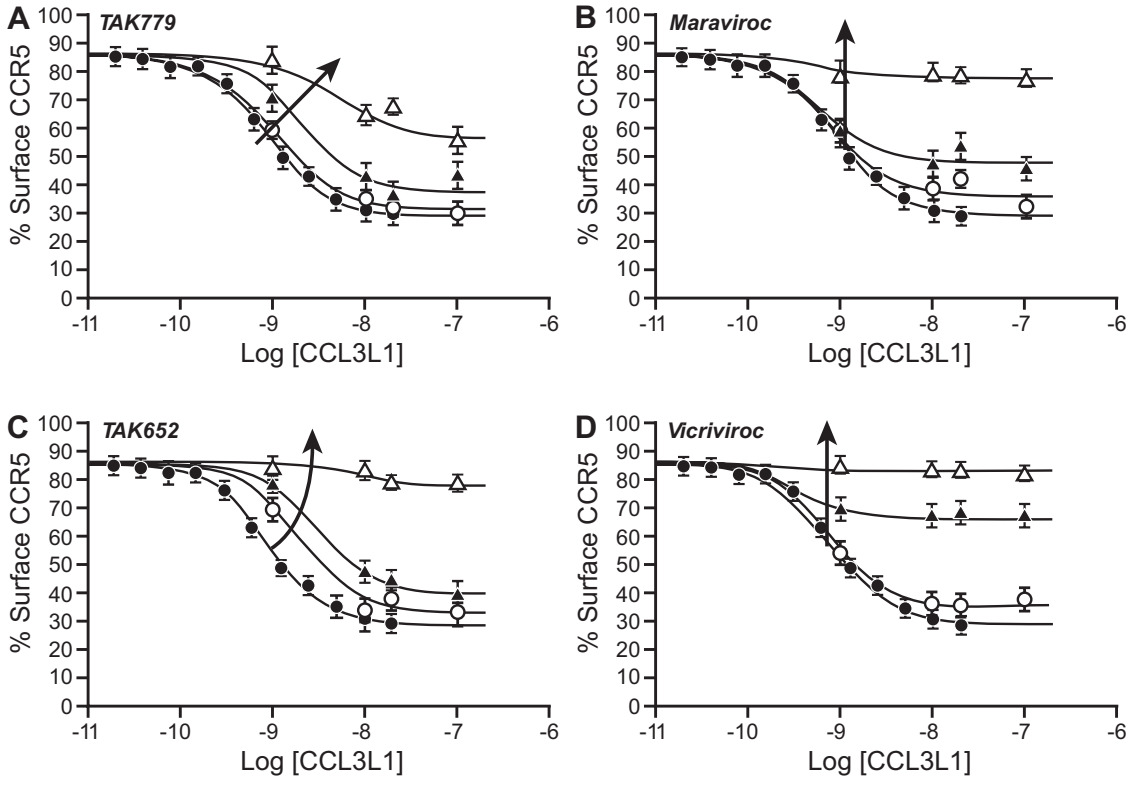

Fig. 4. Concentration-response curves for CCL3L1-mediated internalization of CCR5 in the absence (●) and presence of CCR5 modulators. A, TAK779, 0.64 (○), 3.2 (▲), and 16 (△) nM. B, maraviroc, 0.13 (○), 0.64 (▲), and 3.2 (△) nM. C, TAK652, 0.64 (○), 3.2 (▲), and 16 (△) nM. D, vicriviroc, 0.64 (○), 3.2 (▲), and 16 (△) nM.

TABLE 3
Effects of modulators on CCR5 internalization (pIC_{50})
S.E.M. values calculated based on a pooled estimate of residual standard deviation = 0.2925.

Compound	*N*	Mean	S.D.	S.E.M.
Aplaviroc	17	9.04	0.26	0.071
TAK652	10	8.32	0.298	0.093
Maraviroc	9	9.12	0.168	0.098
TAK779	9	8.18	0.463	0.078
Vicriviroc	14	8.63	0.269	0.078
Sch-C	4	8.86	0.201	0.146

tems of high receptor reserve (high τ values). The fact that there was little dextral displacement observed in Figs. 3 and 4 for aplaviroc, maraviroc, and vicriviroc (full curves were not obtained for Sch-C) is consistent with the notion that these modulators produce very little effect on CCL3L1 affinity. Thus, their main mechanism of action for inhibition of internalization is through blockade of CCL3L1 efficacy for the induction of internalization. Some dextral displacement was observed for TAK779 and TAK652, indicating that these modulators reduce the affinity of CCL3L1 as well as the efficacy.

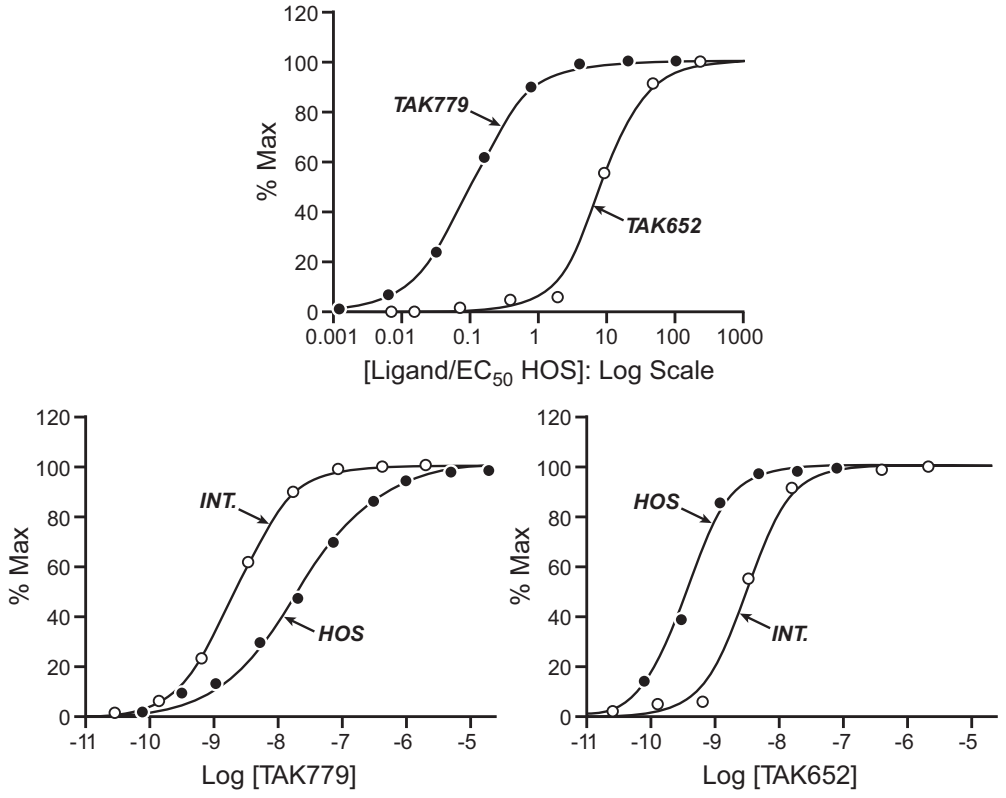

Fig. 5. Relative potency of CCR5 modulators on HIV (in this case, HOS cells) and internalization. Bottom, concentration response curves for TAK652 and TAK779 for inhibition of HIV (●) and internalization (○). It can be seen that the relative potencies for these two modulators reverse for the two activities. Top, curves for inhibition of internalization to TAK779 (●) and TAK652 (○). Ordinates, percentage inhibition of CCL3L1-induced internalization of CCR5. Abscissae, molar concentration of modulator in internalization assay divided by the IC_{50} concentration of modulator required for HOS HIV entry inhibition on a logarithmic scale (relative potency of modulator on internalization versus HOS).

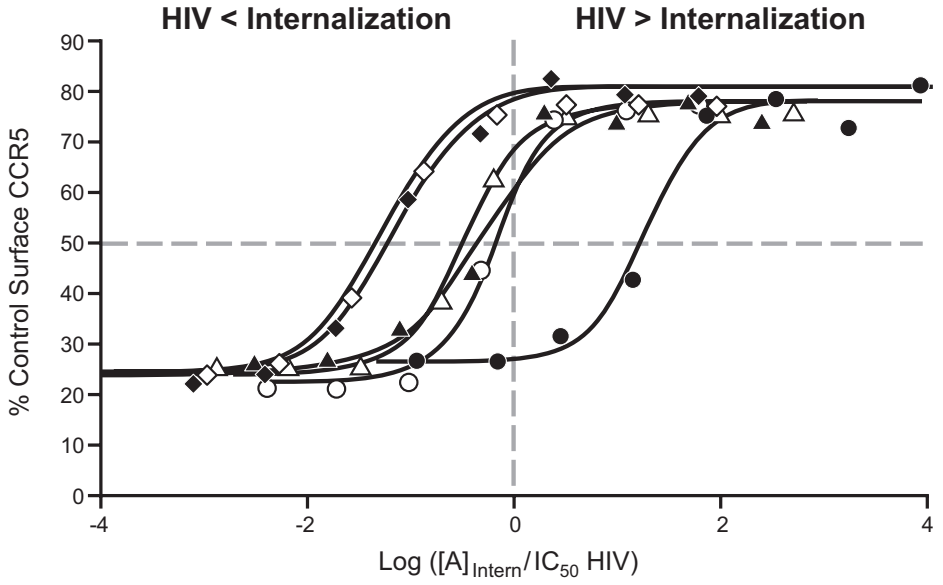

Fig. 6. Relative potency of modulators on internalization versus HIV (PBMC). Ordinates, percentage inhibition of CCL3L1-induced internalization of CCR5. Abscissae, molar concentration of modulator in internalization assay divided by the IC_{50} concentration of modulator required for PBMC HIV entry inhibition on a logarithmic scale (relative potency of modulator on internalization versus PBMC). Curves representing relative activity for Sch-C (◇), TAK779 (♦), maraviroc (△), vicriviroc (▲), aplaviroc (○), and TAK652 (●). Crossed dotted lines represent location parameter of a curve for a compound that is equiactive in both assays. Curve to the left indicates compound that is more potent as an inhibitor of internalization versus HOS; curve to the right indicates a modulator that is relatively more potent as an HIV entry inhibitor over internalization.

Discussion

Numerous lines of evidence from structural and mutagenesis studies (Dragic et al., 2000; Castonguay et al., 2003; Tsamis et al., 2003; Nishikawa et al., 2005; Maeda et al., 2006; Seibert et al., 2006; Westby et al., 2007; Kondru et al., 2008) and biochemical studies (Watson et al., 2005) indicate that these small-molecule inhibitors of CCR5-mediated HIV entry are allosteric modulators. The data describing the allosteric modulation of CCR5 receptors reported here illustrate some further characteristic allosteric properties of these ligands. Specifically, allosteric modulators have three features that distinguish them from orthosteric antagonists. Although the former simply preclude access of the endogenous ligand to the receptor, allosteric modulators cobind with the natural ligand to the receptor and can modify the natural ligands' subsequent actions. This leads to the following three behaviors: 1) modulator effects are saturable (receptor function can be modified but need not be completely blocked), 2) modulators are probe-dependent (different endogenous ligands can be differentially affected), and 3) modulators can affect the affinity and efficacy of ligands to a different extent. This study illustrates two of these three properties: *probe dependence* and *differential alteration of affinity and efficacy*.

The allosteric probe dependence demonstrated in these studies is the differential relative sensitivity of the HIV entry and internalization process, both mediated by CCR5. The highly statistically significant difference in the ratio of IC_{50} values for HIV entry versus internalization within the group of CCR5 modulators is inconsistent with a simple orthosteric binding of these ligands to the receptor. If the interaction were orthosteric, then the antagonism for all pathways mediated by the single receptor would be uniform. This does not necessarily mean that the potency of antagonism for internalization and HIV entry should be the same, because the two assays may be run at differing stimulus inputs. However, any differences seen between the pIC_{50} values for the two processes in an orthosteric environment would be constant for all antagonists, and no texture in *relative* activity would be seen. The fact that there are statistically significant differences in the relative potencies for the blockade of internalization versus HIV entry precludes orthosteric interaction as a mechanism and is consistent with the known allosteric nature of these molecules.

TABLE 4

CCR5 selectivity: CCL3L1-mediated CCR5 internalization versus HOS and PBMC HIV-1 entry

Table shows mean difference in pIC_{50} values, with variance and degrees of freedom, for CCR5 CCL3L1-mediated internalization vs HOS and vs PBMC HIV-entry inhibition. The S.E.M. is calculated using the residual variance estimate pooled across compounds. Selectivity is estimated as 10 to the power of the difference between the mean pIC_{50} for HOS or PBMC HIV-1 entry and mean pIC_{50} for internalization. Also shown are the 95% lower and upper confidence limits for selectivity, which is calculated using critical values from the t-distribution, with degrees of freedom approximated using Satterthwaite's method (Stell et al, 1997).

Compound	Log diff	Variance	*df*	Selectivity
HOS				
Aplaviroc	−0.241	0.00534	64.2	**0.574** 0.42–0.8
TAK652	1.074	0.02537	354.1	**11.865** 5.76–24.4
Maraviroc	−0.135	0.01206	90.8	**0.732** 0.44–1.2
TAK779	−0.281	0.0133	110.1	**0.524** 0.31–0.89
Vicriviroc	0.039	0.0079	94.4	**1.094** 0.73–1.64
Sch-C	−0.689	0.02192	59.8	**0.205** 0.1–0.4
PBMC				
Aplaviroc	−0.237	0.00615	84.7	**0.5** 0.4–0.83
TAK652	1.105	0.02657	380.8	**12.7** 6–26.6
Maraviroc	−0.397	0.01268	100.2	**0.401** 0.24–0.67
TAK779	−0.445	0.01426	207.3	**0.067** 0.036–0.126
Vicriviroc	−0.445	0.01426	263.5	**0.36** 0.21–0.62
Sch-C	−1.35	0.02666	88	**0.045** 0.02–0.09

The second allosteric property exhibited by these molecules interacting with CCR5 is the separate modulation of affinity and efficacy. This has already been demonstrated in a striking manner for aplaviroc, a modulator that completely suppresses the efficacy of RANTES for CCR5 but has insignificant effects on the affinity of RANTES for the receptor. Thus, aplaviroc does not affect RANTES binding but does affect the ability of RANTES to mediate calcium response (Maeda et al., 2004; Watson et al., 2005). In the present studies, the concentration-response curves to CCL3L1 observed in the presence of increasing concentrations of the modulators indicated that three of then (maraviroc, aplaviroc, vicriviroc) produce their blockade of CCL3L1-induced CCR5 internalization through blockade of efficacy (the mechanism of receptor activation leading to internalization) without affecting the receptor affinity for CCL3L1. In contrast, some reduction in the affinity of CCL3L1 was produced by TAK779 and TAK652.

The separate modulation of affinity and efficacy by allosteric ligands is a potentially therapeutically valuable property. For example, if an allosteric antagonist were to increase the affinity of the receptor for the endogenous agonist, then a unique increase in antagonism with increasing agonist stimulus would result. This is because allosteric effects are reciprocal, meaning that if the allosteric modulator increases the affinity of the endogenous agonist, then the endogenous agonist also will increase the affinity of the receptor for the antagonist. Therefore, the higher the level of agonism in the system, the more potent will be the antagonist (assuming it blocks the effect through a suppression of efficacy). Such a stimulus-linked antagonism has been shown for the modulator ifenprodil for *N*-methyl-D-aspartate receptors (Kew et al.,1996) and for the cannabinoid CB1 receptor allosteric modulator Org27569 (Price et al., 2005). In the present case, this would mean that the modulator would increase its ability to block HIV in patients with higher levels of virus.

The second theme of this article relates to the therapeutic ramifications of the allosteric mechanism for these ligands in the blockade of HIV entry. Unlike orthosteric antagonists, which preclude receptor probe (HIV, chemokines) binding to the receptor, allosteric ligands produce permissive effects (Kenakin, 2005) because the modulator and probe bind simultaneously to the receptor. This opens the possibility of a probe dependence that would enable a natural chemokine functional activity for the receptor but preclude use by HIV (see Fig. 9, schematic). It is not completely clear whether sparing natural chemokine function for CCR5 systems would be beneficial or detrimental therapeutically, although there is considerable evidence to allow informed speculation.

The apparent normal health of patients with the Δ32 allele [which prevents functional cellular expression of CCR5 to the cell surface (Dean et al., 1996; Huang et al., 1996)] supports the notion that removal of CCR5 function has no negative effect on normal health. Under these circumstances, there would seem to be no impetus to preserve chemokine function, at least within the context of preventing deleterious effects of removing CCR5 function. However, a closer examination of the Δ32 population reveals a higher incidence of health abnormalities, such as increased severity of symptoms in West Nile Virus disease with concomitant increased risk of death (Glass et al., 2006) and, notably, abnormality in hepatic function. For example, patients with the Δ32 allele have a greater incidence of liver disease and sclerosing cholangitis (Eri et al., 2004). Furthermore, hepatic pathology is found in patients with the Δ32 allele, who have increased vulnerability to liver disease (Welniak et al., 2004) and a greater risk of biliary lesions and mortality after liver transplantation (Moench et al., 2004). In addition, a mild form of immunodeficiency has been associated with loss of CCR5 function (de Silva and Stumpf, 2004; Lederman et al., 2006). These abnormalities in liver function coupled with the observed acute liver toxicity of the CCR5 HIV entry inhibitor aplaviroc (Crabb, 2006) suggest that CCR5 may play a role in normal liver function or protection against liver insult. However, a strong counter to this idea is the observation that no liver toxicity is seen in long-term maraviroc treatment of patients with AIDS in clinical trials. On the strength of this finding alone, it may be premature to seriously consider the loss of CCR5 function as directly deleterious to normal health.

The other possible benefit of sparing normal CCR5 chemokine function, in addition to its role in normal initiation of adaptive immune responses and trafficking of effector cells to sites of infection and inflammation (Lederman et al., 2006), is the potential protective effect of this system against HIV. This possibility is rooted in the knowledge that chemokines block HIV entry in vitro (Simmons et al., 1997; Mack et al., 1998). Likewise, high levels of chemokines have been associated with delay of AIDS progression (Ullum et al., 1998; Garzino-Demo et al., 1999; Shieh et al., 2001; Heredia et al., 2003; Rogez et al., 2003; Xiang et al., 2004). A particularly interesting study of 1064 patients infected with HIV in 57 populations around the world showed a strong inverse correlation between the gene copy number for CCL3L1 (this is variable in humans) and progression to AIDS. Thus, patients with high CCL3L1 gene copy number showed a highly statistically significantly greater survival rate compared with patients with low CCL3L1 gene copy number.

Although some HIV inhibition by chemokines may be due to steric inhibition of HIV binding (Pastore et al., 2003), the most prevalent data indicate that chemokines protect against HIV infection through internalization of the receptor (Alkhatib et al., 1997; Amara et al., 1997; Mack et al., 1998; Pastore et al., 2003). In fact, some modified chemokines, such as PSC-RANTES (Hartley et al., 2004), have been engineered to prevent HIV entry through CCR5 internalization. There-

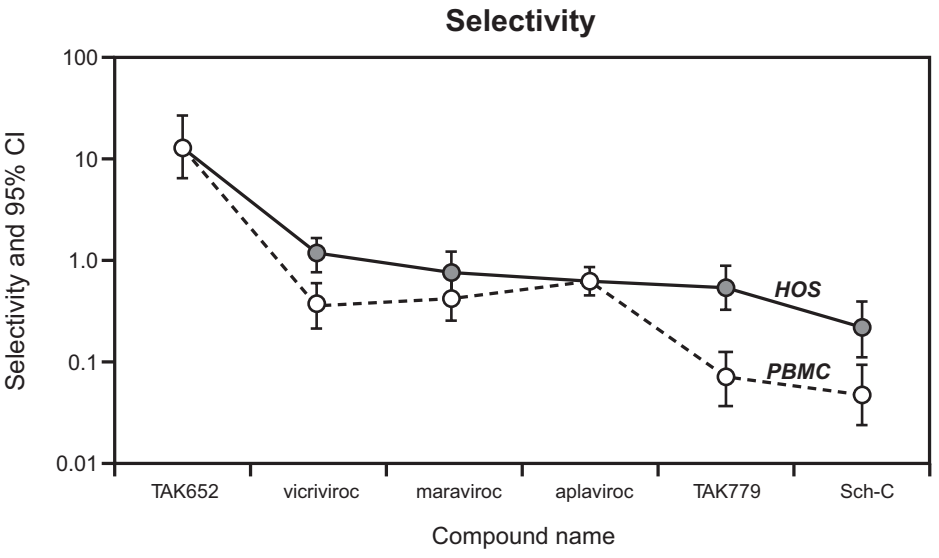

Fig. 7. Ratios (log scale as ordinates) of IC_{50} values for internalization versus HIV entry inhibition in HOS cells (solid lines) and PBMCs (dotted lines). Bars represent 95% confidence limits of the ratio estimates.

HOS

Compound	Selectivity	Connecting letters*		
TAK652	11.9	A		
vicriviroc	1.09		B	
maraviroc	0.732		B	
aplaviroc	0.572		B	
TAK 779	0.524		B	C
Sch-C	0.205			C

PBMC

Compound	Selectivity	Connecting letters*		
TAK652	12.7	A		
aplaviroc	0.580		B	
maraviroc	0.401		B	
vicriviroc	0.359		B	
TAK 779	0.0671			C
Sch-C	0.0445			C

* Compounds that do not share a common letter are significantly different at the 0.01 level

Fig. 8. Comparison of selectivity for different compounds based on the HOS and PBMC assays. Tabulated values are the ratio of IC_{50} values for internalization versus HIV entry inhibition in HOS cells (column 2) and PBMC (column 7). Compounds that do not share a common connecting letter are significantly different at the 0.01 level.

fore, an obvious possible reason for the HIV protective effect of high gene copy number for CCL3L1 is that the patients producing higher levels of CCL3L1 may have lower cell surface levels of CCR5 because of CCL3L1-induced internalization of CCR5. It is noteworthy that an inverse link between CCL3L1 and CCR5 receptor levels has been reported (Ketas et al., 2007). The corollary to this idea is the known faster progression to AIDS of patients infected with HIV who have allelic variants of the CCR5 promoter that cause elevated levels of CCR5 on the cell surface (Martin et al., 1998; McDermott et al., 1998).

As a baseline, a CCR5 HIV treatment that blocks HIV entry and chemokine function should be effective. However, if a functioning chemokine system assists in preventing HIV entry and subsequent progression to AIDS, then a CCR5 HIV-blocking drug that allows chemokines to function normally (Fig. 9) may offer a significant improvement in efficacy. Under these circumstances, the modulator-mediated HIV entry blockade would be assisted by a healthy functioning natural chemokine system (much like the effect seen with CCL3L1 gene copy number).

The previous discussion illustrates the possible advantages of a function-sparing CCR5 allosteric modulator in terms of HIV entry, raising the possibility that there may be some deleterious effects of a functioning CCR5 chemokine system in AIDS as well. This raises the alternative possibility that there might be beneficial aspects of the blockade of CCR5 function by CCR5 entry inhibitor modulators. Specifically, the role of CCR5 in chemokine-induced activation and migration of leukocytes (Beck et al., 1997), the pro-inflammatory effects of CCR5 activation by chemokines (Nardese et al., 2001) and possible role in HIV replication through CCR5 receptor activation, either by chemokines (Kinter et al., 1998; Lin et al., 2006) or HIV envelope (Weissman et al., 1997), could theoretically contribute to complications in the treatment of AIDS. With this in mind, the blockade of the inflammatory effects of CCR5 activation could actually be beneficial. However, these arguments are usually considered when CCR5 agonists such as PSC-RANTES, through their known active CCR5 internalizing properties, are proposed as treatments. Under these circumstances, CCR5 agonism would be a prevalent and constant effect. The scenario with function-sparing CCR5 modulators is quite different in that they would only allow chemokine stimulation due to normal physiological function and would not produce constant agonism. Accordingly, it could be argued that an intermittent CCR5 activation through a naturally operating chemokine system would not produce undue inflammatory agonism. On balance, the idea of preserving natural chemokine function may be so dissimilar to a scenario of powerful synthetically induced CCR5 agonism as to not be relevant to this present discussion.

In summary, it presently is not clear whether CCR5 function-sparing HIV entry inhibition would be useful in the treatment and prevention of AIDS, although the data linking CCL3L1 function to survival is strongly suggestive. It is clear that as more allosteric modulators are introduced into therapy, texture in HIV versus chemokine inhibition, an expected property of allosteric modulation, may be observed. It will be very interesting to compare therapeutic profiles of these

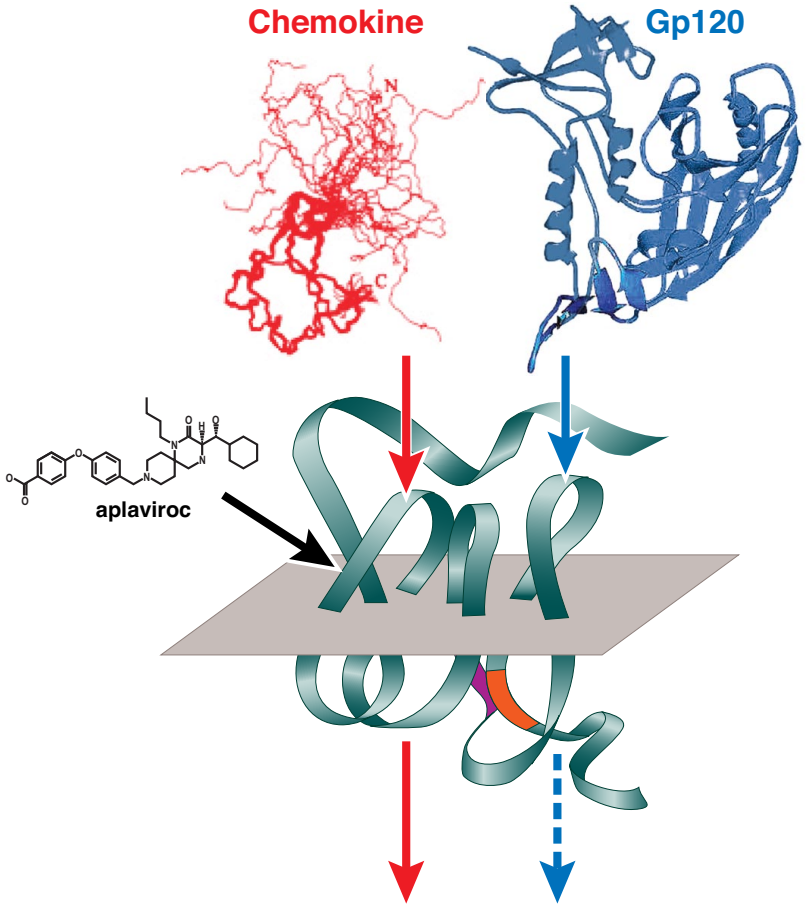

Fig. 9. Schematic diagram of cobinding of a CCR5 modulator, CCL3L1, and the HIV envelope binding protein gp120. The separation of binding sites for these receptor probes leaves open the possibility that an allosteric modulator could bind to the receptor to prevent HIV binding but otherwise not affect the binding and function of CCL3L1 and other chemokines

modulators in the future to determine, through translational medicine, whether such function-sparing effects truly provide a therapeutic advantage.

References

Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, and Berger EA (1996) CC CCR5, a RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272:**1955–1958.

Alkhatib G, Locati M, Kennedy PE, Murphy PM, and Berger EA (1997) HIV-1 coreceptor activity of CCR5 and Its inhibition by chemokines: independence from G protein signaling and importance of coreceptor downmodulation. *Virology* **234:** 340–348.

Amara A, Gall SL, Schwartz O, Salamero J, Montes M, Loetscher P, Baggiolini M, Virelizier JL, and Arenzana-Seisdedos F (1997) HIV Coreceptor downregulation as antiviral principle: SDF-1 α-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. *J Exp Med* **186:**139–146.

Baba M, Nishimura O, Kanzaki N, Okamoto M, Sawada H, Iizawa Y, Shiraishi M, Aramaki Y, Okonogi K, Ogawa Y, et al. (1999) A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. *Proc Natl Acad Sci U S A* **96:**5698–5703.

Baba M, Takashima K, Miyake H, Kanzaki N, Teshima K, Wang X, Shiraishi M, and Iizawa Y (2005) TAK-652 inhibits CCR5-mediated human immunodeficiency virus type 1 infecton in vitro and has favorable pharmacokinetics in humans. *Antimicrob Agents Chemother* **49:**4584–4591.

Baroudy BM, Clader JW, Josien HB, McCombie SW, McKittrick BA, Miller MW, Neustadt BR, Palani A, Smith EM, Steensma R, et al. (2000a), inventors; Schering Corp., Baroudy BM, Clader JW, Josien HB, McCombie SW, McKittrick BA, Miller MW, Neustadt BR, Palani A, Smith EM, Steensma R, et al., assignees. Piperazine derivatives useful as CCR5 antagonists. World Patent no. WO2000066558. 2000 Sep 11.

Baroudy BM, Clader JW, Josien HB, McCombie SW, McKittrick BA, Miller MW, Neustadt BR, Palani A, Steensma R, Tagat JR, et al. (2000b), inventors; Schering Corp., Baroudy BM, Clader JW, Josien HB, McCombie SW, McKittrick BA, Miller MW, Neustadt BR, Palani A, Steensma R, Tagat JR, et al., assignees. Piperidine derivatives useful as CCR5 antagonists. World Patent no. WO2000066559. 2000 Sep 11.

Beck LA, Dalke S, Leiferman KM, Bickel CA, Hamilton R, Rosen H, Bochner BS, and Schleimer RP (1997) Cutaneous injection of RANTES causes eosinophil recruitment: comparison of nonallergic and allergic human subjects. *J Immunol* **159:** 2962–2972.

Black JW and Leff P (1983) Operational models of pharmacological agonist. *Proc R Soc Lond B Biol Sci* **220:**141–162.

Castonguay LA, Weng Y, Adolfsen W, Di Salvo J, Kilburn R, Caldwell CG, Daugherty BL, Finke PE, Hale JJ, Lynch CL, et al. (2003) Binding of 2-aryl-4-(piperidin-1-yl)butanamines and 1,3,4-trisubstituted pyrrolidines to human CCR5: a molecular modeling-guided mutagenesis study of the binding pocket. *Biochemistry* **42:** 1544–1550.

Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, et al. (1996) The β-chemokinereceptors CCR3 and CCR5 facillitate infection by primary HIV-1 isolates. *Cell* **85:**1135–1148.

Christopoulos A and Kenakin T (2002) G-protein coupled receptor allosterism and complexing. *Pharmacol Rev* **54:**323–374.

Crabb C (2006) GlaxoSmithKline ends aplaviroc trials. *AIDS* **20:**641.

Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R, Goedert JJ, Buchbinder SP, Vittinghoff E, Gomperts E, et al. (1996) Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study [published erratum appears in *Science* **274:**1069, 1997]. *Science* **273:**1856–1862.

Demarest JS, Shibayama R, Ferris C, Vavro M, St Clair M, and Boone M (2004) A novel CCR5 antagonist, 873140, exhibits potent in vitro anti-HIV activity. XV International AIDS Conference; Bangkok, Thailand; 2004 July 11–16. Abstract WeOrA1231.

Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, et al. (1996) Identification of a major coreceptor for primary isolates of HIV-1. *Nature* **381:**661–666.

de Silva E and Stumpf MP (2004) HIV and the CCR5-Δ32 resistance allele. *FEMS Microbiol Lett* **241:**1–12.

Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, et al. (1996) HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381:**667–673.

Dragic T, Trkola A, Thompson DA, Cormier EG, Kajumo FA, Maxwell E, Lin SW, Ying W, Smith SO, Sakmar TP, et al. (2000) A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. *Proc Natl Acad Sci U S A* **97:**5639–5644.

Ehlert FJ (1988) Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. *Mol Pharmacol* **33:**187–194.

Eri R, Jonsson JR, Pandeya N, Purdie DM, Clouston AD, Martin N, Duffy D, Powell EE, Fawcett J, Florin TH, et al. (2004) CCR5-Delta32 mutation is strongly associated with primary sclerosing cholangitis. *Genes Immun* **5:**444–450.

Fätkenheuer G, Pozniak AL, Johnson MA, Plettenberg A, Staszewski S, Hoepelman AI, Saag MS, Goebel FD, Rockstroh JK, Dezube BJ, et al. (2005) Efficacy of short-term monotherapy with maraviroc, a new CCR antagonist, in patients infected with HIV-1. *Nat Med* **11:**1170–1172.

Garzino-Demo A, Moss RB, Margolick JB, Cleghorn F, Sill A, Blattner WA, Cocchi F, Carlo DJ, DeVico AL, and Gallo RC (1999) Spontaneous and antigen-induced production of HIV-inhibitory β-chemokines are associated with AIDS-free status. *Proc Natl Acad Sci U S A* **96:**11986–11991.

Glass WG, McDermott DH, Lim JK, Lekhong S, Yu SF, Frank WA, Pape J, Cheshier RC, and Murphy PM (2006) CCR5 deficiency increases risk of symptomatic West Nile virus infection. *J Exp Med* **203:**35–40.

Gonzalez E, Kulkarni H, Bolivar H, Mangano A, Sanchez R, Catano G, Nibbs RJ, Freedman BI, Quinones MP, Bamshad MJ, et al. (2005) The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. *Science* **307:**1434–1440.

Hartley O, Gaertner H, Wilken J, Thompson D, Fish R, Ramos A, Pastore C, Dufour B, Cerini F, Melotti A, et al. (2004) Medicinal chemistry applied to a synthetic protein: development of highly potent HIV entry inhibitors. *Proc Natl Acad Sci U S A* **101:**16460–16465.

Hazen R, Harvey R, Ferris R, Craig C, Yates P, Griffin P, Miller J, Kaldor I, Ray J, Samano V, et al. (2007) In vitro antiviral activity of the novel, tyrosyl-based human immunodeficiency virus (HIV) type 1 protease inhibitor brecanavir (GW640385) in combination with other antiretrovirals and against a panel of protease inhibitor-resistant HIV. *Antimicrob Agents Chemother* **51:**3147–3154.

Heredia A, Davis C, Amoroso A, Dominique JK, Le N, Klingebiel E, Reardon E, Zella D, and Redfield RR (2003) Induction of G_1 cycle arrest in T lymphocytes results in increased extracellular levels of β-chemokines: a strategy to inhibit R5 HIV-1. *Proc Natl Acad Sci U S A* **100:**4179–4184.

Huang Y, Paxton WA, Wolinsky SM, Neumann AU, Zhang L, He T, Kang S, Ceradini D, Jin Z, Yazdanbakhsh K, et al. (1996) The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nat Med* **2:**1240–1243.

Jakubík J, Bacáková L, El-Fakahany EE, and Tucek S (1997) Positive cooperativity of acetylcholine and other agonists with allosteric ligands on muscarinic acetylcholine receptors. *Mol Pharmacol* **52:**172–179.

Jenkinson S, McCoy D, Kerner S, Ferris R, Lawrence W, Fox T, and Smith C (2003) Development of a high-throughput viral-free assay for the measurement of CCR5-mediated HIV/cell fusion. *Receptors Channels* **9:**117–123.

Kazmierski W, Bifulco N, Yang H, Boone L, DeAnda F, Watson C, and Kenakin TP (2003) Recent progress in discovery of small-molecule CCR5 chemokine receptor ligsnds as HIV-1 inhibitors. *Bioorg Med Chem* **11:**2663–2676.

Kazmierski W, Peckham JP, Duan M, Kenakin TP, Jenkinson S, Gudmundsson KS, Piscitelli SC, and Feldman PL (2005) Recent progress in the discovery of new CCR5 and CXCR4 chemokine receptor antagonists as inhibitors of HIV-1 entry. Part 2. *Curr Med Chem Anti-Infective Agents* **4:**133–152.

Kazmierski W, Gudmundsson KS, and Piscitelli SC (2007) Small molecule CCR5 and CXCR4-based viral entry inhibitors for anti-HIV therapy currently in development. *Annu Rep Med Chem* **42:**301–320.

Kenakin TP (2005) New concepts in drug discovery: collateral efficacy and permissive antagonism. *Nat Rev Drug Discov* **4:**919–927.

Kenakin T, Jenkinson S, and Watson C (2006) Determining the potency and molecular mechanism of action of insurmountable antagonists. *J Pharmacol Exp Ther* **319:**710–723.

Ketas TJ, Kuhmann SE, Palmer A, Zurita J, He W, Ahuja SK, Klasse PJ, and Moore JP (2007) Cell surface expression of CCR5 and other host factors influence the inhibition of HIV-1 infection of human lymphocytes by CCR5 ligands. *Virology* **364:**281–290.

Kew JN, Trube G, and Kemp JA (1996) A novel mechanism of activity-dependent NMDA receptor antagonism describes the effects of ifenprodil in rat cultured cortical neurons. *J Physiol* **497:**761–772.

Kinter A, Catanzaro A, Monaco J, Ruiz M, Justement J, Moir S, Arthos J, Oliva A, Ehler L, Mizell S, et al. (1998) CC-chemokines enhance the replication of T-tropic strains of HIV-1 in $CD4^+$ T cells: role of signal transduction. *Proc Natl Acad Sci U S A* **95:**11880–11885.

Kondru R, Zhang J, Ji C, Mirzadegan T, Rotstein D, Sankuratri S, and Dioszegi M (2008) Molecular interactions of CCR5 with major classes of small-molecule anti-HIV CCR5 antagonists. *Mol Pharmacol* **73:**789–800.

Lederman MM, Penn-Nicholson A, Cho M, and Mosier D (2006) Biology of CCR5 and its role in HIV infection and treatment. *JAMA* **296:**815–826.

Lin YL, Mettling C, Portalès P, Réant B, Robert-Hebmann V, Reynes J, Clot J, and Corbeau P (2006) The efficiency of R5 HIV-1 infection is determined by CD4 T-cell surface CCR5 density through G[alpha]i-protein signalling. *AIDS* **20:**1369–1377.

Mack M, Luckow B, Nelson PJ, Cihak J, Simmons G, Clapham PR, Signoret N, Marsh M, Stangassinger M, Borlat F, et al. (1998) Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. *J Exp Med* **187:**1215–1224.

Maeda K, Nakata H, Koh Y, Miyakawa T, Ogata H, Takaoka Y, Shibayama S, Sagawa K, Fukushima D, Moravek J, et al. (2004) Spirodiketopiperazine-based CCR5 inhibitor which preserves CC-chemokine/CCR5 interactions and exerts potent activity against R5 human immunodeficiency virus type 1 in vitro. *J Virol* **78:**8654–8662.

Maeda K, Das D, Ogata-Aoki H, Nakata H, Miyakawa T, Tojo Y, Norman R, Takaoka Y, Ding J, Arnold GF, et al. (2006) Structural and molecular interactions of CCR5 inhibitors with CCR5. *J Biol Chem* **281:**12688–12698.

Martin MP, Dean M, Smith MW, Winkler C, Gerrard B, Michael NL, Lee B, Doms RW, Margolick J, Buchbinder S, et al. (1998) Genetic variation of AIDS progression by a promoter variant of CCR5. *Science* **282:**1907–1911.

McDermott DH, Zimmerman PA, Guignard F, Kleeberger CA, Leitman SF, and Murphy PM (1998) CCR5 promoter polymorphism and HIV-1 disease progression. *Lancet* **352:**866–870.

Mitsuya H, Maeda K, Shibayama S, Takaoka Y (2002) inventors; ONO Pharmaceuticals, Mitsuya H, Maeda K, Shibayama S, Takaoka Y, assignees. Drugs containing triazaspiro[5.5]undecane derivatives as the active ingredient. World Patent no. WO2002074769. 2002 Sep 26.

Moench C, Uhrig A, Lohse AW, and Otto G (2004) CC chemokine receptor 5delta32 polymorphism—a risk factor for ischemic-type biliary lesions following orthotopic liver transplantation. *Liver Transpl* **10:**434–439.

Nardese V, Longhi R, Polo S, Sironi F, Arcelloni C, Paroni R, DeSantis C, Sarmientos P, Rizzi M, Bolognesi M, et al. (2001) Structural determinants of CCR5 recognition and HIV-1 blockade in RANTES. *Nat Struct Biol* **8:**611–615.

Nishikawa M, Takashima K, Nishi T, Furuta RA, Kanzaki N, Yamamoto Y, and Fujisawa J (2005) Analysis of binding sites for the new small-molecule CCR5 antagonist TAK-220 on human CCR5. *Antimicrob Agents Chemother* **49:**4708–4715.

Pastore C, Picchio GR, Galimi F, Fish R, Hartley O, Offord RE, and Mosier DE (2003) Two mechanisms for human immunodeficiency virus type 1 inhibition by n-terminal modifications of RANTES. *Antimicrob Agents Chemother* **47:**509–517.

Perros M, Price DA, Stammen BL, and Wood A (2001) inventors; Pfizer, Inc., Perros M, Price DA, Stammen BL, and Wood A, assignees. Preparation of therapeutic tropane derivatives. World Patent no. WO2001090106. 2001 Nov 29.

Price MR, Baillie GL, Thomas A, Stevenson LA, Easson M, Goodwin R, McLean A, McIntosh L, Goodwin G, Walker G, et al. (2005) Allosteric modulation of the cannabinoid CB1 receptor. *Mol Pharmacol* **68:**1484–1495.

Rogez C, Martin M, Dereuddre-Bosquet N, Martal J, Dormont D, and Clayette P (2003) Anti-human immunodeficiency virus activity of tau interferon in human macrophages: involvement of cellular factors and β-chemokines. *J Virol* **77:**12914–12920.

Schols D (2006) HIV co-receptor inhibitors as novel class of anti-HIV drugs. *Antivir Res* 71:216–226.

Seibert C, Ying W, Gavrilov S, Tsamis F, Kuhmann SE, Palani A, Tagat JR, Clader JW, McCombie SW, Baroudy BM, et al. (2006) Interaction of small molecule inhibitors of HIV-1 entry with CCR5. *Virology* **349:**41–54.

Shieh B, Yan YP, Ko NY, Liau YE, Liu YC, Lin HH, Chen PP, and Li C (2001) Detection of elevated serum beta chemokine levels in seronegative Chinese individuals exposed to human immunodeficiency virus type 1. *Clin Infect Dis* **33:**273–279.

Shiraishi M, Baba M, Aikawa K, Kanzaki N, Seto M, and Iizawa Y (2003), inventors; Takeda Chemical Industries, Ltd., Shiraishi M, Baba M, Aikawa K, Kanzaki N, Seto M, and Iizawa Y, assignees. Bicyclic compound, production and use as HIV inhibitors. World Patent no. WO2003014105. 2003 Feb 20.

Simmons G, Clapham PR, Picard L, Offord RE, Rosenkilde MM, Schwartz TW, Buser R, Wells TN, and Proudfoot AE (1997) Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science* **276:**276–279.

Steel RG, Torrie JH, and Dickey DA (1997) *Principles and Procedures of Statistics: A Biometrical Approach*, 3rd ed, McGraw-Hill, Boston, MA.

Tsamis F, Gavrilov S, Kajumo F, Seibert C, Kuhmann S, Ketas T, Trkola A, Palani A, Clader JW, Tagat JR, et al. (2003) Analysis of the mechanism by which small molecule CCR5 antagonists SCH-3,51,125 and SCH-3,50,581 inhibit human immunodeficiency virus type 1 entry. *J Virol* **77:**5201–5208.

Ullum H, Cozzi Lepri A, Victor J, Aladdin H, Phillips AN, Gerstoft J, Skinhøj P, and Pedersen BK (1998) Production of β-chemokines in human immunodeficiency virus (HIV) infection: evidence that high levels of macrophage inflammatory protein-1β are associated with a decreased risk of HIV disease progression. *J Infect Dis* **177:**331–336.

Watson C, Jenkinson S, Kazmierski W, and Kenakin TP (2005) The CCR5 receptor-based mechanism of action of 873140, a potent allosteric non-competitive HIV entry-inhibitor. *Mol Pharmacol* **67:**1268–1282.

Weissman D, Rabin RL, Arthos J, Rubbert A, Dybul M, Swofford R, Venkatesan S, Farber JM, and Fauci AS (1997) Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor. *Nature* **389:**981–985.

Welniak LA, Wang Z, Sun K, Kuziel W, Anver MR, Blazar BR, and Murphy WJ (2004) An absence of CCR5 on donor cells results in acceleration of acute graft-vs-host disease. *Exp Hematol* **32:**318–324.

Westby M, Smith-Burchnell C, Mori J, Lewis M, Mosley M, Stockdale M, Dorr P, Ciaramella G, and Perros M (2007) Reduced maximal inhibition in phenotypic susceptibility studies indicates that viral strains resistant to the CCR5 antagonist maraviroc utilize inhibitor-bound receptor for entry. *J Virol* **81:**2359–2371.

Xiang J, George SL, Wünschmann S, Chang Q, Klinzman D, and Stapleton JT (2004) Inhibition of HIV-1 replication by GB virus C infection through increases in RANTES, MIP-1α, MIP-1β, and SDF-1. *Lancet* **363:**2040–2046.

Address correspondence to: Terry Kenakin, Department of Biochemical Reagents and Assay Development, GlaxoSmithKline Research and Development, 5 Moore Drive, Research Triangle Park, NC 27709. E-mail: terry.p.kenakin@gsk.com