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Structural Basis for the Interaction of CCR5 with a Small Molecule, Functionally Selective CCR5 Agonist

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The chemokine receptor CCR5 is an attractive target for HIV-1 drug development, as individuals whose cells lack surface CCR5 expression are highly resistant to HIV-1 infection. CCR5 ligands, such as CCL5/RANTES, effectively inhibit HIV-1 infection by competing for binding opportunities to the CCR5 and inducing its internalization. However, the inherent proinflammatory activity of the chemotactic response of CCR5 ligands has limited their clinical use. In this study, we found that a novel small molecule, functionally selective CCR5 agonist, 2,2-dichloro-1-(triphenylphosphonio)vinyl formamide perchlorate (YM-370749), down-modulates CCR5 from the cell surface without inducing a chemotactic response and inhibits HIV-1 replication. In molecular docking studies of YM-370749 and a three-dimensional model of CCR5 based on the rhodopsin crystal structure as well as binding and functional studies using various CCR5 mutants, the amino acid residues necessary for interaction with YM-370749 were marked. These results provide a structural basis for understanding the activation mechanism of CCR5 and for designing functionally selective agonists as a novel class of anti-HIV-1 agents. *The Journal of Immunology,* **2006, 177: 3116–3122.**

The introduction of highly active antiretroviral therapy (HAART),[2] comprising two nucleoside/nucleotide reverse-transcriptase inhibitors plus a nonnucleoside reverse-transcriptase inhibitor or a protease inhibitor, has markedly decreased HIV-associated morbidity and mortality (1). However, there are still several problems with HAART. First, complicated regimens with large numbers of pills reduce long-term adherence to HAART. Second, adverse drug-associated effects require treatment modification or cessation (2). Finally, incomplete eradication of HIV-1 favors the emergence of multidrug-resistant strains (3). Therefore, the discovery of novel anti-HIV-1 agents with new mechanisms of action is still needed to increase the efficacy with which HIV replication is inhibited and delay the emergence of drug-resistant variants.

The β-chemokine receptor CCR5 belongs to the rhodopsin-type, G protein-coupled receptor (GPCR) superfamily (4) and is a major coreceptor for HIV-1 (5–10). Because individuals who are homozygous for the *CCR5Δ32* mutation do not express CCR5 on the cell surface and are highly resistant to macrophage-tropic HIV-1 infection (11, 12), CCR5 seems to be an attractive target for anti-HIV-1 drug development (13). In fact, low molecular weight CCR5 antagonists inhibited HIV-1 replication by disturbing the HIV adsorption step (14). Alternatively, CCR5 agonists such as CCL5/RANTES also inhibited HIV replication by two mechanisms: disturbing HIV adsorption to CCR5 and inducing CCR5 internalization (15, 16). Although peptide agonists for CCR5 are candidates for a new class of anti-HIV agent, they are not orally available and, moreover, have undesirable proinflammatory activities that are likely to induce adverse side effects (17).

In this study, we show that a small molecule compound, 2,2-dichloro-1-(triphenylphosphonio)vinyl formamide perchlorate (YM-370749), did not stimulate undesirable chemotactic activity but induced internalization of CCR5 from the cell surface. These results suggest that CCR5 active states induced by YM-370749 are different from those induced by CCL5. Our molecular model of the YM-370749–CCR5 complex provides a structural basis for understanding the activation mechanism of CCR5 as well as for designing a novel anti-HIV-1 agent.

Materials and Methods

Wild-type and mutant CCR5 expression

The murine pre-B cell line B300-19 was cultured in RPMI 1640 medium containing 10% FBS, 50 μM 2-mercaptethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. The expression vector pEF-BOS-Neo (18), carrying full-length CCR5 cDNA, was transfected into B300-19 cells by electroporation, and G418-resistant stable transformants were isolated. CCR5 mutants were generated by PCR using primers bearing the relevant specific mutations.

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) elevation assay

Cells were incubated with 5 μM fura-2 acetoxymethyl ester (Dojindo Laboratories) in HEPES-buffered salt solution (20 mM HEPES (pH 7.4), 140 mM NaCl, 4 mM KCl, 1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 0.05% BSA) at 37°C for 45 min. After washing, $[Ca^{2+}]_i$ was analyzed using CAF-110 (Jasco). Pertussis toxin (Alexis Biochemicals) was used at a concentration of 100 ng/ml and added 17 h before the agonists. The CCR5-selective antagonist SCH-351125 (19) was synthesized at Astellas Pharma and added 2 min before the agonists.

[^{35}S]GTPγS-binding assay

Cell membranes (5 μg/ml protein) were incubated at 25°C for 1.5 h with 150 pM [^{35}S]GTPγS (Amersham Biosciences), 5 mg/ml wheat germ agglutinin scintillation proximity assay beads, and 2 μM GDP in the presence

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[2] Abbreviations used in this paper: HAART, highly active antiretroviral therapy; B300-19/CCR5, B300-19 cells expressing CCR5; βGal, β-galactosidase; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; EGFP, enhanced GFP; FluV-A, influenza virus type A; GPCR, G protein-coupled receptor; LTR, long-terminal repeat; PHAM, PHA M; TM, transmembrane; YM-370749, 2,2-dichloro-1-(triphenylphosphonio)vinyl formamide perchlorate; WT, wild type.

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or absence of various concentrations of unlabelled ligands in incubation buffer (20 mM HEPES-NaOH (pH 7.05), 100 mM NaCl, 5 mM $MgCl_2$, and 0.2% (w/v) BSA). Radioactivity was counted using a TopCount scintillation counter (Packard Biosciences).

Flow cytometric analysis of CCR5 internalization

Cells were incubated for 3 h with different concentrations of CCL5 (PeproTech) or YM-370749 (Sigma-Aldrich Library of Rare Chemicals), immediately placed on ice, and washed with ice-cold FACS buffer (PBS, 1% FBS, and 0.1% NaN_3). The cells were stained with anti-CCR5 Ab (2D7; BD Pharmingen) or an isotype-matched control Ab, placed on ice for 30 min, washed twice with FACS buffer, and analyzed for levels of surface expression of CCR5 using a flow cytometer (FACSCalibur; BD Biosciences). Data analysis was conducted using CellQuest software (BD Pharmingen). Relative fluorescence intensity was calculated as follows: [mean channel fluorescence (ligands) − mean channel fluorescence (negative control)]/[mean channel fluorescence (medium} − mean channel fluorescence (negative control)].

Fluorescence microscopy

A PCR-synthesized β-arrestin-2 cDNA insert deleted from the stop codon was fused to the enhanced GFP (EGFP; BD Clontech) in the pEF-BOS-Neo expression vector. FuGENE 6 transfection reagent (Roche) caused the transient expression of CCR5, β-arrestin-2-EGFP, and G protein receptor kinase 2 in COS-1 cells. The cells were incubated in DMEM containing 0.1% (w/v) BSA for 12 h before treatment with 100 nM CCL5 or 30 μM YM-370749 for 1 h at 37°C. After being washed with ice-cold PBS, the cells were fixed in PBS containing 3% paraformaldehyde for 30 min. The samples were analyzed using confocal laser-scanning microscopy and a LSM5 PASCAL system (Zeiss).

Chemotaxis assay

Chemotaxis assays were performed using 96-well chemotaxis chambers (Neuro Probe) with 5-μm pore, polyvinylpyrrolidone-free, polycarbonate filters for 3 h at 37°C in a humidified 5% CO_2 incubator. Cells suspended at 5×10^6 cells/ml in RPMI 1640 supplemented with 0.1% (w/v) BSA were applied to the upper wells, with various concentrations of CCL5 or YM-370749 in the lower wells. Cell numbers migrating to the lower chambers were quantified by bioluminescent assay (ATP-Lite; PerkinElmer).

Antiviral assays

PBMC were stimulated for 3 days with PHA M (PHAM; 10 μg/ml; Sigma-Aldrich). PHAM-PBMC (10^6 cells/ml) were infected with HIV-1_{BaL} (R5 strain) or HIV-1_{HXB2} (X4 strain) equivalent to 1 ng/ml p24 in the presence of various concentrations of YM-370749. After 7 days incubation, the amounts of p24 Ag produced by the cells into the culture medium were determined by an enzyme immunoassay kit (Cellular Products).

CD4, CCR5, and long-terminal repeat (LTR)-controlled β-galactosidase (βGal) expression vectors were transfected into human glioma-derived NP-2 cells (NP-2/CD4/CCR5-LTR-βGal cells). NP-2/CD4/CCR5-LTR-βGal cells were infected with HIV-1_{BaL} or four clinical isolates (401, 409, 411, and 415) as described previously (20) and cultured in the presence of various concentrations of YM-370749. Forty-eight hours after viral exposure, all of the cells stained blue with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were counted. Cytotoxicity was determined by the MTT method as previously described (21).

Anti-viral activity against influenza virus type A (FluV-A) (Ishikawa/7/82 (H3N2); Rational Drug Design Laboratories) and HSV-1 (KOS strain; Rational Drug Design Laboratories) were determined using plaque-reduction assays (22). Madin-Darby canine kidney cells for anti-FluV-A assay and Vero cells for anti-HSV-1 assay were grown in Eagle's MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The culture medium was removed and replaced with a maintenance medium consisting of Eagle's MEM supplemented with 2% FBS and antibiotics, and viruses (100 PFU) were added. After a 1-h virus adsorption period, the cells were washed and filled with maintenance medium containing 0.8% methylcellulose. After a 4-day incubation for anti-influenza virus assay and a 3-day incubation for anti-HSV-1 assay, the number of plaques was counted. YM-370749 was included throughout the assay.

YM-370749 and CCL5 binding studies

Cell membranes (5 μg/ml protein) were incubated at 25°C for 3 h with 50 pM ^{125}I-labeled CCL3 (PerkinElmer) and 5 mg/ml wheat germ agglutinin scintillation proximity assay beads (Amersham Biosciences) in the presence or absence of various concentrations of unlabeled ligands in incubation buffer containing 50 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% (w/v) BSA. Radioactivity was counted using a TopCount scintillation counter (Packard Biosciences). Nonspecific binding was determined in the presence of 50 nM CCL3. Absolute inhibition constant (K_i) values were calculated from the IC_{50} using the equation of Cheng and Prusoff (23).

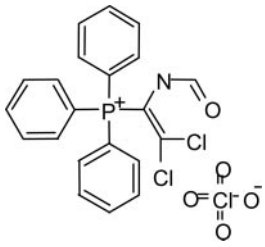

FIGURE 1. Two-dimensional structure of YM-370749. Molecular weight, 501; purity, 99% as determined by HPLC.

Molecular modeling and docking study

We created a three-dimensional model of the human CCR5 based on the sequence alignment and the crystal structure of bovine rhodopsin (24) using the MOE software package (Chemical Computing Group). The docking of YM-370749 with this model was performed using the GOLD program (25).

Results

YM-370749 is a functionally selective CCR5 agonist

To identify small molecule CCR5 agonists that induced CCR5 internalization, but not chemotaxis, in CCR5-expressing B300-19

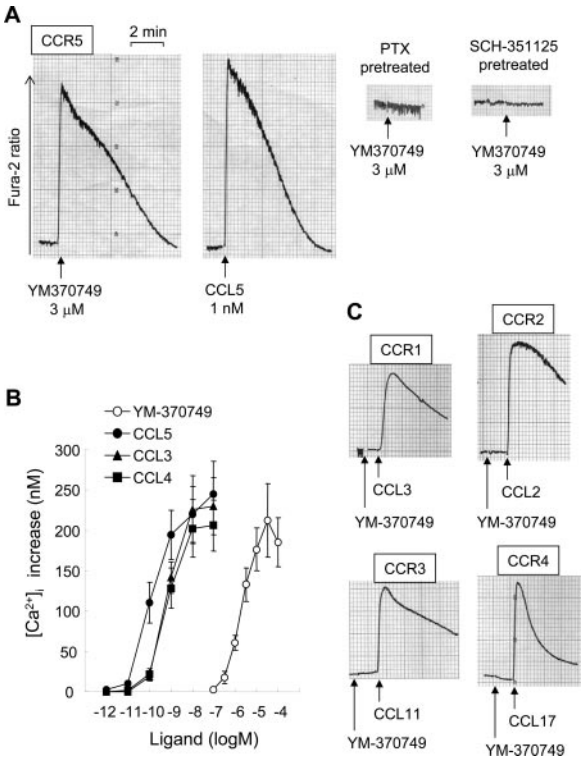

FIGURE 2. Increase in CCR5-mediated $[Ca^{2+}]_i$ caused by YM-370749. *A,* $[Ca^{2+}]_i$ increase in B300-19/CCR5 cells. YM-370749 or CCL5 were added to the B300-19/CCR5 cells, and $[Ca^{2+}]_i$ was measured using fura-2 fluorometry. Pertussis toxin (PTX; 100 ng/ml) and SCH-351125 (1 μM) were added 17 h and 2 min, respectively, before the addition of YM-370749. *B*, Concentration dependence of the initial transient increase in $[Ca^{2+}]_i$. Values are the mean ± SE of five separate experiments. *C*, $[Ca^{2+}]_i$ increase in B300-19/CCR1, CCR2, CCR3, or CCR4 cells. YM-370749 (30 μM) and the natural ligands (1 nM) were added.

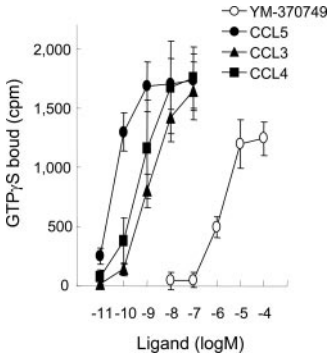

FIGURE 3. Concentration dependence of GTPγS binding. Values are the mean ± SE of three separate experiments.

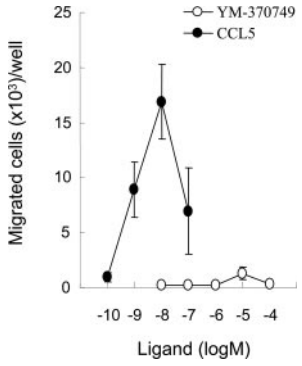

FIGURE 5. Chemotactic effects of CCL5 and YM-370749 on B300-19/CCR5 cells. Values are the mean ± SE of four separate experiments.

(B300-19/CCR5) cells, we conducted a large-scale, high-throughput screening of in-house chemical libraries by measuring $[Ca^{2+}]_i$ and discovered that YM-370749 (Fig. 1) had the required properties. A low molecular weight compound, YM-370749, elevated $[Ca^{2+}]_i$ in B300-19/CCR5 cells, which was also the case with the natural CCR5 ligand CCL5 (Fig. 2*A*). The $[Ca^{2+}]_i$ increase elicited by YM-370749 was completely inhibited by pretreatment with 100 ng/ml pertussis toxin, indicating that this compound signaled through a G_i protein-coupled receptor, and it was also inhibited by the CCR5-selective antagonist SCH-351125 (Fig. 2*A*). The increase of $[Ca^{2+}]_i$ by YM-370749 was concentration-dependent with an EC_{50} of 2.1 μM (Fig. 2*B*). This compound at 30 μM had no effect against CCR1-, CCR2-, CCR3-, or CCR4-expressing cells (Fig. 2*C*). YM-370749 also induced GTPγS binding to B300-19/CCR5 cell membranes in a concentration-dependent manner with an EC_{50} of 1.4 μM (Fig. 3).

It is well known that chemokines induce the internalization of their receptors. We analyzed the effects of YM-370749 on CCR5 internalization in B300-19/CCR5 cells. The cell surface CCR5 was measured by FACS using the CCR5-specific mAb 2D7. As shown in Fig. 4, *A* and *B*, YM-370749 induced CCR5 internalization in a concentration-dependent manner. YM-370749 did not affect the cell surface CCR5 level when the experiments were done at 4°C, which suggests that YM-370749 did not compete for 2D7 binding to CCR5. Next, YM-370749-induced CCR5 internalization was assessed by fluorescent microscopy using COS-1 cells transiently expressing CCR5, β-arrestin-2-EGFP, and G protein receptor kinase 2 (Fig. 4, *C–F*). It has been reported that β-arrestin-2 mediates CCR5 internalization (26) and that activation of CCR5 by chemokines results in the colocalization of the receptor and β-arrestin-2 (27). In the absence of ligands, the fluorescence was detected mainly at the cell surface (Fig. 4*C*). As is the case for CCL5, the addition of YM-370749 induced a profound redistribution of the staining that is consistent with intracellular accumulation of the receptor (Fig. 4, *D* and *E*). The effect of YM-370749 was inhibited by pretreating the cells with the CCR5 antagonist SCH-351125 (Fig. 4*F*).

In contrast to CCL5, YM-370749 did not stimulate significant chemotaxis in B300-19/CCR5 cells at any of the concentrations tested (Fig. 5). Pretreating the cells with YM-370749 was shown to

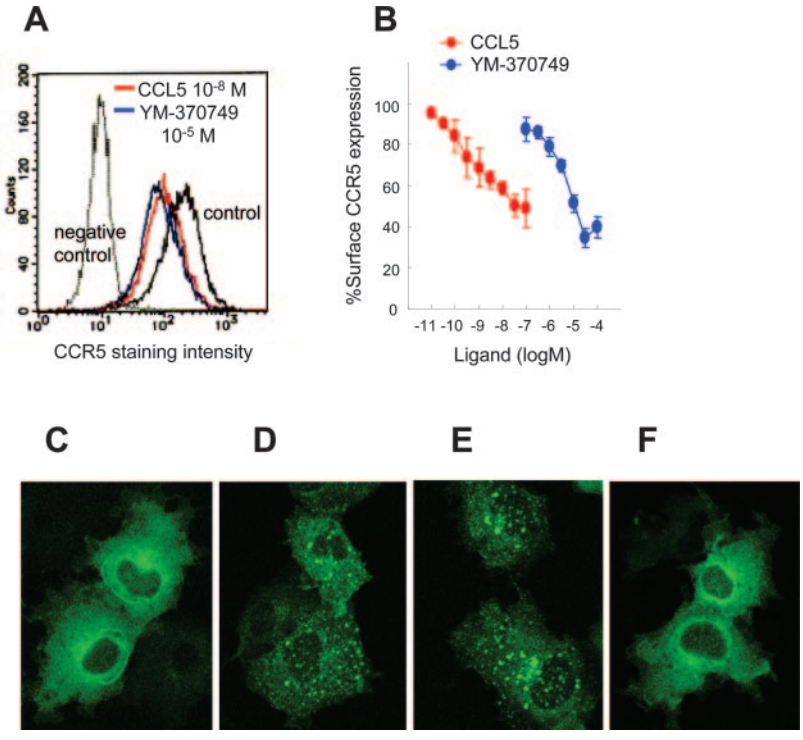

FIGURE 4. Down-modulation of CCR5 from the cell surface. *A*, Detection of cell surface CCR5 on B300-19/CCR5 cells using FACS. An isotype-matched mAb was used as the negative control. *B*, Concentration dependence of CCR5 down-modulation in B300-19/CCR5 cells. Values are the mean ± SE of three separate experiments. *C–F*, Fluorescence detection of β-arrestin-2-EGFP using confocal laser scanning microscopy. *C*, Untreated control. *D*, Cells treated with 100 nM CCL5. *E*, Cells treated with 30 μM YM-370749. *F*, Cells pretreated with 1 μM SCH-351125 for 15 min before the addition of 30 μM YM-370749.

Table I. *Anti-viral activities of YM-370749*[a]

Virus Type	Assay	IC_{50} (μM)	CC_{50}[b] (μM)
HIV-1 *BaL*	p24 ELISA	0.20	2.2
HIV-1 *HXB2*	p24 ELISA	>2.0	
HIV-1 *BaL*	β-Galactosidase	1.0 ± 0.05	16 ± 3.6
HIV-1 *401*	β-Galactosidase	1.1 ± 0.04	
HIV-1 *409*	β-Galactosidase	1.5 ± 0.15	
HIV-1 *411*	β-Galactosidase	0.74 ± 0.06	
HIV-1 *415*	β-Galactosidase	0.50 ± 0.08	
FluV-A *Ishikawa782*	Plaque reduction	>30	>30
HSV-1 *KOS*	Plaque reduction	>30	>30

[a] PBM were stimulated with PHAM (10 μg/ml) for 3 days and then infected with R5 strain BaL or X4 strain HXB2 in the presence of various concentrations of YM-370749. Viral production was determined 7 days after the infection by measuring p24 in culture supernatants using ELISA kit. The data are derived from a single assay. NP-2/CD4/CCR5-LTR-βGal cells were exposed to either HIV-1_{BaL} or four clinical isolates (401, 409, 411, and 415) in the presence of various concentrations of YM-370749. Viral infection was confirmed 2 days after exposure to the virus by counting the cells stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Values are the mean ± SE of three separate experiments. Madin-Darby canine kidney and Vero cells were infected with FluV-A and HSV-1, respectively. The virus was washed away after 1 h. After incubation for 4 days for the anti-FluV-A assay and incubation for 3 days for the anti-HSV-1 assay, the number of plaques was counted. YM-370749 was included throughout the assay.

[b] Cytotoxic concentrations of a compound that reduces the number of cells by 50%.

inhibit CCL5-induced chemotaxis (data not shown). We determined whether YM-370749 had any potential cytostatic or cytotoxic effects in the chemotaxis assay. The cells were incubated in RPMI 1640 medium supplemented with 0.1% BSA for 3 h in the presence or absence of 10^{-4} M YM-370749 under the same conditions as those used in chemotaxis assay. The cells were then cultured in RPMI 1640 supplemented with 10% FBS for 24 h before determination of the viable cell number. YM-370749 did not affect the cell growth. Taken together, these results suggest that YM-370749 is a functionally selective CCR5 agonist.

YM-370749 selectively inhibits the replication of R5 strain HIV-1

We examined the antiviral activities of YM-370749 against several HIV-1 strains including BaL, HXB2, four clinical isolates (401, 409, 411, and 415), FluV-A, and HSV-1. In the p24 ELISA using PHAM-PBMC, YM-370749 selectively inhibited the replication of the R5 strain HIV-1_{BaL} (IC_{50} = 0.20 μM), which uses CCR5 as a coreceptor. It did not, however, inhibit the X4 strain HIV-1_{HxB2}, which uses CXCR4 as a coreceptor (Table I). In the βGal assay, which does not allow the infection of X4 strain viruses (20), YM-370749 inhibited the infection of NP-2/CD4/CCR5-LTR-βGal cells by HIV-1_{BaL} and the four clinical isolates with similar potency (IC_{50} = 0.50–1.5 μM). As expected, at concentrations up to 30 μM YM-370749 did not exhibit any antiviral activity against FluV-A or HSV-1. These results suggest that the anti-HIV-1 activity of YM-370749 is specifically represented as interaction with CCR5.

Molecular model of the YM-370749–CCR5 complex

Structural analysis of the molecular complex between YM-370749 and human CCR5 will provide useful information for understanding the mechanism of CCR5 activation and the future drug design of a novel anti-HIV agent. We initially compared the binding affinity of YM-370749, CCL5, and CCL3 for both human and rhesus monkey CCR5. Rhesus CCR5 differs from human CCR5 by eight substitutions of the 352 total amino acid residues. We found that CCL5 and CCL3 bound to both human and rhesus monkey CCR5 with the same affinity, whereas YM-370749 bound to rhesus CCR5 11 times more weakly than to human CCR5 (Table II). To determine which amino acids were involved in YM-370749 binding, we created eight human CCR5 mutants, each containing one of the eight amino acid substitutions found in rhesus monkey CCR5: I9T, N13D, M49I, I52V, F78L, V130I, K171R, or I198M. The expression level of these mutated receptors on the B300-19 cell surface, as determined by FACS analysis, was comparable to that of wild-type (WT) CCR5, and a global conformational change caused by these mutations is unlikely given that that all mutants are equally recognized by four different mAbs specific to CCR5 (data not

Table II. *Binding affinities of YM-370749, CCL5, and CCL3 for human WT, rhesus WT, and various mutant human CCR5 receptors*

	YM-370749[a]		CCL5[a]		CCL3[a]	
Mutants	K_i (μM)	Ratio	K_i (pM)	Ratio	K_i (pM)	Ratio
Human WT	2.7 ± 0.16	1.0	48 ± 7.3	1.0	200 ± 14	1.0
Rhesus WT	29 ± 2.7[b]	11	21 ± 2.5	0.44	180 ± 16	0.9
Human I9T	2.7 ± 0.29	1.0	18 ± 4.8	0.37	240 ± 23	1.2
Human N13D	4.1 ± 0.46	1.5	52 ± 42	1.1	210 ± 17	1.1
Human M49I	3.0 ± 0.10	1.1	18 ± 5.3	0.38	230 ± 4.2	1.2
Human I52V	2.1 ± 0.21	0.79	24 ± 7.9	0.50	200 ± 13	1.0
Human F78L	2.6 ± 0.23	0.99	18 ± 6.5	0.37	190 ± 5.1	0.95
Human V130I	3.2 ± 0.17	1.2	12 ± 3.4	0.25	220 ± 13	1.1
Human K171R	4.6 ± 0.67	1.7	42 ± 5.3	0.87	190 ± 15	0.95
Human I198M	19 ± 2.5[b]	7.0	49 ± 14	1.0	220 ± 4.1	1.1

[a] K_i values are given for YM-370749, CCL5, and CCL3 inhibition of ^{125}I-labeled CCL3 binding to B300-19/CCR5 cell membranes. K_i values represent the mean ± SE of three experiments. The ratio given is relative to the K_i for human wild-type CCR5.

[b] Significant differences from human WT CCR5 with $p < 0.01$ (Dunnet's multiple comparison test).

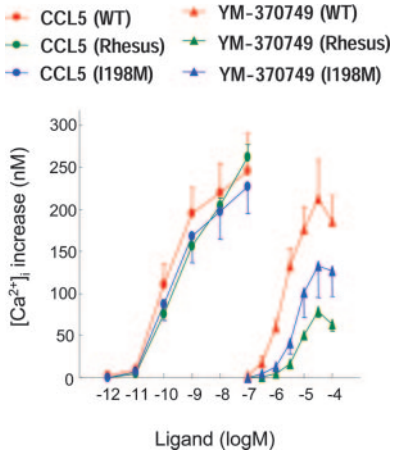

FIGURE 6. Comparison of $[Ca^{2+}]_i$ increase by YM-370749 in B300-19/human CCR5, rhesus CCR5, and human CCR5-I198M cells. Concentration dependence of an initial transient increase in $[Ca^{2+}]_i$ induced by YM-370749 and CCL5 in B300-19/human CCR5 (WT), B300-19/rhesus CCR5 (Rhesus), and B300-19/human-I198M cells (I198M) are shown. Values are the mean ±SE of five separate experiments.

shown). The binding affinities of CCL5 and CCL3 for all of the mutants were similar to or slightly higher than that of WT CCR5. The binding affinities of YM-370749 for these mutants were also comparable to that of the WT receptor, with the exception of CCR5-I198M; the affinity of YM-370749 for CCR5-I198M was 19 μM as compared with 2.7 μM for WT CCR5, which was a significant 7.0-fold reduction (Table II). We next examined the $[Ca^{2+}]_i$ elevation activity of YM-370749 in B300-19/CCR5, B300-19/CCR5-I198M, and B300-19/rhesus CCR5 cells (Fig. 6). The $[Ca^{2+}]_i$ elevation activity of CCL5 in B300-19/CCR5 cells was almost similar to those in B300-19/CCR5-I198M, and B300-19/rhesus CCR5 cells. However, YM-370749 could not reach the same maximum efficacy in B300-19/CCR5-I198M (62% elevation) or B300-19/rhesus CCR5 cells (37% elevation) as in B300-19/CCR5 cells, and it was less potent in B300-19/CCR5-I198M

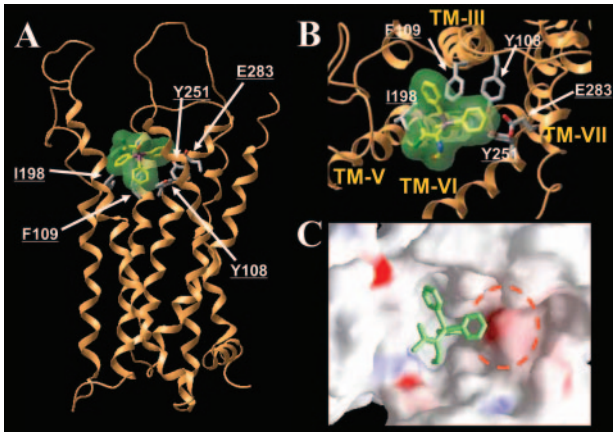

FIGURE 7. Structural model of the human CCR5-YM-370749 complex. *A,* Ribbon diagram. Arrows indicate the side chains of the key residues Tyr^{108} (Y108), Phe^{109} (F109), Ile^{198} (I198), Tyr^{251} (Y251), and Glu^{283} (E283). YM-370749 is shown as a stick model with the carbon atoms colored yellow and the van der Waals surface colored light green. *B*, Close-up view along the helical axes from the extracellular side. *C*, Surface representation of the binding pocket of human CCR5. The surface is colored according to the local electrostatic potential (56) (negative in red and positive in blue). The dashed red circle indicates the negatively charged region around Glu^{283}.

(EC_{50} = 4.7 μM) and B300-19/rhesus CCR5 cells (EC_{50} = 6.2 μM) than in B300-19/CCR5 cells (EC_{50} = 2.1 μM). These results implied that the Ile^{198} in CCR5 is an important residue for the interaction with YM-370749.

To further investigate the molecular interaction between YM-370749 and CCR5, we conducted a docking study with YM-370749 and a three-dimensional model of CCR5, which was based on the rhodopsin crystal structure. As shown in Fig. 7*A*, the proposed binding site for YM-370749 is located in a pocket surrounded by the human CCR5 transmembrane (TM) helices III, V, VI, and VII. Each of the three hydrophobic benzene groups of YM-370749 seem to contribute to the van der Waals interactions with the side chains of the hydrophobic residues, contacting Tyr^{108} and Phe^{109} in TM-III, Ile^{198} in TM-V, and Tyr^{251} in TM-VI (Fig. 7*B*). The importance of Ile^{198} was supported by the CCR5 mutational analysis described above. The docking study also pointed to an electrostatic interaction between the partially positively charged phenyl ring in YM-370749 and the negatively charged carboxyl group of Glu^{283} in TM-VII (Fig. 7*C*).

Discussion

To date, small molecule agonists to GPCR for small peptide ligands (<20 amino acid residues) such as cholecystokinin (28), angiotensin (29), somatostatin (30), and opioid (31), were reported. However, small molecule agonists to GPCR for high molecular weight protein ligands, such as chemokines (66, 69, and 68 amino acid residues for CCL3, CCL4, and CCL5, respectively) have not been reported. In this study, we showed that YM-370749 exerted several agonistic activities to CCR5, including intracellular Ca^{2+} elevation, GTPγS binding, and receptor down-modulation (Figs. 2–4). These agonistic activities by YM-370749 were mediated directly through CCR5, as evidenced by the observations that YM-370749 did not show any activity in B300-19/CCR1–4, and the agonistic activities of YM-370749 in B300-19/CCR5 such as $[Ca^{2+}]_i$ elevation and receptor down-modulation were completely inhibited by the CCR5-selective antagonist SCH-351125 (Figs. 2*A* and 4*F*). This is the first report regarding a small molecule agonist to GPCR for large peptides like chemokine.

Interestingly, in contrast to CCL5, YM-370749 did not stimulate undesirable chemotaxis. The fact that YM-370749 induced pertussis toxin-sensitive Ca^{2+} elevation, but not chemotaxis, indicated that the level of G_i protein activation was not sufficient for chemotaxis. These results suggest that YM-370749 and CCL5 stabilized different active conformations of CCR5, which then coupled with different intracellular signaling cascades. YM-370749 has been reported to be a kind of active state selective agonist (32) for mAbs against CCR5 (33), calcitonin ligands (34), and cholecystokinin analog (35). Because chemokine receptor activation stimulates various intracellular signaling events, including those involving the Jak/signaling transducer and transcription activator (36), PI3K (37), Syk (38), and Pyk2 (39), it will be interesting to see whether YM-370749 could stimulate these signaling events as well.

CCR5 antagonists that act by inhibiting the binding of HIV to CCR5 could induce drug-resistant variants in vitro (40). If a functionally selective agonist like YM-370749 showed anti-HIV activity by inducing receptor internalization, the rapid emergence of drug-resistant HIV variants might be avoided. Additionally, it has been reported that the cell surface CCR5 density is critical for HIV-1 infection (41, 42) and that receptor internalization makes the greater contribution to antiviral activity by CCR5 ligands (43). Compounds that promote internalization of CCR5 without undesirable proinflammatory activity are ideal agents against HIV-1 infection. YM-370749 selectively inhibited the replication of R5

strain HIV-1 but did not inhibit the X4 strain HIV-1 (Table I). These results suggest that YM-370749 inhibited HIV-1 replication by inducing CCR5 internalization and/or inhibiting HIV binding. Although the extent of CCR5 internalization caused by YM-370749 in B300-19/CCR5 cells was modest (Fig. 4*B*), YM-370749 may induce CCR5 internalization more efficiently in PBM or NP-2 cells. Internalization of CCR5 has been reported to be affected by the expression levels of the cellular β-arrestins and G protein receptor kinase (26). Alternatively, the anti-HIV-1 activity might be attributable to the modest effect on CCR5 internalization. This is because the reduced expression level of CCR5 in *CCR5Δ32* heterozygotes is known to be responsible for the slower progression of HIV-1 infection (44). Alternatively, Reeves et al. (45) reported that cell susceptibility to R5 virus infection in other in vitro systems is unaffected by changes in CCR5 density >10-fold. Further study will be required to clarify whether the antiviral activity of YM-370749 is dependent on CCR5 internalization. The IC_{50} value (0.2 μM) obtained for the antiviral assay using PHAM-PBMC was lower than the IC_{50} values (0.5–1.5 μM) obtained for the antiviral assay using NP-2/CD4/CCR5-LTR-βGal cells and the EC_{50} values obtained for the functional assays using B300-19/CCR5 cells (2.1 and 1.4 μM for the $[Ca^{2+}]_i$ and GTPγS assays, respectively). Lengthy incubation periods with YM-370749 and/or a lower CCR5 density on the PBM cell surface compared with that on NP-2/CD4/CCR5-LTR-βGal cells and B300-19/CCR5 cells might cause a more effective down-modulation and/or occupation of CCR5 by YM-370749. However, cytotoxic (concentrations of 2.2 μM for PHAM-PBMC and 16 μM for NP-2/CD4/CCR5; see Table I), cytostatic, or other effects caused by YM-370749 might affect the antiviral activity. Further study will be required to clarify that the antiviral activity of YM-370749 is directly mediated through CCR5.

Structural analysis is useful for understanding the activation mechanism of CCR5 and for designing the novel anti-HIV-1 agent. The binding and $[Ca^{2+}]_i$ study using the I198M mutant clearly showed that Ile^{198} was involved in the interaction with YM-370749 (Table II and Fig. 6). Billick et al. (46) reported that I198M mutation abrogated HIV-1 entry inhibition by SCH-351125 and suggested that the region of CCR5 near Ile^{198} has an important influence on the conformational state of this receptor. It is tempting to speculate that interaction of YM-370749 with Ile^{198} induces a conformational change of CCR5 that leads to the activation.

Docking studies using YM-370749 and a three-dimensional model of CCR5 showed that the binding site of YM-370749 is located in a pocket surrounded by a transmembrane domain (Fig. 7, *A* and *B*). Considering that CCL5 interacts with the N-terminal extracellular domain and the second extracellular loop of CCR5 (47), our results suggest that the binding site of YM-370749 is quite different from that of CCL5, and it would explain the functional difference between YM-370749 and CCL5. The docking studies also pointed out that the binding of YM-370749 is mediated by an electrostatic interaction with Glu^{283} in TM-VII and by hydrophobic interactions with the Tyr^{108} and Phe^{109} in TM-III, Ile^{198} in TM-V, and Tyr^{251} in TM-VI on CCR5. Among the five amino acid residues at which we showed the interaction with YM-370749 in the docking studies, the Phe^{109} is not conserved in other chemokine receptor subtypes (Tyr in CCR1, His in CCR2 and CCR3, and Leu in CCR4). The Phe^{109} might therefore contribute to the chemokine receptor selectivity of YM-370749.

The Glu^{283} is well conserved among chemokine receptors and is involved in CCR5 antagonist binding (48, 49). Analogous glutamate residues within CCR1, CCR2, and CXCR4 have been shown to be important for small molecule antagonist binding (50–52). Our results suggest that YM-370749 also uses common electrostatic interactions between the antagonist and a common acidic amino acid in TM-VII of the receptors. The crystal structure of the transcription regulator BmrR complex with tetraphenylphosphonium, the structure of which is similar to that of YM-370749 (53), will explain that the positive charge on the phenyl ring in YM-370749 is due to the phosphorus atom and makes an electrostatic interaction with Glu^{283}.

Govaerts et al. (54, 55) has reported that mutations of Tyr^{108} and other aromatic residues located in CCR5 TM-II and TM-III differentially affected the functional response to various natural ligands without significantly altering their binding affinity and suggested that the amino acid residues were involved in the activation of the receptor. YM-370749 may exert its agonistic activity through the interaction with Tyr^{108}. Further studies will be needed to understand more precisely the molecular details of CCR5 active states induced by YM-370749.

In summary, YM-370749 is a small molecule, functionally selective agonist for the human CCR5 that can inhibit the replication of HIV-1. Insights into the molecular basis for the interaction of CCR5 with YM-370749 will provide structural basis for understanding the CCR5 activation mechanism and for designing ideal anti-HIV-1 agents that promote internalization of CCR5 without proinflammatory activity.

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References

1. Carpenter, C. C., M. A. Fischl, S. M. Hammer, M. S. Hirsch, D. M. Jacobsen, D. A. Katzenstein, J. S. Montaner, D. D. Richman, M. S. Saag, R. T. Schooley, et al. 1998. Antiretroviral therapy for HIV infection in 1998: updated recommendations of the International AIDS Society-USA panel. *J. Am. Med. Assoc.* 280: 78–86.
2. Montessori, V., N. Press, M. Harris, L. Akagi, and J. S. Montaner. 2004. Adverse effects of antiretroviral therapy for HIV infection. *Can. Med. Assoc. J.* 170: 229–238.
3. Siliciano, J. D., J. Kajdas, D. Finzi, T. C. Quinn, K. Chadwick, J. B. Margolick, C. Kovacs, S. J. Gange, and R. F. Siliciano. 2003. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting $CD4^+$ T cells. *Nat. Med.* 9: 727–728.
4. Strader, C. D., T. M. Fong, M. R. Tota, D. Underwood, and R. A. Dixon. 1994. Structure and function of G protein-coupled receptors. *Annu. Rev. Biochem.* 63: 101–132.
5. Alkhatib, G., C. Combadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger. 1996. CC CKR5: a RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 272: 1955–1958.
6. Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, et al. 1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381: 661–666.
7. Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P. D. Ponath, L. Wu, C. R. Mackay, G. LaRosa, W. Newman, et al. 1996. The β-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 85: 1135–1148.
8. Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger. 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 272: 872–877.
9. Doranz, B. J., J. Rucker, Y. Yi, R. J. Smyth, M. Samson, S. C. Peiper, M. Parmentier, R. G. Collman, and R. W. Doms. 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the β-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* 85: 1149–1158.
10. Dragic, T., V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore, and W. A. Paxton. 1996. HIV-1 entry into $CD4^+$ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 381: 667–673.
11. Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. R. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Koup, and N. R. Landau. 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* 86: 367–377.
12. Samson, M., F. Libert, B. J. Doranz, J. Rucker, C. Liesnard, C. M. Farber, S. Saragosti, C. Lapoumeroulie, J. Cognaux, C. Forceille, et al. 1996. Resistance

to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* 382: 722–725.
13. Kazmierski, W., N. Bifulco, H. Yang, L. Boone, F. DeAnda, C. Watson, and T. Kenakin. 2003. Recent progress in discovery of small-molecule CCR5 chemokine receptor ligands as HIV-1 inhibitors. *Bioorg. Med. Chem.* 11: 2663–2676.
14. Tsamis, F., S. Gavrilov, F. Kajumo, C. Seibert, S. Kuhmann, T. Ketas, A. Trkola, A. Palani, J. W. Clader, J. R. Tagat, et al. 2003. Analysis of the mechanism by which the small-molecule CCR5 antagonists SCH-351125 and SCH-350581 inhibit human immunodeficiency virus type 1 entry. *J. Virol.* 77: 5201–5208.
15. Cocchi, F., A. L. DeVico, A. Garzino-Demo, S. K. Arya, R. C. Gallo, and P. Lusso. 1995. Identification of RANTES, MIP-1 α, and MIP-1 β as the major HIV-suppressive factors produced by $CD8^+$ T cells. *Science* 270: 1811–1815.
16. Mack, M., B. Luckow, P. J. Nelson, J. Cihak, G. Simmons, P. R. Clapham, N. Signoret, M. Marsh, M. Stangassinger, F. Borlat, et al. 1998. Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. *J. Exp. Med.* 187: 1215–1224.
17. Beck, L. A., S. Dalke, K. M. Leiferman, C. A. Bickel, R. Hamilton, H. Rosen, B. S. Bochner, and R. P. Schleimer. 1997. Cutaneous injection of RANTES causes eosinophil recruitment: comparison of nonallergic and allergic human subjects. *J. Immunol.* 159: 2962–2972.
18. Mizushima, S., and S. Nagata. 1990. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* 18: 11.
19. Strizki, J. M., S. Xu, N. E. Wagner, L. Wojcik, J. Liu, Y. Hou, M. Endres, A. Palani, S. Shapiro, J. W. Clader, et al. 2001. SCH-C (SCH 351125), an orally bioavailable, small molecule antagonist of the chemokine receptor CCR5, is a potent inhibitor of HIV-1 infection in vitro and in vivo. *Proc. Natl. Acad. Sci. USA* 98: 12718–12723.
20. Soda, Y., N. Shimizu, A. Jinno, H. Y. Liu, K. Kanbe, T. Kitamura, and H. Hoshino. 1999. Establishment of a new system for determination of coreceptor usages of HIV based on the human glioma NP-2 cell line. *Biochem. Biophys. Res. Commun.* 258: 313–321.
21. Kodama, E. I., S. Kohgo, K. Kitano, H. Machida, H. Gatanaga, S. Shigeta, M. Matsuoka, H. Ohrui, and H. Mitsuya. 2001. 4′-Ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants in vitro. *Antimicrob. Agents Chemother.* 45: 1539–1546.
22. Suzutani, T., H. Machida, and T. Sakuma. 1988. Efficacies of antiherpesvirus nucleosides against two strains of herpes simplex virus type 1 in Vero and human embryo lung fibroblast cells. *Antimicrob. Agents Chemother.* 32: 1046–1052.
23. Cheng, Y., and W. H. Prusoff. 1973. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* 22: 3099–3108.
24. Palczewski, K., T. Kumasaka, T. Hori, C. A. Behnke, H. Motoshima, B. A. Fox, I. Le Trong, D. C. Teller, T. Okada, R. E. Stenkamp, et al. 2000. Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289: 739–745.
25. Jones, G., P. Willett, and R. C. Glen. 1995. Molecular recognition of receptor sites using a genetic algorithm with a description of desolvation. *J. Mol. Biol.* 245: 43–53.
26. Aramori, I., S. S. Ferguson, P. D. Bieniasz, J. Zhang, B. Cullen, and M. G. Caron. 1997. Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 co-receptor. [Published erratum appears in 1997 EMBO J. 16: 6055.] *EMBO J.* 16: 4606–4616.
27. Mueller, A., E. Kelly, and P. G. Strange. 2002. Pathways for internalization and recycling of the chemokine receptor CCR5. *Blood* 99: 785–791.
28. Aquino, C. J., D. R. Armour, J. M. Berman, L. S. Birkemo, R. A. Carr, D. K. Croom, M. Dezube, R. W. Dougherty, Jr., G. N. Ervin, M. K. Grizzle, et al. 1996. Discovery of 1,5-benzodiazepines with peripheral cholecystokinin (CCK-A) receptor agonist activity. 1. Optimization of the agonist "trigger." *J. Med. Chem.* 39: 562–569.
29. Perlman, S., H. T. Schambye, R. A. Rivero, W. J. Greenlee, S. A. Hjorth, and T. W. Schwartz. 1995. Nonpeptide angiotensin agonist. Functional and molecular interaction with the AT1 receptor. *J. Biol. Chem.* 270: 1493–1496.
30. Emery, P. T., N. B. Higgs, A. C. Warhurst, G. L. Carlson, and G. Warhurst. 2002. Anti-secretory properties of non-peptide somatostatin receptor agonists in isolated rat colon: luminal activity and possible interaction with P-glycoprotein. *Br. J. Pharmacol.* 135: 1443–1448.
31. Kaczor, A., and D. Matosiuk. 2002. Non-peptide opioid receptor ligands - recent advances. Part I — agonists. *Curr. Med. Chem.* 9: 1567–1589.
32. Kenakin, T. 2003. Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol. Sci.* 24: 346–354.
33. Blanpain, C., J. M. Vanderwinden, J. Cihak, V. Wittamer, E. Le Poul, H. Issafras, M. Stangassinger, G. Vassart, S. Marullo, D. Schlndorff, et al. 2002. Multiple active states and oligomerization of CCR5 revealed by functional properties of monoclonal antibodies. *Mol. Biol. Cell* 13: 723–737.
34. Watson, C., G. Chen, P. Irving, J. Way, W. J. Chen, and T. Kenakin. 2000. The use of stimulus-biased assay systems to detect agonist-specific receptor active states: implications for the trafficking of receptor stimulus by agonists. *Mol. Pharmacol.* 58: 1230–1238.
35. Roettger, B. F., D. Ghanekar, R. Rao, C. Toledo, J. Yingling, D. Pinon, and L. J. Miller. 1997. Antagonist-stimulated internalization of the G protein-coupled cholecystokinin receptor. *Mol. Pharmacol.* 51: 357–362.
36. Rodriguez-Frade, J. M., A. J. Vila-Coro, A. Martin, M. Nieto, F. Sanchez-Madrid, A. E. Proudfoot, T. N. Wells, A. C. Martinez, and M. Mellado. 1999. Similarities and differences in RANTES- and (AOP)-RANTES-triggered signals: implications for chemotaxis. *J. Cell Biol.* 144: 755–765.
37. Turner, L., S. G. Ward, and J. Westwick. 1995. RANTES-activated human T lymphocytes. A role for phosphoinositide 3-kinase. *J. Immunol.* 155: 2437–2444.
38. Ganju, R. K., S. A. Brubaker, R. D. Chernock, S. Avraham, and J. E. Groopman. 2000. β-Chemokine receptor CCR5 signals through SHP1, SHP2, and Syk. *J. Biol. Chem.* 275: 17263–17268.
39. Ganju, R. K., P. Dutt, L. Wu, W. Newman, H. Avraham, S. Avraham, and J. E. Groopman. 1998. β-Chemokine receptor CCR5 signals via the novel tyrosine kinase RAFTK. *Blood* 91: 791–797.
40. Trkola, A., S. E. Kuhmann, J. M. Strizki, E. Maxwell, T. Ketas, T. Morgan, P. Pugach, S. Xu, L. Wojcik, J. Tagat, et al. 2002. HIV-1 escape from a small molecule, CCR5-specific entry inhibitor does not involve CXCR4 use. *Proc. Natl. Acad. Sci. USA* 99: 395–400.
41. Platt, E. J., K. Wehrly, S. E. Kuhmann, B. Chesebro, and D. Kabat. 1998. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J. Virol.* 72: 2855–2864.
42. Lin, Y. L., C. Mettling, P. Portales, J. Reynes, J. Clot, and P. Corbeau. 2002. Cell surface CCR5 density determines the postentry efficiency of R5 HIV-1 infection. *Proc. Natl. Acad. Sci. USA* 99: 15590–15595.
43. Pastore, C., G. R. Picchio, F. Galimi, R. Fish, O. Hartley, R. E. Offord, and D. E. Mosier. 2003. Two mechanisms for human immunodeficiency virus type 1 inhibition by N-terminal modifications of RANTES. *Antimicrob. Agents Chemother.* 47: 509–517.
44. Visco-Comandini, U., C. Hultgren, C. Brostrom, M. Birk, S. Kim, and M. Sallberg. 1998. Human immunodeficiency virus type 1 disease progression, CCR5 genotype, and specific immune response. *Clin. Diagn. Lab. Immunol.* 5: 463–466.
45. Reeves, J. D., S. A. Gallo, N. Ahmad, J. L. Miamidian, P. E. Harvey, M. Sharron, S. Pohlmann, J. N. Sfakianos, C. A. Derdeyn, R. Blumenthal, et al. 2002. Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. *Proc. Natl. Acad. Sci. USA* 99: 16249–16254.
46. Billick, E., C. Seibert, P. Pugach, T. Ketas, A. Trkola, M. J. Endres, N. J. Murgolo, E. Coates, G. R. Reyes, B. M. Baroudy, et al. 2004. The differential sensitivity of human and rhesus macaque CCR5 to small-molecule inhibitors of human immunodeficiency virus type 1 entry is explained by a single amino acid difference and suggests a mechanism of action for these inhibitors. *J. Virol.* 78: 4134–4144.
47. Navenot, J. M., Z. X. Wang, J. O. Trent, J. L. Murray, Q. X. Hu, L. DeLeeuw, P. S. Moore, Y. Chang, and S. C. Peiper. 2001. Molecular anatomy of CCR5 engagement by physiologic and viral chemokines and HIV-1 envelope glycoproteins: differences in primary structural requirements for RANTES, MIP-1 α, and vMIP-II binding. *J. Mol. Biol.* 313: 1181–1193.
48. Castonguay, L. A., Y. Weng, W. Adolfsen, J. Di Salvo, R. Kilburn, C. G. Caldwell, B. L. Daugherty, P. E. Finke, J. J. Hale, C. L. Lynch, 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.
49. Dragic, T., A. Trkola, D. A. Thompson, E. G. Cormier, F. A. Kajumo, E. Maxwell, S. W. Lin, W. Ying, S. O. Smith, T. P. Sakmar, and J. P. Moore. 2000. A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. *Proc. Natl. Acad. Sci. USA* 97: 5639–5644.
50. de Mendonca, F. L., P. C. da Fonseca, R. M. Phillips, J. W. Saldanha, T. J. Williams, and J. E. Pease. 2005. Site-directed mutagenesis of CC chemokine receptor 1 reveals the mechanism of action of UCB 35625, a small molecule chemokine receptor antagonist. *J. Biol. Chem.* 280: 4808–4816.
51. Mirzadegan, T., F. Diehl, B. Ebi, S. Bhakta, I. Polsky, D. McCarley, M. Mulkins, G. S. Weatherhead, J. M. Lapierre, J. Dankwardt, et al. 2000. Identification of the binding site for a novel class of CCR2b chemokine receptor antagonists: binding to a common chemokine receptor motif within the helical bundle. *J. Biol. Chem.* 275: 25562–25571.
52. Rosenkilde, M. M., L. O. Gerlach, J. S. Jakobsen, R. T. Skerlj, G. J. Bridger, and T. W. Schwartz. 2004. Molecular mechanism of AMD3100 antagonism in the CXCR4 receptor: transfer of binding site to the CXCR3 receptor. *J. Biol. Chem.* 279: 3033–3041.
53. Zheleznova, E. E., P. N. Markham, A. A. Neyfakh, and R. G. Brennan. 1999. Structural basis of multidrug recognition by BmrR, a transcription activator of a multidrug transporter. *Cell* 96: 353–362.
54. Govaerts, C., C. Blanpain, X. Deupi, S. Ballet, J. A. Ballesteros, S. J. Wodak, G. Vassart, L. Pardo, and M. Parmentier. 2001. The TXP motif in the second transmembrane helix of CCR5. A structural determinant of chemokine-induced activation. *J. Biol. Chem.* 276: 13217–13225.
55. Govaerts, C., A. Bondue, J. Y. Springael, M. Olivella, X. Deupi, E. Le Poul, S. J. Wodak, M. Parmentier, L. Pardo, and C. Blanpain. 2003. Activation of CCR5 by chemokines involves an aromatic cluster between transmembrane helices 2 and 3. *J. Biol. Chem.* 278: 1892–1903.
56. Nicholls, A., K. A. Sharp, and B. Honig. 1991. Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins* 11: 281–296.