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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-mercaptoethanol, 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 Ca2+ concentration ([Ca2+]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 KHPO4, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, and 0.05% BSA) at 37°C for 45 min. After washing, [Ca2+]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-351215 (19) was synthesized at Astellas Pharma and added 2 min before the agonists.

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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 MgCl₂, 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% NaN₃). 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 expression of CCR5, Neo expression vector. FLUOR 6 transfection reagent (Roche) caused the 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)].

Chemotaxis assay

Chemotaxis assays were performed using 96-well chemotaxis chambers (Neuro Probe) with 5-μm pore, polycarbonate filters for 3 h at 37°C in a humidified 5% CO₂ incubator. Cells suspended at 5 x 10⁵ 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 stimlated for 3 days with PHA M (PHAM; 10 μg/ml; Sigma-Aldrich). PHAM-PBMC (10⁶ cells/ml) were infected with HIV-1 R5 (strain) or HIV-1 X4 (X4 strain) equivalent to 1 ng/ml p24 in the presence of various concentrations of YM-370749. After 7 days incubation, the cells were harvested, 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)].

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

CaCl₂, and 0.1% (w/v) BSA. Radioactivity was counted using a TopCount scintillation counter (Packard Biosciences). Non-specific binding was determined in the presence of 50 nM CCL3. Absolute inhibition constant (Kᵢ) values were calculated from the IC₅₀ using the equation of Cheng and Prusoff (23).
(B300-19/CCR5) cells, we conducted a large-scale, high-throughput screening of in-house chemical libraries by measuring \([\text{Ca}^{2+}]_i\) and discovered that YM-370749 (Fig. 1) had the required properties. A low molecular weight compound, YM-370749, elevated \([\text{Ca}^{2+}]_i\) in B300-19/CCR5 cells, which was also the case with the natural CCR5 ligand CCL5 (Fig. 2A). The \([\text{Ca}^{2+}]_i\) increase elicited by YM-370749 was completely inhibited by pretreatment with 100 ng/ml pertussis toxin, indicating that this compound signaled through a Gi protein-coupled receptor, and it was also inhibited by the CCR5-selective antagonist SCH-351125 (Fig. 2A). The increase of \([\text{Ca}^{2+}]_i\) by YM-370749 was concentration-dependent with an EC\(_{50}\) of 2.1 \(\mu\text{M}\) (Fig. 2B). This compound at 30 \(\mu\text{M}\) had no effect against CCR1-, CCR2-, CCR3-, or CCR4-expressing cells (Fig. 2C).

YM-370749 also induced GTP\(\gamma\text{S}\) binding to B300-19/CCR5 cell membranes in a concentration-dependent manner with an EC\(_{50}\) of 1.4 \(\mu\text{M}\) (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, \(\beta\)-arrestin-2-EGFP, and G protein receptor kinase 2 (Fig. 4, C–F). It has been reported that \(\beta\)-arrestin-2 mediates CCR5 internalization (26) and that activation of CCR5 by chemokines results in the colocalization of the receptor and \(\beta\)-arrestin-2 (27). In the absence of ligands, the fluorescence was detected mainly at the cell surface (Fig. 4C). 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. 4F).

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...
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 R5 strain HIV-1BaL (IC_{50} = 0.20 \mu M), which uses CCR5 as a coreceptor. It did not, however, inhibit the X4 strain HIV-1HXB2, 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-1BaL and the four clinical isolates with similar potency (IC_{50} = 0.50–1.5 \mu M). As expected, at concentrations up to 30 \mu M 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. 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: 9T, 13D, 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 all mutants are equally recognized by four different mAbs specific to CCR5 (data not shown).

### Table I. Anti-viral activities of YM-370749

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Assay</th>
<th>IC_{50} (\mu M)</th>
<th>CC_{50} (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 BaL</td>
<td>p24 ELISA</td>
<td>0.20</td>
<td>2.2</td>
</tr>
<tr>
<td>HIV-1 HXB2</td>
<td>p24 ELISA</td>
<td>&gt;2.0</td>
<td></td>
</tr>
<tr>
<td>HIV-1 BaL</td>
<td>β-Galactosidase</td>
<td>1.0 ± 0.05</td>
<td>16 ± 3.6</td>
</tr>
<tr>
<td>HIV-1 401</td>
<td>β-Galactosidase</td>
<td>1.1 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>HIV-1 409</td>
<td>β-Galactosidase</td>
<td>1.5 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>HIV-1 411</td>
<td>β-Galactosidase</td>
<td>0.74 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>HIV-1 415</td>
<td>β-Galactosidase</td>
<td>0.50 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>FluV-A Ishikawa782</td>
<td>Plaque reduction</td>
<td>&gt;30</td>
<td>&gt;30</td>
</tr>
<tr>
<td>HSV-1 KOS</td>
<td>Plaque reduction</td>
<td>&gt;30</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

*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-1BaL 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-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.

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

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

<table>
<thead>
<tr>
<th>Mutants</th>
<th>YM-370749^a</th>
<th>CCL5^a</th>
<th>CCL3^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_i (\mu M)</td>
<td>Ratio</td>
<td>K_i (pM)</td>
</tr>
<tr>
<td>Human WT</td>
<td>2.7 ± 0.16</td>
<td>1.0</td>
<td>48 ± 7.3</td>
</tr>
<tr>
<td>Rhesus WT</td>
<td>29 ± 2.7^b</td>
<td>11</td>
<td>21 ± 2.5</td>
</tr>
<tr>
<td>Human 9T</td>
<td>2.7 ± 0.29</td>
<td>1.0</td>
<td>18 ± 4.8</td>
</tr>
<tr>
<td>Human N13D</td>
<td>4.1 ± 0.46</td>
<td>1.5</td>
<td>52 ± 4.2</td>
</tr>
<tr>
<td>Human M49I</td>
<td>3.0 ± 0.10</td>
<td>1.1</td>
<td>18 ± 5.3</td>
</tr>
<tr>
<td>Human I52V</td>
<td>2.1 ± 0.21</td>
<td>0.79</td>
<td>24 ± 7.9</td>
</tr>
<tr>
<td>Human F78L</td>
<td>2.6 ± 0.23</td>
<td>0.99</td>
<td>18 ± 6.5</td>
</tr>
<tr>
<td>Human V130I</td>
<td>3.2 ± 0.17</td>
<td>1.2</td>
<td>12 ± 3.4</td>
</tr>
<tr>
<td>Human K171R</td>
<td>4.6 ± 0.67</td>
<td>1.7</td>
<td>42 ± 5.3</td>
</tr>
<tr>
<td>Human I198M</td>
<td>19 ± 2.5^b</td>
<td>7.0</td>
<td>49 ± 14</td>
</tr>
</tbody>
</table>

^a K_i values are given for YM-370749, CCL5, and CCL3 inhibition of ^125I-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).
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 [Ca²⁺]i elevation activity of YM-370749 in B300-19/CCR5, B300-19/CCR5-I198M, and B300-19/CCR5 cells (Fig. 6). The [Ca²⁺]i elevation activity of CCL5 in B300-19/CCR5 cells was almost similar to those in B300-19/CCR5-I198M, and B300-19/CCR5 cells. However, YM-370749 could not reach the same maximum efficacy in B300-19/CCR5-I198M (62% elevation) or B300-19/CCR5 cells (37% elevation) as in B300-19/CCR5 cells, and it was less potent in B300-19/CCR5-I198M (EC₅₀ = 4.7 μM) and B300-19/rhesus CCR5 cells (EC₅₀ = 6.2 μM) than in B300-19/CCR5 cells (EC₅₀ = 2.1 μM). These results implied that the Ile¹⁹⁸ 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. 7A, 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 Tyr¹⁰⁸ and Phe¹⁰⁹ in TM-III, Ile¹⁹⁸ in TM-V, and Tyr²⁵¹ in TM-VI (Fig. 7B). The importance of Ile¹⁹⁸ 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 Glu²⁸³ in TM-VII (Fig. 7C).

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 Ca²⁺ 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 [Ca²⁺]i elevation and receptor down-modulation were completely inhibited by the CCR5-selective antagonist SCH-351125 (Figs. 2A and 4F). 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 Ca²⁺ elevation, but not chemotaxis, indicated that the level of G₂ 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. 4B), 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 CCR5Δ2 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 IC₅₀ value (0.2 μM) obtained for the antagonistic assay using PHAM-PBMC was lower than the IC₅₀ values (0.5–1.5 μM) obtained for the antiviral assay using NP-2/CD4/CCR5-LTR-βGal cells and the EC₅₀ values obtained for the functional assays using B300-19/CCR5 cells (2.1 and 1.4 μM for the [Ca²⁺]ᵢ and GTPY Sachs assays, respectively). Lengthy incubation periods with YM-370749 and/or a lower CCR5 density on the PBMC 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 [Ca²⁺]ᵢ study using the I198M mutant clearly showed that Ile₁⁰⁸ 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 Ile₁⁰⁸ has an important influence on the conformational state of this receptor. It is tempting to speculate that interaction of YM-370749 with Ile₁⁰⁸ 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 Glu²⁸³ in TM-VII and by hydrophobic interactions with the Tyr¹⁰⁸ and Phe¹⁰⁹ in TM-III, Ile¹⁰⁸ in TM-V, and Tyr²⁵¹ 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 Phe¹⁰⁹ is not conserved in other chemokine receptor subtypes (Tyr in CCR1, His in CCR2 and CCR3, and Leu in CCR4). The Phe¹⁰⁹ might therefore contribute to the chemokine receptor selectivity of YM-370749.

The Glu²⁸³ 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 Glu²⁸³.

Govaerts et al. (54, 55) has reported that mutations of Tyr¹⁰⁸ 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 Tyr¹⁰⁸. 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
to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor. Nature 382: 722–725.


