Allosteric and Orthosteric Sites in CC Chemokine Receptor (CCR5), a Chimeric Receptor Approach

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Stefanie Thiele‡, Anne Steen†, Pia C. Jensen‡, Jacek Mokrosinski‡, Thomas M. Frimurer§, and Mette M. Rosenkilde††
From the ‡Laboratory for Molecular Pharmacology, Department of Neuroscience and Pharmacology and §The Novo Nordisk Foundation Center for Protein Research, Faculty of Health Sciences, University of Copenhagen, Blegdamsvej 3, DK-2200, Copenhagen, Denmark

Background: Characterization of 7TM biology and chemistry is needed generally and within chemokine receptors.

Results: A CCR5-CCR2 receptor chimera was constructed by transferring all extracellular regions of CCR2 to CCR5. CCR2 chemokine binding was maintained and so was small molecule CCR5 agonists and antagonists.

Conclusion: Orthosteric and allosteric sites could be structurally separated and still act together.

Significance: New basic knowledge to be used in drug development.

Chemokine receptors play a major role in immune system regulation and have consequently been targets for drug development leading to the discovery of several small molecule antagonists. Given the large size and predominantly extracellular receptor interaction of endogenous chemokines, small molecules often act more deeply in an allosteric mode. However, opposed to the well described molecular interaction of allosteric modulators in class C 7-transmembrane helix (7TM) receptors, the interaction in class A, to which the chemokine receptors belong, is more sparsely described. Using the CCR5 chemokine receptor as a model system, we studied the molecular interaction and conformational interchange required for proper action of various orthosteric chemokines and allosteric small molecules, including the well known CCR5 antagonists TAK-779, SCH-C, and aplaviroc, and four novel CCR5 ago-allosteric molecules. A chimera was successfully constructed between CCR5 and the closely related CCR2 by transferring all extracellular regions of CCR2 to CCR5, i.e. a Trojan horse that resembles CCR2 extracellularly but signals through a CCR5 transmembrane unit. The chimera bound CCR2 (CCL2 and CCL7), but not CCR5 chemokines (CCL3 and CCL5), with CCR2-like high affinities and potencies throughout the CCR5 signaling unit. Concomitantly, high affinity binding of small molecule CCR5 agonists and antagonists was retained in the transmembrane region. Importantly, whereas the agonistic and antagonistic properties were preserved, the allosteric enhancement of chemokine binding was disrupted. In summary, the Trojan horse chimera revealed that orthosteric and allosteric sites could be structurally separated and still act together with transmission of agonism and antagonism across the different receptor units.

With several hundred members divided into four main groups, 7 transmembrane-helix (7TM) receptors constitute the largest group of proteins in the human genome and are the targets for the majority of current drugs (1). Their great number reflects the multitude of chemically diverse ligands, ranging from photons over small molecule ligands to peptides and proteins. The diversity of the ligands is huge. This is also the case for the ligand-binding site, which may be located within the transmembrane area and/or the extracellular domains (2). The binding site of the endogenous ligand is referred to as the orthosteric binding site. Allosteric compounds have a binding site that is topographically distinct from the orthosteric site. They have been identified for numerous receptors and have in general become increasingly acknowledged in drug development for their higher specificity, preventing overdosing and, provided they are neutral by themselves, dependence on the presence of the endogenous ligand, therefore only taking effect at the site of interest (where the endogenous ligand is located) (3).

Chemokine receptors belong to the class A subfamily of 7TM receptors. They are involved in the regulation and development of the immune system by guiding leukocyte migration and development. The chemokine system includes more than 19 receptors and 50 ligands and is characterized by promiscuity and redundancy for the majority of its members. According to the number and spacing of the first two of usually four conserved cysteines, chemokines and their receptors are divided into four groups as follow: the CC, CXC, XC, and CX3C groups (4). It is generally accepted that the chemokine core binds to the extracellular domain of a given receptor, whereas the N terminus may interact with residues in the transmembrane area (5).

The central role of the chemokines in immune system regulation combined with the control of angiogenesis, cancer growth and metastasis, and HIV cell entry have facilitated drug development programs and thereby the identification of numerous small molecule antagonists and agonists (6). These

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† To whom correspondence should be addressed: Blegdamsvej 3, Bldg. 18.5, Copenhagen, DK-2200, Denmark. Tel.: 45-30604608; Fax: 45-35327610; E-mail: rosenkilde@sund.ku.dk.

‡ The abbreviations used are: 7TM, 7-transmembrane helix; TM, transmembrane; IP, inositol phosphate; ZnBip, ZnPhe, CuBip, CuPhe, Zn2 + or Cu2 + in complex with bipyridine or phenanthroline.
small molecules usually have similar pharmacophores with an elongated structure, one or two, more or less centrally located positively charged amines and flanking aromatic residues (7, 8). The positively charged amines anchor to GluVII:063 on top of TM-VII, a residue that is conserved in 74% of all chemokine receptors compared with <1% in nonchemokine 7TM receptors (7). This residue divides the main ligand binding crevice into a minor (delimited by TM-I, -II, -III, and -VII) and a major binding pocket (delimited by TM-III, -IV, -V, -VI, and -VII) (10). The flanking aromatic moieties of the small molecules interact with residues in the major and minor binding pocket, as described, e.g. for the CCR5 antagonists aplaviroc and SCH-C (7, 11–14). Interestingly, agonists also display a similar pharmacophore with a central positive charge and flanking aromatic moieties, as observed for CCR5 and CCR8 agonists (15, 16). However, recently, a novel series of metal ion chelator-based, and thus structurally different, CCR1 agonists were identified (17). These compounds also anchored to GluVII:06 and acted as allosteric enhancers of CCL3 (MIP-1α), but not of CCL5 (regulated on activation normal T cell expressed and secreted) binding (17).

7TM receptor drugs have traditionally been developed based on the structure of the endogenous ligand and consequently target the orthosteric site. It has, however, become increasingly acknowledged that 7TM receptors have allosteric binding sites with obvious advantages over the orthosteric binding site from a drug-action point of view (as discussed above). Thus, e.g. class C 7TM receptors have large N-terminal domains that contain the orthosteric site, and the receptors can therefore be modulated via a clearly separated allosteric site within the transmembrane region (18). On the contrary, many class A receptors contain the orthosteric binding site within the transmembrane region, as described for rhodopsin, adenosine, acetylcholine, and other monoamine receptors, whereas the allosteric binding sites are located in the extracellular regions (3, 19). In contrast to the cognate ligands of these receptors, chemokines are large molecules and somewhat exceptional with their orthosteric binding site located entirely in the extracellular domains, and the allosteric binding sites are located deeper in the transmembrane region (5, 8).

CCR5 shares the chemokine ligands CCL3 and CCL5 with CCR1, and in this study we find that CCR5, like CCR1 (17), is activated by metal ion chelator-based compounds. CCR2, the receptor most similar to CCR5 despite no overlapping chemokine binding profiles (20), is however not activated by these small molecule agonists. Until now, several series of small molecule antagonists have been described for CCR5, as for instance aplaviroc and SCH-C (both CCR5-specific) and the dual CCR5/CCR2 antagonist TAK-779 (21–23). The small molecule agonists for CCR5 (but not CCR2) identified in this study combined with the previously identified small molecule antagonists with different degrees of CCR5 selectivity over CCR2 and two sets of completely selective chemokines for CCR5 and CCR2 inspired us to study the molecular interaction of orthosteric and allosteric ligands, especially the conformational interchange in CCR5. Therefore, we constructed a “Trojan horse” chimeric receptor, CCR5-CCR2(all), that consisted of the CCR5 receptor scaffold but with the extracellular domains being “shaved off” and replaced by the corresponding extracellular domains from CCR2. Construction of chimeric receptors has been applied earlier (24–26), also for the analysis of CCR5 and CCR2 (27–30). However, the majority of these were built in a “centaur” way with the N-terminal part stemming from one receptor and the C-terminal part from another receptor, therefore being sequence-related but not domain-related as in our Trojan horse chimera. CCR5-CCR2(all) recognized CCR2 but not CCR5 chemokines, and at the small molecule level, the receptor bound the CCR5-selective nonpeptide agonists and most antagonists (except for aplaviroc). Importantly, for all ligand classes, the functional properties (agonism and antagonism) were maintained indicating that despite the different origin of the extracellular (CCR2) and transmembrane and intracellular (CCR5) domains, the receptor acted as one functional unit with transmission of activity between the domains of different origin. For aplaviroc, the C-terminal part of ECL-2 (ECL-2b) was additionally needed for maintained binding, but importantly, this was sufficient for high potency action of this antagonist. All together, by using ligands with different chemical and pharmacological properties, we studied ligand binding and receptor function across heterogeneous receptor domains in the Trojan horse CCR5-CCR2(all) chimeric receptor that was created successfully without destroying the overall conformation and function of the receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—The human chemokines CCL3, CCL5, CCL2, and CCL7 were purchased from PeproTech. TAK-779, SCH-C, and aplaviroc were kindly provided by Gary Bridger (AnorMED). The highest concentration of metal ion chelator complexes were made from 0.2 M ZnCl2 in water, 0.2 M CuSO4 in water, 0.4 M phenanthroline in 70% ethanol, 0.4 M bipyridine in DMSO and were supplemented with 10% DMSO, water, and 70% ethanol. Dilutions were made in water. The concentration of chelator was double the concentration of the ions, which in turn determines the concentration of the complex, to provide optimal 1:1 complexation of the ions by chelators. The human CCR5 was cloned in-house from a leukocyte cDNA library, while CCR2 cDNA was kindly provided by Tim Wells (Glaxo-SmithKline, UK). The chimeric receptors CCR5-CCR2(all) and CCR5-CCR2(an2b) were designed in-house and purchased from GenScript. The promiscuous G protein Gαo4myr (abbreviated as Gαi4myr) was kindly provided by Evi Kostenis (University of Bonn, Germany). myo-[3H]inositol (PT6-271), Bolton-Hunter reagent, and iodinated chemokines 125I-CCL3, 125I-CCL5, and 125I-CCL2 were purchased from PerkinElmer Life Sciences. 125I-CCL7 was Bolton-Hunter-labeled and HPLC-purified in-house (31). AG 1-X8 anion exchange resin was from Bio-Rad.

**Site-directed Mutagenesis**—Point mutations were introduced in the receptors by the polymerase chain reaction over-
lap extension technique using WT CCR5, CCR2, or CCR5-CCR2(all) as templates. All reactions were carried out using Pfu polymerase (Stratagene) under conditions recommended by the manufacturer. The mutations were cloned into the eukaryotic expression vector pcDNA3.1+ and were verified by restriction endonuclease digestion and DNA sequencing (Eurofins MWG Operon, Germany).

Transfections and Tissue Culture—COS-7 cells were grown at 10% CO2 and 37 °C in Dulbecco’s modified Eagle’s medium with GlutaMAX (Invitrogen) adjusted with 10% fetal bovine serum, 180 units/ml penicillin, and 45 μg/ml penicillin/streptomycin. Transfection of the COS-7 cells was performed by the calcium phosphate precipitation method (32).

Inositol Phosphate Turnover (IP Turnover)—COS-7 cells were transfected according to the procedure mentioned above. Briefly, 6 × 10⁶ cells were transfected with 20 μg of receptor cDNA in addition to 30 μg of the promiscuous chimeric G protein, Goq/αqmyr, which turns the Goq-coupled signal, the most common pathway for endogenous chemokine receptors, into the Gq pathway (phospholipase C activation measured as IP turnover) (32, 33). One day after transfection, COS-7 cells (1.5 × 10⁵ cells/well) were incubated for 24 h with 2 μCi of [3H]inositol in 0.3 ml of growth medium per well. Cells were washed twice in PBS and were incubated in 0.2 ml of Hank’s balanced salt solution (Invitrogen) supplemented with 10 mM LiCl at 37 °C in the presence of various concentrations of ligands. Cells were extracted by addition of 1 ml of 10 mM formic acid to each well followed by incubation on ice for 30–60 min. The generated [3H]inositol phosphates were purified on AG 1-X8 anion exchange resin. Determinations were made in duplicates. This readout has previously been used with success in other chemokine receptors (16, 17). For the test of the antagonists, the agonist was given after a 10-min preincubation with the antagonists and in concentrations that lead to ~80% of maximal activation for the respective ligand and receptor.

Binding Experiments—6 × 10⁶ COS-7 cells were transfected with 40 μg of receptor cDNA and transferred to culture plates 1 day after transfection. The number of cells seeded per well was determined by the apparent expression efficiency of the receptors and was aimed at obtaining 5–10% specific binding of the added radioactive ligand. Two days after transfection, cells were assayed by competition binding for 3 h at 4 °C using 10–15 pm ¹²⁵I-CCL3, ¹²⁵I-CCL5, ¹²⁵I-CCL2, or ¹²⁵I-CCL7 plus unlabeled ligand in 0.2 ml of 50 mM Hepes buffer, pH 7.4, supplemented with 1 mM CaCl₂, 5 mM MgCl₂, and 0.5% (w/v) bovine serum albumin. After incubation, cells were washed quickly two times in 4 °C binding buffer supplemented with 0.5 mM NaCl except for ¹²⁵I-CCL7 binding to CCR2, where the washing was performed in binding buffer without NaCl. Non-specific binding was determined in the presence of 0.1 μM unlabeled CCL3, CCL5, CCL2, or CCL7, respectively. Determinations were made in duplicates.

Receptor Surface Expression by Enzyme-Linked Immunosorbent Assay (ELISA)—COS-7 cells were transiently transfected with the N-terminal FLAG-tagged receptor variants. The cells were washed once in TBS (50 mM Tris base, 150 mM NaCl, pH 7.6), fixed in 4% glutaraldehyde for 15 min following three washes in TBS, and incubated in blocking solution (2% bovine serum albumin in TBS) for 30 min at room temperature. The cells were subsequently incubated 2 h with anti-FLAG (M1) antibody (2 μg/ml) in TBS supplemented with 1% bovine serum albumin and 1 mM CaCl₂ at room temperature. After three washes in TBS with 1 mM CaCl₂, the cells were incubated for 1 h with goat anti-mouse horseradish peroxidase-conjugated antibody in the same buffer as the anti-FLAG antibody. After three washes in TBS supplemented with 1 mM CaCl₂, the immune reactivity was revealed by the addition of horseradish peroxidase substrate according to the manufacturer’s instruction.

Generation of Stably Transfected L1.2 Cells—Receptor constructs were cloned into the bicistronic vector pIRE2-ZsGreen1 (Clontech), which results in bicistronic gene expression of receptor and GFP located on one mRNA, but translated as two proteins. L1.2 cells were grown at 5% CO₂, 37 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.2 mM glutamine, 180 units/ml penicillin, and 45 μg/ml penicillin/streptomycin. L1.2 cells were transfected as described by the manufacturer using Nucleofector V solutions and program X-001 of the Nucleofector-2b-Device (Lonza, Switzerland). 48 h after transfection, G418 was added at 1 mg/ml and L1.2 cells were seeded in a 96-well plate at 25,000 cells per well. After a further 17 days, pool clones from CCR2 and CCR5-CCR2(all) were analyzed in FACS and sorted for further cultivation before the migration experiments. For CCR5, inserted into pcDNA3.1, 10 clones were picked and tested in a migration assay. The three clones with highest expression were used for the migration experiments.

Migration—ChemoTx 96-well plates (3.2 mm diameter, 5 μm pore size, and 30-μl well volume (Neuroprobe)) were used for migration experiments of stably transfected L1.2 cells. In short, the lower wells were filled with buffer (Hepes-modified RPMI 1640 medium, 0.1% BSA) and ligands, and the filter was applied. 20-μl cell suspension drops containing 200,000 cells/drop were placed on top of the filter for migration into the lower wells. After a 5-h incubation at 5% CO₂, 37 °C, the drops were scraped off, and the filter was removed. The lower well solution was transferred to a white 96-well plate, and 20 μl of CellTiter-Glo® (Promega) solution was added. The luminescence was measured in a Wallac Envision 2104 Multilabel Reader (PerkinElmer Life Sciences).

Sequence Analysis—Protein sequences of 286 class A 7TM receptors were aligned in Geneious 5.3 software using Clustal W multiple sequence alignment algorithm (34). For the alignment, BLOSUM cost matrix was employed, and gap open and gap penalty costs were 10 and 0.1, respectively. Alignment results were verified for proper overlap of highly conserved NPXXY domain specific for TM-VII of class A 7TM receptors. Gaps within TM-VII upstream from NPXXY motive were manually removed. 10 receptor sequences were excluded from the analysis due to lack of similarity in TM-VII region.

Calculations—IC₅₀ and EC₅₀ values were determined by nonlinear regression using the GraphPad-Prism4 software (GraphPad Software, San Diego).
RESULTS

Transfer of a Functional Orthosteric Site from CCR2 to the Signaling Scaffold of CCR5—The functional properties of the Trojan horse chimera, CCR5-CCR2(all) (sequence presented in Fig. 1) were tested in parallel with the two WT receptors (CCR5 and CCR2) using the measurement of IP turnover in transiently transfected COS-7 cells. CCR5-CCR2(all) was activated by the CCR2 chemokines CCL2 and CCL7 with EC50 values of 4.9 and 0.35 nM, respectively, thus with 1.6–4.5-fold higher potencies as compared with CCR2 WT (EC50 values of 7.9 and 1.6 nM, respectively; Fig. 1, B and C, and Table 1). In contrast, the CCR5 chemokines CCL3 and CCL5 had no activity on the chimera (Fig. 1, C and Table 1), whereas they both activated CCR5 WT in the nanomolar range as published previously (Fig. 1, A and Table 1) (35). To assess the domain swap in a more natural signaling experiment, we tested whether the chimera CCR5-CCR2(all) showed the same phenotype in migration assays using L1.2 cells stably transfected with CCR5, CCR2, and CCR5-CCR2(all). Importantly, the migration studies confirmed the successful

TABLE 1

Activation of CCR5, CCR2, and CCR5-CCR2(all) by chemokines and metal ion chelators

| Ligand | CCR5 wt | CCR2 wt | CCR5-CCR2(all) |
|--------|---------|---------|---------------|
|        | EC50 ± SEM | EC50 | Potency | EC50 ± SEM | EC50 | Potency | Fmut |
|        | (nM) | (nM) | (nM) | (nM) | (nM) | (nM) | (fold) |
| CCL3   | -7.8 ± 0.06 | 17 (24) | no activation | \(\text{EC}_{50}\) | \(\text{EC}_{50}\) | \(\text{EC}_{50}\) | \(\text{F}_{\text{mut}}\) |
| CCL5   | -8.7 ± 0.05 | 19 (26) | no activation | \(\text{EC}_{50}\) | \(\text{EC}_{50}\) | \(\text{EC}_{50}\) | \(\text{F}_{\text{mut}}\) |
| CCL2   | no activation | (5) | \(\text{EC}_{50}\) | \(\text{EC}_{50}\) | \(\text{EC}_{50}\) | \(\text{EC}_{50}\) | \(\text{F}_{\text{mut}}\) |
| CCL7   | no activation | (4) | \(\text{EC}_{50}\) | \(\text{EC}_{50}\) | \(\text{EC}_{50}\) | \(\text{EC}_{50}\) | \(\text{F}_{\text{mut}}\) |

Interchange in 7TMD Receptors, Implications for Future Drugs
domain swap, as CCL2 and CCL7 activated the chimera with similar potencies as CCR2 WT, whereas CCL3 and CCL5 only activated CCR5 WT (Fig. 1, D–F). Additional chimeras with single exchanged extracellular domains were not activated by the CCR2-specific chemokines indicating that more than one domain was needed for proper binding of CCL2 and CCL7 (supplemental Fig. 1). In summary, construction of the Trojan horse CCR5-CCR2(all) successfully led to an active receptor ligands activated CCR5-CCR2(all) with similar or even higher potencies (EC50 values from 4.5 to 23 μM) as observed for CCR1 (17). The interaction of these ligands with the structural similarity and considerable overlap between endogenous ligands for CCR1 and CCR5 (both are activated by—Because of the structural similarity and considerable overlap between endogenous ligands for CCR1 and CCR5 (both are activated by metal ions nor chelators alone showed any activity on CCR5 (data not shown). Conclusively, although the orthosteric chemokine-binding site could be transferred from CCR2 onto a CCR5 scaffold (and is thereby linked to the extracellular domains of CCR2), the small molecule activity was retained within the transmembrane regions of CCR5 and was unaffected by the exchange of the extracellular regions with those of CCR2.

**Positive Cooperative Effects of Small Molecule Agonists in CCR5 Could Not Be Transferred to CCR5-CCR2(all)**—In addition to acting as agonists, the metal ion chelators were able to enhance the binding of 125I-CCL3 to CCR5 up to 7-fold above maximum specific binding in the absence of unlabeled ligand, thus acting allosteric in relation to CCL3 similar to the previously described action in CCR1 (17). The binding affinities of metal ion chelators as measured against 125I-CCL3 were in the same range with their agonistic potencies (Ki from 7.3 to 93 μM, Fig. 3A), again as observed in CCR1 (17). However, compared with the high affinity displacement of CCL5 from CCR1 (17), a 10–30-fold lower affinity was observed for metal ion chelators in displacing 125I-CCL5 from CCR5 (Ki from 197 to 620 μM, Fig. 3B). This indicates an allosteric binding mode in CCR5 related to CCL5, in contrast to a competition situation in CCR1 with overlapping binding sites between metal ion chelators and CCL5. As only CCR2-specific chemokines bound to CCR5-CCR2(all), we tested whether the allosteric enhancing effect on 125I-CCL3 binding to CCR5 could be transferred from the CCR5 transmembrane unit (binding the allosteric compounds) to the CCR2 extracellular interface. Despite the maintained agonism of the small molecules in the chimeric CCR5-CCR2(all) (Fig. 2C and Table 1), no allosteric enhancement of 125I-L-CCL2 or 125I-L-CCL7 binding was observed. In contrast, a low affinity displacement with Ki values from 162 to 528 μM for 125I-L-CCL2 and from 90 to 267 μM for 125I-L-CCL7 was observed (Fig. 3, C and D), i.e. with Ki values lying in the same range as observed for 125I-L-CCL5 binding to CCR5 (Fig. 3B).

**Action of Small Molecule Agonists Depends upon Acidic Residues in the Top of TM-VII**—To investigate the extension and possible overlap of the binding sites for chemokines and small molecules in CCR2, CCR5, and CCR5-CCR2(all), we initially focused on two acidic residues in TM-VII, Asp in position VII:02 and Glu in position VII:06. Besides being anchor for many small molecule agonists and antagonists, the chemokine receptor-conserved GluVII:06 is also the anchor point for many chemokines (7, 17). AspVII:02 is also found predominantly among chemokine receptors (~55% among these) as compared with ~6% among nonchemokine class A 7TM receptors and
AspVII:-02, located two helical turns above, contributes to a molecule agonists completely depends on GluVII:06, whereas seen in Fig. 4, the activity (Fig. 4, with CCR5 WT (2.7–13-fold decreased potencies), whereas a impaired the activity of small molecule agonists as compared earlier studies (12). For CCL7, however, GluVII:06 seemed more important (63-fold decreased potency upon Ala substitution) than AspVII:02 (5.6-fold decreased potency) (Fig. 5B). In CCR5-CCR2(all), a much lower dependence was observed for both acidic residues, as Ala substitution of GluVII:06 only resulted in 3.6-fold decreased CCL7 potency and 4.4-fold decreased CCL2 potency, whereas the impact of AspVII:02 was even smaller (1.1–3.2-fold decrease) (Fig. 5, C and D). Changes in receptor surface expression could not explain the changes in potencies, as all Ala substitutions were found to be

### TABLE 2

Surface expression of mutations AspVII:-02Ala and GluVII:06Ala in CCR5, CCR2, and CCR5-CCR2(all) and their effect on chemokine and metal ion chelator-mediated receptor activation.

| Mutation | Surface Expression | CCL2 | CCL3 | CCL5 | CCL7 | ZnBip | ZnPhe |
|----------|-------------------|------|------|------|------|-------|-------|
|          |                   | EC50 | EC50 | EC50 | EC50 | EC50  | EC50  |
| WT       |                   |      |      |      |      |       |       |
| C276A    |                   |      |      |      |      |       |       |
| C284A    |                   |      |      |      |      |       |       |
| E283A    |                   |      |      |      |      |       |       |
| D276A    |                   |      |      |      |      |       |       |
| E291A    |                   |      |      |      |      |       |       |

**Different Interaction of CCL2 and CCL7 with CCR2 WT as Compared with Chimeric Receptor**—Contrary to the similar dependence of the small molecule agonists on acidic residues in CCR5 WT and in CCR5-CCR2(all), the pattern differed for the CCR2 chemokine agonists (CCL2 and CCL7). Thus, CCL2 was highly dependent on GluVII:06 and AspVII:02 in CCR2 WT as >13-fold decreased potency was observed for Ala substitution of either of these residues (Fig. 5A and Table 2), as reported in earlier studies (12). For CCL7, however, GluVII:06 seemed more important (63-fold decreased potency upon Ala substitution) than AspVII:02 (5.6-fold decreased potency) (Fig. 5B). In CCR5-CCR2(all), a much lower dependence was observed for both acidic residues, as Ala substitution of GluVII:06 only resulted in 3.6-fold decreased CCL7 potency and 4.4-fold decreased CCL2 potency, whereas the impact of AspVII:02 was even smaller (1.1–3.2-fold decrease) (Fig. 5, C and D). Changes in receptor surface expression could not explain the changes in potencies, as all Ala substitutions were found to be

![Diagram](image-url)
equal to or even higher expressed than the respective WT receptors as determined by ELISA (Table 2). Furthermore, homologous competition binding experiments uncovered unchanged CCR2 WT-like affinities for CCL2 and CCL7 on both Ala substitutions in CCR2 (Table 3), despite the decreases in potencies (Table 2). In contrast, the two Ala substitutions in the CCR5-CCR2(all) background resulted in minor decreases in affinities (1.9–4.0-fold, see Table 3), which for both ligands were similar to the observed decreases in potencies (1.1–4.4-fold, see Table 2). Thus, it is concluded that the two acidic residues at the top of TMVII (Asp284 and Glu291) are of huge importance for the activation but not the binding of CCL2 and CCL7 on CCRT2 WT, although this dependence for chemokine binding to CCR5 and CCR5-CCR2(all) were 100-fold reduced (Fig. 6). On the contrary, the affinities of the potencies of CCL2 and CCL7 at CCR5-CCR2(all) were up to 4.4-fold, see Table 3). Thus, it is concluded that the two acidic residues at the top of TMVII (Asp284 and Glu291) are of huge importance for the activation but not the binding of CCL2 and CCL7 on CCR2 WT, although this dependence for chemokine-mediated receptor activation is weakened in the CCR5-CCR2(all) chimera.

Allosteric Mechanisms for the Small Molecule Agonists in CCR5 WT and CCR5-CCR2(all)—To further investigate the action of the small molecule agonists, their effects on chemokine potency and affinity were assessed. Although only small decreases in potencies were observed for CCL3 and CCL5 activating CCR5 WT in the presence of small molecule agonists, the potencies of CCL2 and CCL7 at CCR5-CCR2(all) were up to 100-fold reduced (Fig. 6). On the contrary, the affinities of chemokine binding to CCR5 and CCR5-CCR2(all) were unchanged in the presence of small molecule agonists, as observed in competition binding experiments (Table 4). Conclusively, as ligands with overlapping binding sites (i.e., competitive ligands) are expected to primarily affect the affinity of an orthosteric ligand, these ligand co-administration experiments clearly suggest an allosteric binding mode of the small molecule agonists and thereby confirm the binding and functional data in Figs. 1–5.

Retained Activity of CCR5 Nonpeptide Antagonists in the CCR5-CCR2(all) Chimera—As a third pharmacological aspect, we investigated the effect of the domain swapping in the CCR5-CCR2(all) chimera on the CCR5 antagonists SCH-C and aplaviroc, which completely lost its antagonistic functionality in the CCR5-CCR2(all) chimera. As expected from the dual nature of TAK-779, the antagonistic property was retained in the chimera. Interestingly, the activity of SCH-C was also retained and thereby independent of the extracellular CCR2 domains. This was not observed for aplaviroc, which completely lost its antagonistic functionality in CCR5-CCR2(all) chimera due to the absence of these particular residues in ECL-2b of CCR2 and consequently in CCR5-CCR2(all).

Rescue of Aplaviroc Activity by Reintroducing ECL-2b from CCR5 into CCR5-CCR2(all)—To further investigate the activity with an IC50 of 10 and 29 nm, respectively, i.e. with

### Table 3

| Homologous chemokine binding to CCR2, CCR5, and CCR5-CCR2(all) with or without Ala substitutions of acidic residues in TMVII |
|---------------------------------------------------------------|
| **IC50 ± SEM** | **IC50** (log) | **IC50** (nM) | **Fold** (n) |
| 125I-CCL3 binding | CCR5 | D276A VII:-02 | -8,4 ± 0,05 | 4,1 | 1,0 (3) |
| | E283A VII:06 | -8,5 ± 0,16 | 3,1 | 0,8 (3) |
| 125I-CCL7 binding | CCR5-CCR2(all) | D284A VII:-02 | -8,2 ± 0,14 | 7 | 1,0 (5) |
| | E291A VII:06 | -8,5 ± 0,18 | 3,5 | 4,0 (5) |

**FIGURE 5.** Effect on chemokine ligands of the Ala substitutions of AspVII:-02 and GluVII:06 in CCR2 WT and in the chimeric receptor. The activation of WT receptors and chimera (stippled), and the Ala substitutions of AspVII:-02 (□) and GluVII:06 (●) in WT CCR2 (A and B) and CCR5-CCR2(all) (D and E) was tested in an IP turnover assay in COS-7 cells as described in legend to Fig. 1. The activation curves are shown for CCL2 (left panel) and CCL7 (right panel). (n = 2–15).
Chemokine binding to CCR5, CCR5-CCR2(all), and CCR2 in the absence and presence of small molecule agonists

The competition binding experiments were performed in transiently transfected COS-7 cells using 125I-CCL3, 125I-CCL2, and 125I-CCL7 as radioligands. The log IC50 values for the homologous binding curves in the absence and presence of the small molecule agonists are shown together with the average fold increase in Bmax to CCR5-CCR2(all). The potencies of the metal ion chelators were ranging from 1.3 μM for ZnPhe, over 3.1 μM for CuPhe and 11 μM for ZnBip to 29 μM for CuBip. This indicates that the absence of ECL-2b from CCR2 makes no difference for proper chemokine or small molecule-based activation.

**TABLE 4**

Chemokine binding to CCR5, CCR5-CCR2(all), and CCR2 in the absence and presence of small molecule agonists

The competition binding experiments were performed in transiently transfected COS-7 cells using 125I-CCL3, 125I-CCL2, and 125I-CCL7 as radioligands. The log IC50 values for the homologous binding curves in the absence and presence of the small molecule agonists are shown together with the average fold increase in Bmax calculated from each assay. The number of experiments (n) is given. MC stands for metal ion chelator.

**FIGURE 6.** Effect of small molecule agonists on the potency of chemokines at CCR5 and CCR5-CCR2(all). The activation of CCR5 by CCL3 and CCL5 (A) and CCR5-CCR2(all) by CCL2 and CCL7 (B) in the absence and presence of ZnBip, ZnPhe, CuPhe, and CuBip was determined in an IP turnover assay in COS-7 cells. The concentrations of the small molecule agonists were chosen to reach ~ 20% and 60% of maximal chemokine activation and were as follows: CCR5, ZnBip/CuBip 10 and 32 μM and ZnPhe/CuPhe 3.2 and 10 μM; CCR5-CCR2(all), ZnBip/CuBip 3.2 and 10 μM and ZnPhe/CuPhe 1.0 and 3.2 μM. The fold decrease of potency as compared with the absence of small molecule agonist is shown.

**Figure 6: Effect of small molecule agonists on the potency of chemokines at CCR5 and CCR5-CCR2(all).** The activation of CCR5 by CCL3 and CCL5 (A) and CCR5-CCR2(all) by CCL2 and CCL7 (B) in the absence and presence of ZnBip, ZnPhe, CuPhe, and CuBip was determined in an IP turnover assay in COS-7 cells. The concentrations of the small molecule agonists were chosen to reach ~ 20% and 60% of maximal chemokine activation and were as follows: CCR5, ZnBip/CuBip 10 and 32 μM and ZnPhe/CuPhe 3.2 and 10 μM; CCR5-CCR2(all), ZnBip/CuBip 3.2 and 10 μM and ZnPhe/CuPhe 1.0 and 3.2 μM. The fold decrease of potency as compared with the absence of small molecule agonist is shown.
Interchange in 7TM Receptors, Implications for Future Drugs

(CCR6-CCR5(all)), did not succeed in maintaining CCR5 chemokine binding (37). This could be due to the low identity between these two receptors (33%) as compared with 74% between CCR5 and CCR2 and thereby a larger degree of structural changes applied to the CCR6-CCR5 chimera. Importantly, our data confirm the current understanding of chemokine binding to the extracellular receptor regions. A two-state model has been proposed for the action of CCL2 with CCR2, stating that an initial high affinity interaction of the chemokine core with the extracellular domains of the receptor (especially the N terminus) is followed by a receptor-activating interaction of the chemokine N terminus with residues in the transmembrane helices. Thus, CCL2 was shown to interact with a DYDY motif in the CCR2 N terminus (42, 43), followed by interaction of the chemokine N terminus with AspVII-02 (Asp284) and GluVII:06 (Glu291) in TM-VII (12). These data are indeed confirmed here by the unchanged high affinity but very low potencies of CCL2 and CCL7 in the Ala substitutions of these two acidic residues in CCR2 (Fig. 5 and Table 3). In addition, ECL-2 has earlier been shown to be important for CCL2 activity (29), again in agreement with our finding that transfer of a single region from CCR2 to CCR5 (N terminus or any of the ECLs) is insufficient for proper CCL2 or CCL7 action (supplemental Fig. 1). In CCR5, we confirm earlier studies showing that especially ECL-2 (29) and, in the case of CCL3, also ECL-3 are highly important, as we find that their replacement with CCR2 counterparts abolished CCL3 and CCL5 activity (supplemental Fig. 1). Altogether, our chimera confirms that chemokines interact with and recognize their receptors via the extracellular domains (Fig. 9).

Identification of a Novel Class of Small Molecule Agonists for CCR5—Metal ion chelators (Zn^{2+} or Cu^{2+} ions in complex with bipyridine or phenanthroline, Fig. 2) have earlier been shown to be complex ago-allosteric modulators of CCR1 by acting as agonists with micromolar potencies and concomitantly allosterically enhancing the binding of CCL3, although they displaced CCL5 with micromolar affinities indicating partly overlapping binding sites in the area around GluVII:06, as Ala substitution in this position resulted in huge and similar decreases in potencies for CCL5 and metal ion chelators (but not CCL3) (17). In this study, we find that also CCR5 is activated by these molecules that, as in CCR1, depend on GluVII:06 (Table 2). Like in CCR1, the small molecule agonists enhance the binding of CCL3 (act allosterically) but do not displace CCL5 with the same high affinity as observed in CCR1. This could be due to a lesser degree of overlap between small molecule agonists and CCL5-binding sites in CCR5 compared with CCR1, as clearly indicated by the lower impact of Ala substitution of GluVII:06 in CCR5 (5.1-fold decrease in potency for CCL5, Table 2) as compared with 26-fold decrease in CCL5 potency in CCR1 (17). Our test of the impact of small molecule agonists on the potency (Fig. 6) and affinity (Table 4) of the chemokine ligands confirms the allosteric mode of action as no changes in the chemokine affinities were observed in the presence of small molecule agonists, although their potencies were slightly reduced at CCR5. In conclusion, CCL5 and the metal ion chelator-based agonists do not overlap in CCR5, which in turn also confirms that CCL5 interacts differently with CCR5 compared with CCR1, as suggested previously (27, 44).

Separation of Small Molecule and Chemokine-binding Sites—Interestingly, the activity of small molecule agonists was retained in CCR5-CCR2_{all} and could therefore be assigned to the transmembrane region of CCR5. However, as the loops of CCR5 and CCR2 are similar to a certain extent, it cannot be excluded that also residues in the extracellular domains contribute to the small molecule agonist site. Thus, in both receptors, ECL-2, which is linked to the top of TM-III via a conserved disulfide bridge to CysIII:01, harbors a Glu in position Cys^{-6} (6 residues prior to the conserved Cys in ECL-2) and an aromatic residue in position Cys^{+4}. Furthermore, ECL-2 varies greatly in length and sequence throughout class A 7TM receptors, and with its position on top of the major binding pocket, with a lid function in some receptors, ECL-2 contributes to ligand specificity in some class A 7TM receptors (45). Indeed, we find that

![FIGURE 7. Activity of small molecule antagonists on WT CCR5, WT CCR2, and CCR5-CCR2_{all}.](image-url)
aplaviroc loses its antagonistic property in CCR5-CCR2(all) but could be rescued by reintroducing ECL-2b, which is in good agreement with earlier studies (14). Interestingly, the presently available crystal structures of 7TM receptors have uncovered several different conformations of ECL-2 ranging from $\alpha$-sheets with the receptor N terminus (rhodopsin) or ECL-1 (adenosine 2A receptor) to small $\alpha$-helices (1- and 2-adrenoceptors) (46–54). In CXCR4, an anti-parallel $\alpha$-sheet is formed, which in one case is extended with another $\alpha$-strand from the co-crystallized peptide antagonist (36). In addition to the small molecule agonists, the activity of the antagonist SCH-C was also retained in the chimera. Thus, it can be concluded that we successfully generated a receptor with two distinct sites that bind ligands independently of each other (Fig. 9).

As expected from studies in CCR1 (17), the small molecule agonists anchored to GluVII:06 in CCR5-CCR2(all) and in WT CCR5 (Table 2). CCL2 and CCL7, however, have a reduced (or even lost) dependence of both GluVII:06 and AspVII:02 in the chimera as compared with CCR2. Thus, even though both chemokines bind to the extracellular CCR2 domains in the chimera, the interaction with GluVII:06 and AspVII:02 was weakened and was not sufficient to compete for the small molecule agonists, presumably because of a slightly altered (although still high affine) binding of the chemokines in the extracellular domains (Fig. 9). Consequently, CCL2 and CCL7 are only displaced by the small molecule agonists from CCR5-CCR2(all) with rather low affinity, but neither is their binding enhanced, indicating that the positive allosteric cross-talk from the CCR5 transmembrane region (binding the ago-allosteric molecules) with the CCR5 extracellular domains (binding CCL3), could not be established in CCR5-CCR2(all). Thus, positive allosteric modulation not only requires that similar active conformations are stabilized but also maintained conformational interchange and communication between domains, properties that could not be established in the chimera.

Ago-allosteric Ligands in CCR5 and CCR5-CCR2(all) - Allosteric modulators can be neutral, i.e. do not signal on their own, or they can be agonists or antagonists for a given receptor. However, independent of the efficacy, the binding of an allosteric modulator leads to changes in the balance between different
CCR5-CCR2(all) with very low affinities (Fig. 3, B–D), which importantly were much lower compared with the affinities for the enhancement of \(^{125}\text{I}-\text{CCL3}\) binding (Fig. 3A) and to the potencies as agonists (Fig. 2, A and C). These low affinity displacements and the decreased potencies of CCL2 and CCL7 at CCR5-CCR2(all) in the presence of small molecule agonists (Fig. 6B) correlate well with the different dependence of chemokines and small molecule ligands on Asp\(^{284}\) and Glu\(^{291}\) in CCR5-CCR2(all). Thus, because CCL2 and CCL7 do not compete with small molecule agonists for Asp\(^{284}\) and Glu\(^{291}\), higher concentrations are needed to overcome the small molecule agonist-bound state to further activate the receptor, resulting in the observed decrease in potencies in the presence of small molecule agonists. Different and equally complex kinds of allosteric behaviors have earlier been observed in e.g. the action of L-692,429 at the ghrelin receptor (55) or the actions of Org-27569, Org-27759, and Org-29647 at the CB\(_2\) receptor (56).

We conclude that Trojan horse-based chimeric receptors constitute a powerful pharmacological tool to study allosteric phenomena in 7TM receptors. The construction and combination of receptors in the chimera can be chosen so that not only a separation of the orthosteric and allosteric binding sites is achieved but also a separation between the ligand-binding and receptor-activating domains. This also emphasizes the module-like construction of 7TM receptors and confirms the understanding that agonists may activate certain (or different) receptors in different ways (2). Furthermore, because of their advantages such as the higher specificity and dependence on the orthosteric ligand, allosteric modulators and the understanding of their action will become increasingly important in drug development.

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**FIGURE 8.** Rescued aplaviroc activity in CCR5-CCR2(all). A schematic drawing of CCR5 in black (left), CCR5-CCR2(all) (middle), and CCR5-CCR2(all,Delta2b) (right) with CCR2-derived parts shown in gray. The activity of aplaviroc on WT CCR5 (stippled), CCR5-CCR2(all) ( ), and CCR5-CCR2(all,Delta2b) ( ) was tested in an IP turnover assay in COS-7 cells. A, antagonism against CCL2-activated chimeras and CCL3-activated CCR5 (dashed line). B, antagonism against CCL7-activated chimeras and CCL5-activated CCR5 (dashed line). All curves are normalized to the maximal chemokine activation of the respective curve (n = 2–6).

**FIGURE 9.** Schematic overview of chemokine and small molecule interaction with CCR5 WT, CCR2 WT, and CCR5-CCR2(all). A schematic drawing of CCR5 WT (left, gray), CCR2 WT (middle, blue), and chimeric CCR5-CCR2(all) receptor (right, gray and blue) as seen from the plane of the membrane and with the extracellular loops and N terminus (EC1) visualized at the top and the intracellular domains (ICD) at the bottom, separating the transmembrane area (TM), with TM-VII accented in darker color. All three receptors contain AspVII:-02 and GluVII:06 (highlighted as yellow points in TM-VII). In WT CCR5 and CCR2, chemokine interaction is dependent on the acidic residues, although this interaction is weakened in the chimeric receptor (middle row). Also small molecules (metal ion chelators) interact with the two acidic residues in CCR5 and CCR5-CCR2(all), but are not binding to CCR2.

ent receptor conformations, which in turn are likely to change the affinity and/or function of the orthosteric ligands. In CCR5, we identified the metal ion chelator complexes to be agonists and positive allosteric modulators toward CCL3 (ago-allosteric ligands). The allosteric nature is furthermore underlined by the fact that these compounds competed against \(^{125}\text{I}-\text{CCL5}\) binding to CCR5 and \(^{125}\text{I}-\text{CCL2}\) and \(^{125}\text{I}-\text{CCL7}\) binding to CCR5.
