Structure of CC chemokine receptor 2 with orthosteric and allosteric antagonists

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CC chemokine receptor 2 (CCR2) is one of 19 members of the chemokine receptor subfamily of human class A G-protein-coupled receptors. CCR2 is expressed on monocytes, immature dendritic cells, and T-cell subpopulations, and mediates their migration towards endogenous CC chemokine ligands such as CCL2 (ref. 1). CCR2 and its ligands are implicated in numerous inflammatory and neurodegenerative diseases1 including atherosclerosis, multiple sclerosis, asthma, neuropathic pain, and diabetic nephropathy, as well as cancer2. These disease associations have motivated numerous preclinical studies and clinical trials4 (see http://www.clinicaltrials.gov) in search of therapies that target the CCR2–chemokine axis. To aid drug discovery efforts5, here we solve a structure of CCR2 in a ternary complex with an orthosteric (BMS-681 (ref. 6)) and allosteric (CCR2-RA-[R]7) antagonist. BMS-681 inhibits chemokine binding by occupying the orthosteric pocket of the receptor in a previously unseen binding mode. CCR2-RA-[R] binds in a novel, highly druggable pocket that is the most intracellular allosteric site observed in class A G-protein-coupled receptors so far; this site spatially overlaps the G-protein-binding site in homologous receptors. CCR2-RA-[R] inhibits CCR2 non-competitively by blocking activation-associated conformational changes and formation of the G-protein-binding interface. The conformational signature of the conserved microwatch residues observed in double-antagonist-bound CCR2 resembles the most inactive G-protein-coupled receptor structures solved so far. Like other protein–protein interactions, receptor–chemokine complexes are considered challenging therapeutic targets for small molecules, and the present structure suggests diverse pocket epitopes that can be exploited to overcome obstacles in drug design.

A ternary complex between an engineered construct of human CCR2 isoform B (further referred to as CCR2-T4L or simply CCR2), an orthosteric antagonist BMS-681 (compound 13d in ref. 6), and an allosteric antagonist CCR2-RA-[R]7 is crystallized using the lipidic cubic phase (LCP) method8, and the structure was determined to 2.8 Å resolution (Extended Data Table 1 and Extended Data Fig. 1). Simultaneous addition of two compounds markedly stabilized detergent-solubilized CCR2-T4L compared with twice the concentration of each compound individually (Fig. 1a), suggesting concurrent binding of CCR2-RA-[R] and BMS-681 to the receptor. The presence of both compounds was critical for crystallization.

In the structure, CCR2 adopts the canonical fold of class A G-protein-coupled receptors (GPCRs) with seven transmembrane (TM) helices connected by three extracellular (EC) and three intracellular (IC) loops (Fig. 1b). Both compounds are visible in the electron density (Fig. 1b–d); BMS-681 binds in the extracellular orthosteric pocket (Fig. 1b, c) while CCR2-RA-[R] is located more than 30 Å away (Fig. 1b, d), in a site that is the most intracellular allosteric pocket observed in class A GPCRs so far (Fig. 1e). The binding site of CCR2-RA-[R] spatially overlaps with the G-protein-binding site in homologous receptors (Fig. 1f). As for other chemokine receptors9–12, CCR2 is expected to have two conserved disulfide bonds in its extracellular domains, with Cys32–Cys277 connecting the amino (N) terminus to ECL3 (NT–ECL3), and Cys113–Cys190 connecting TM3 to ECL2. Electron density is apparent for the ECL2–TM3 disulfide bond but not for the N-terminal residues 1–36 or the NT–ECL3 disulfide bond (Fig. 1b, c). Because the NT–ECL3 disulfide bond has been shown to be important for CCR2 signalling13, its absence is unlikely to be an inherent feature of the receptor; instead, it might be caused by strain of the bond in the ligand-bound state of the receptor14, possibly exacerbated by solvent exposure and radiation damage of the crystals15.

As with other chemokine receptors, the extracellular orthosteric pocket of CCR2 can be divided into a major and a minor subpocket, defined by helices III–VII, and helices I–III and VII, respectively, and separated by residues Y1203.32 and E2917.39 (superscript indicates residue number according to Ballesteros–Weinstein nomenclature). BMS-681 binds predominantly in the minor subpocket (Fig. 2a, b) and buries 366.3 Å² of surface area. The 6-trifluoromethyl quinazoline moiety protrudes between helices I and VII towards the lipid bilayer, while the tri-substituted cyclohexane packs against W982.60. The γ-lactam secondary exocyclic amine forms a hydrogen bond with the hydroxyl of T2927.40, which is critical for binding of chemically related compounds such as BMS-558 (compound 22 in ref. 16) and the Teijin lead series17,18. This amine is also within hydrogen-bonding distance from the backbone carbonyl of Q2887.36. The carbonyl oxygen of the γ-lactam forms a hydrogen bond with Y491.39, which itself is hydrogen-bonded to the side chain of T2927.40. The N1 nitrogen of the quinazoline is within 4 Å of the Q2887.36 side chain. The protonated tertiary amine on the cyclohexane ring is proximal to a structured water molecule in the binding site. Some CCR2 antagonists, particularly those containing a basic amine, are known to depend on the conserved E2917.39 in the receptor19; however, no direct interaction is observed between E2917.39 and BMS-681. The receptor-bound, bioactive conformation of BMS-681 is strikingly similar to the crystallographic conformation of free BMS-681 (Fig. 2c and Extended Data Table 2), suggesting the absence of internal strain in the bound state.

BMS-681 engages several residues that are critical for CCL2 binding and/or activation of CCR2 (refs 17, 18) including Y491.39, W982.60, Y1203.32, and T2927.40. Thus, it seems to directly compete with chemokine binding to the orthosteric pocket. Additionally, by inserting between helices I and VII, BMS-681 may put strain onto residues C32–V37 connecting TM1 to ECL3, destabilize the conserved NT–ECL3 disulfide bond (absent in the structure), and prevent the N terminus and TM1 from adopting a productive chemokine binding conformation observed in homologous receptor–chemokine structures11,12 (Extended Data Fig. 2).
On the opposite side of the receptor, CCR2-RA-[R] is caged by the intracellular ends of helices I–III and VI–VIII and buries 297.8 Å² of surface area. The inner hydrophilic part of the cage is made by V63, L67, L81, L134, A241, V244, I245, Y305, F312, while the outer (cytosol-facing) polar part consists of T77, R138, G309, K311, and Y315 (Fig. 2d, e), as well as the backbones of engineered R237 and K240. The binding pocket of CCR2-RA-[R] is highly enclosed and possesses a balanced combination of hydrophobic and polar features, all of which favours pocket ‘druggability’. Owing to the lack of a side-chain on G309, the hydroxyl and pyrrolone carbonyl groups of CCR2-RA-[R] can hydrogen-bond to the exposed backbone amides of E310, K311, and F312 (Fig. 2d, e). The acetyl group of the compound resides near the terminal amine of K311. The critical roles of V244, Y305, K311, and F312 in CCR2-RA-[R] binding were established by an earlier mutagenesis study. Because homologues of several residues in the CCR2-RA-[R] binding pocket directly couple to the G protein in bovine rhodopsin and the β3 adrenergic receptor (β3AR) structures (Extended Data Fig. 3), CCR2-RA-[R] appears to sterically interfere with G-protein binding to CCR2.

The structure suggests an interesting symmetrical mechanism for the concurrent antagonistic action of the two compounds. BMS-681 interferes with chemokine binding directly and with G-protein coupling indirectly, by stabilizing an inactive, presumably G-protein-incompatible, conformation of the receptor. Conversely, CCR2-RA-[R] directly prevents G-protein coupling and allosterically inhibits binding of the CCL2 chemokine, which, like most GPCR agonists, requires an active, G-protein-associated receptor for high affinity binding. Bi-directional allosteric communication between the extra- and intracellular sides of the receptor is reminiscent of that previously observed in adenosine A2A receptor (AA2AR) structures and in a two-dimensional schematic depiction. Of the two conformers in the free BMS-681 structure, one is almost identical to the CCR2-complexed conformation. A thermal denaturation study revealed an active, G-protein-associated receptor for high affinity binding and transducin peptide bound to rhodopsin (PDB accession numbers 3SN6 and 4X1H).

**Figure 1** | Structure of a complex between CCR2, BMS-681 and CCR2-RA-[R] and comparison with other allosteric modulators of class A GPCRs. **a.** Thermal denaturation curves demonstrate higher stability of CCR2-T4L in the presence of both BMS-681 and CCR2-RA-[R] compared with each compound individually. Data are representative of three independent experiments conducted on different days. b. Overall view of double-antagonist-bound CCR2. c, d, Structure viewed from the extracellular (c) and intracellular (d) side with simulated annealing omit maps of BMS-681 (c) and CCR2-RA-[R] (d) shown at 3σ. e, CCR2-RA-[R] compared with other allosteric ligands crystallized with class A GPCRs (Protein Data Bank (PDB) accession numbers 4MBS, 4XNV, 4PHU, and 4MOT). f, CCR2-RA-[R] compared with the carboxy (C)-terminal helix of Gαs bound to the β3 adrenergic receptor and transducin peptide bound to rhodopsin (PDB accession numbers 3SN6 and 4X1H).

**Figure 2** | Ligand binding sites and receptor interactions. **a,** BMS-681 interactions with CCR2 viewed in three dimensions from the extracellular side (a) and in a two-dimensional schematic depiction (b). c, Of the two conformers in the free BMS-681 structure, one is almost identical to the CCR2-complexed conformation. d, e, CCR2-RA-[R] interactions viewed in three dimensions along the plane of the membrane and from the intracellular side (d), and in a two-dimensional depiction (e). In b, e, polar and non-polar residue contacts are shown as blue and green, respectively. Bulk solvent and lipid are represented by blue and yellow shading, respectively.
(ref. 23) (orthosteric) and [3H]CCR2-RA (allosteric). In equilibrium competition binding assays on wild-type CCR2, both INCB-3344 and CCR2-RA-[R] displaced their homologous radioligand with half-maximum inhibitory concentration (IC50) values of 17 and 13 nM, respectively (Extended Data Table 3), comparable to previously reported values23. Compared to wild-type CCR2, the affinity of both antagonists towards CCR2-T4L was improved by approximately twofold, suggesting a slight engineering-related shift towards the inactive state. BMS-681 fully displaced [3H]INCB-3344 with nanomolar affinities for both constructs, but did not displace [3H]CCR2-RA. Instead, at 1 μM concentration it enhanced the binding of [3H]CCR2-RA by >30% (Extended Data Fig. 4a, b and Extended Data Table 3).

In kinetic radioligand experiments, the presence of BMS-681 also increased total binding of [3H]CCR2-RA to both wild-type CCR2 and CCR2-T4L, with the increase as high as 62% in the case of CCR2-T4L (Extended Data Fig. 4c, d and Extended Data Table 4). BMS-681 (1 μM) decreased the dissociation rate constant of [3H]CCR2-RA, while producing a slight increase (wild-type CCR2) or no change (CCR2-T4L) in the observed association rate constants. Moreover, for CCR2-T4L, the presence of BMS-681 changed the biphasic dissociation profile of [3H]CCR2-RA to monophasic, suggesting stabilization of the receptor population in a homogeneous conformational state (Extended Data Table 4). Along with the stability and equilibrium binding data, these results further corroborate the hypothesis that BMS-681 and CCR2-RA-[R] cooperatively stabilize a preferred inactive conformation of CCR2-T4L.

We next analysed the structure of double-antagonist-bound CCR2-T4L to better understand this conformation. The plethora of existing class A GPCR structures suggests a conserved conformational signature of an active receptor state26. This signature involves increased separation between the intracellular end of helix VI and the rest of the TM bundle, an inward repositioning and rotation of helix VII, and concerted repacking of the highly conserved microswitches R3.50 (of the DR3.50Y motif), Y5.58, and Y7.53 (of the NPxY7.53 motif) (Fig. 3a, b) to form an intracellular binding interface for G protein. Furthermore, rather than adopting either an ‘on’ or ‘off’ state, receptors can occupy an ensemble of intermediate conformations27. The active state signature is fully represented in US28, the only agonist-bound chemokine receptor crystalized so far12 (Fig. 3a–c). By contrast, the double-antagonist-bound CCR2 structure appears to occupy the opposite end of the activation spectrum as it shares the conformational microswitch signatures of the most inactive GPCR structures observed thus far (Fig. 3a–e).

As in the inactive CCR5–maraviroc complex10, the intracellular ends of CCR2 helices III and VI are close together, and the conserved R3.50 interacts with D3.49 and T2.39, effectively disrupting the G-protein-binding pocket (Fig. 3b, d, e). Similarly, in both CCR2 and CCR5 structures, the intracellular end of helix VII is in the inactive outward-facing conformation with Y7.53 pointing towards helix II rather than the centre of the bundle. However, in CCR5, Y5.58 is oriented towards the centre of the bundle, whereas in the present CCR2 structure, it faces the lipid and is sterically blocked from approaching R3.50 and Y7.53 by F6.38 (Fig. 3d, e). The net result of these interactions is that the crystallographically observed conformation of CCR2 appears to be even more inactive than that of CCR5 and most similar to dark rhodopsin28 (Fig. 3f).

The allosteric pocket possesses a balanced combination of hydrophobic and polar features, making it a promising target for drug development.
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Author Contributions I.K. and T.M.H. designed the study and coordinated all experiments. V.Y. designed and engineered protein constructs, performed crystallization experiments, collected the diffraction data, and determined the structure. I.Q., M.G., and C.Z. assisted with protein engineering and crystallization. G.W.H. assisted with protein terminal domain of CCR5 and refinement. A.P.I. and L.H.H. designed, and N.V.O.Z. and H.d.V. performed, equilibrium and kinetics binding experiments. I.K. performed computational and bioinformatics analyses. R.J.C., P.C., and A.T. synthesized, characterized, and crystallized the BMS-681 compound analogues. M.D. assisted with compound crystallization, D.S. assisted with the allosteric compound characterization, R.A. assisted with structure analysis, V.C. and R.C.S. assisted with crystallography, Y.Z., N.V.O.Z., and T.M.H. wrote the paper.

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Research Letter

**METHODS**

Design and expression of CCR2-T4L fusion constructs. The sequence of human CCR2 isoform B (Uniprot ID P41597-2) was engineered for crystallization by truncation of C-terminal residues 329–360 and by grafting T4 lysozyme (T4L) into the ICL3. In the process of construct optimization, the native CCR2 residues between L226-62 and R240-32 (L226-62: KTLLRCRNEKKHR-R240-32) were removed and replaced with corresponding residues from the crystallized structure of M2 muscarinic acetylcholine receptor (PDB accession number 3U0N, resulting amino-acid sequence 522-547: RAKSRL-T4L-PPPSREK-522-547) and 2-hydroxy-2-methyl-1-propanone (CHS). The presence of T4L in ICL3 is expected to prevent receptor activation; however the similar affinities of BMS-681 and CCR2-RA-[R] for both wild-type (WT) CCR2 and CCR2-T4L (Extended Data Fig. 4a, b and Extended Data Table 3) suggest that the fusion construct is a good surrogate of WT CCCR for understanding ligand recognition.

The CCR2-T4L coding sequence was cloned into a modified FstBac1 (Invitrogen) vector with an HA signal sequence followed by a Tag at the N terminus and a PreScission protease site provided by a l × His tag and another Flag tag at the C terminus. The receptor was expressed in Spodoptera frugiperda (Spf) cells. High titre recombinant baculovirus (>10^8 viral particles per millilitre) was obtained using the Bac-to-Bac Baculovirus Expression System (Invitrogen) as previously described. Sf9 cells at a cell density of (2–3) x 10^6 cells per millilitre were infected with P1 virus at a multiplicity of infection of 5. Cells were harvested at centrifugation 48 h after infection and stored at −80 °C until use.

**Purification of CCR2-T4L**. Insect cell membranes were prepared by thawing frozen cell pellets in a hypotonic buffer containing 10 mM HEPES (pH 7.5), 10 mM MgCl2, 20 mM KCl, and EDTA-free complete protease inhibitor cocktail tablets (Roche). Extensive washing of the raw membranes was performed by repeated douncing and centrifugation in the same hypotonic buffer (two or three times) and then a high osmotic buffer containing 10 mM NaCl, 10 mM HEPES (pH 7.5), 10 mM MgCl2, 20 mM KCl and EDTA-free complete protease inhibitor cocktail tablets (three or four times), thereby separating soluble and membrane-associated proteins from integral membrane proteins. Stock solutions (40 mM) of BMS-681 and CCR2-RA-[R] were made in isopropanol. Washed membranes were resuspended into a buffer containing 50 mM BMS-681, 2 mg/ml iodoacetamide, and EDTA-free complete protease inhibitor cocktail tablets, and incubated at 4 °C for 1 h before solubilization. The membranes were then solubilized in 50 mM HEPES (pH 7.5), 400 mM NaCl, 1% (w/v) n-dodecyl-β-D-maltopyranoside (DDM, Anatrace), 0.2% (w/v) cholesteryl hemimuccinate (CHS, Sigma) at 4 °C for 3 h. The supernatant was isolated by centrifugation at 50,000g for 30 min, and incubated in 20 mM HEPES (pH 7.5), 400 mM NaCl with TALON IMAC resin (Clontech) overnight at 4 °C. After binding, the resin was washed without addition of ligands with ten column volumes of Wash 1 Buffer (50 mM HEPES (pH 7.5), 400 mM NaCl, 10% (v/v) glycerol, 0.1% (w/v) DDM, 0.02% (w/v) CHS, 100 mM imidazole), followed by four column volumes of Wash 2 Buffer (50 mM HEPES (pH 7.5), 400 mM NaCl, 10% (v/v) glycerol, 0.02% (w/v) DDM, 0.01% (w/v) CHS, 250 mM imidazole), PD MiniTrap G-25 columns (GE Healthcare) were used to remove imidazole. The protein was then treated overnight with His-tagged PreScission protease to cleave the C-terminal His-tag and Flag-tag. PreScission protease and the cleaved C-terminal fragment were removed by binding to TALON IMAC resin for 2 h at 4 °C. The protein was collected as the TALON IMAC column flow-through. The protein was then treated overnight with His-tagged PreScission protease to cleave the C-terminal fragment and a PreScission protease site followed by a 10 amino-acid sequence S2265.62-RASKSRI-T4L-PPPSREK-K2406.32. The presence of M2 muscarinic acetylcholine receptor (PDB accession number 3UON, resulting amino-acid sequence 522-547: RAKSRL-T4L-PPPSREK-522-547) and 2-hydroxy-2-methyl-1-propanone (CHS) was verified by X-ray fluorescence scans (Extended Data Fig. 5). The zinc ion is coordinated by a water molecule as well as side chains of H144, E128, E238, and E1005. Data collection and refinement statistics are shown in Extended Data Table 1.

**Crystallography and structure determination of BMS-681.** BMS-681 was dissolved in a minimal amount of CH3CN and then 1% v/v water was added. After standing overnight, the resulting crystals were collected. Data were obtained on a Bruker-AXS X8-Proteum Kappa goniometer and APEXII detector. Intensities were measured using Cu Kα radiation (λ = 1.5418 Å) with the crystal kept at a constant temperature using an Oxford cryo system during data collection. Indexing and processing of the measured intensity data were performed with the SAINT software. Structure solution with SHELXTL-97, and structure refinement with SHELXL-97.

The derived atomic coordinates (parameters and temperature factors) were refined through full matrix least-squares. The function minimized in the refinement was Σ(|Fobs|−|Fcalc|)^2 while R = Σ(|Fobs|−|Fcalc|)/Σ|Fcalc| and w = |Fobs|/(|Fobs|^2+|Fcalc|^2) where w is an appropriate weighting function based on errors in the observed intensities. Hydrogens were introduced in idealized positions with isotropic temperature factors, but no hydrogen parameters were varied. It should be noted that the refinement model illustrates disorder and partial occupancy factors of ‘guest’ solvent/water molecules within the crystalline lattice. The atomic positions of these disordered molecules were taken from the CSD map analysis, which showed peaks of electron density of varying intensities at the refined positions representing the disordered solvent/water molecules. Data collection and refinement statistics are shown in Extended Data Table 2.

Cell culture and transfections. CHO cells (provided by H. den Dulk, Leiden University, The Netherlands; originally obtained and certified by American Type Culture Collection) were cultured in Dulbecco’s Modified Eagle Medium/F-12 Nutrient Mixture (DMEM/F-12) supplemented with 10% (v/v) newborn calf serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin; they were maintained at 37 °C and in 5% CO2. Cells were subcultured twice a week at a ratio of 1:30 to 1:50 by trypsinization. Transient transfection of CHO cells with WT CCR2 and CCR2-T4L constructs was performed using a polyethyleneimine method, as described previously.

Briefly, CHO cells were grown on plates (diameter 15 cm) to around 50% confluence and then transfected with a DNA/polycation mixture containing 10 μg plasmid DNA—previously diluted in 150 mM NaCl solution—mixed with polyethyleneimine solution (1 mg/ml) at a 1:6 DNA:polyethylenimine mass ratio. Before adding 1 ml of the transfection mixture to each plate, the culture medium of the cells was refreshed and the mixture incubated for 20 min at room temperature. Following transfection, cells were incubated for 48 h at 37 °C and 5% CO2 before membrane preparation.

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Twenty-four hours after transfection, sodium butyrate was added to each plate at a final concentration of 3 mM to increase receptor expression. CHO cells were tested for mycoplasma contamination before use, the outcome of which was negative.

**Membrane preparation.** Membranes from CHO cells transiently expressing the WT CCR2 or CCR2-T4L were prepared as described previously. Briefly, cells were detached from plates (diameter 15 cm) using 5 mL of phosphate-buffered saline and centrifuged for 5 min at 3000g. The membranes were separated from the cytosolic fractions by several centrifugation and homogenization steps. First, the pellets were resuspended and homogenized in ice-cold membrane buffer (50 mM Tris-HCl buffer, supplemented with 5 mM MgCl₂, pH 7.4) using an Ultra Turrax Homogenizer (IKA-Werke, Staufen, Germany). Homogenized membranes were then centrifuged in an Optima LE-80 K ultracentrifuge (Beckman Coulter, Fullerton, California, USA) at 31,000g for 20 min at 4°C. The final membrane pellet was resuspended also in ice-cold membrane buffer and aliquoted before storage. Membrane aliquots were stored at −80°C and protein concentrations were measured using a standard BCA protein determination assay (Pierce Chemical Company, Rockford, Illinois, USA).

**Radioligand binding assays.** [³H]INCB-3344 (specific activity 32 Ci mmol⁻¹) and [³H]CCR2-RA ([³H] activity 63 Ci mmol⁻¹) were custom-labelled by Vitrax (Placentia, California, USA). INJ-27141491 was synthesized as described and [³H]CCR2-RA (specific activity 63 Ci mmol⁻¹) was deposited in the Protein Data Bank (PDB) under accession number 5T1A. The structure of free BMS-681 is deposited in the Protein Data Bank (PDB) under accession number 4G33. The structure of the BMS-681–CCR2-RA–[R] complex have been deposited in the Protein Data Bank under accession number 5T1A. The structure of free BMS-681 is deposited in the Cambridge Crystallographic Data Centre (http://www.ccdc.cam.ac.uk/) under accession number 1479580. All other data are available from the corresponding authors upon reasonable request.

Power calculation. The sample size of 20 wells per experimental condition was adequate to detect a 3-fold change in the ability of a new inhibitor to bind to CCR2. For competition binding assays with [³H]INCB-3344, a concentration of 5 nM [³H]INCB-3344 was used, and non-specific binding was determined with 10μM of INCB-3344. In the case of [³H]CCR2-RA competition binding assays, a radioligand concentration of 3 nM was used and non-specific binding was determined with 10μM of INJ-27141491. In all cases, homologous or competition displacement assays were performed using six increasing concentrations of competing ligands. Kinetic experiments were also performed at 25°C using 7 nM [³H]CCR2-RA and 30μg of membrane protein in a 100μL reaction volume. For association experiments, CHO-CCR2 or CHO-CCR2-T4L membranes were added to the reaction at eight different time points, in the absence or presence of 1μM BMS-681. For dissociation experiments, membranes were first incubated with radioligand for 90 min; dissociation was then initiated by addition of 10μM of CCR2-RA–[R] at 12 different time points, in the presence or absence of 1μM BMS-681. More time points were used in the dissociation assays, to characterize the biphasic profile of [³H]CCR2-RA dissociation. In all cases, total radioligand binding did not exceed 10% of the total radioligand added to avoid ligand depletion. For all experiments, incubation was terminated by dilution with ice-cold wash buffer (50mM Tris-HCl buffer (pH 7.4), 5 mM MgCl₂, 0.05% CHAPS). Separation of bound from free radioligand was achieved by rapid filtration over a 96-well GF/B filter plate using a Perkin Elmer Filtermate-harvester (Perkin Elmer, Groningen, The Netherlands) and filter-bound radioactivity was determined in a Perkin Elmer 2450 Microbeta2 plate counter after addition of 25μL Microscint scintillation cocktail per well (Perkin-Elmer, Groningen, The Netherlands).

**Statistical methods.** No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. All radioligand binding data were analysed using Prism 6.0 and 7.0 (GraphPad Software, San Diego, California, USA). The pIC₅₀ values were obtained by nonlinear regression analysis of competition displacement assays. Apparent association rate constants (kₐss) and maximum binding (B_max) used to calculate %B/B_max were determined by fitting the association data to a one-phase exponential association function. Dissociation rate constants were determined by fitting the dissociation data to a monophasic (kₐff) or biphasic (kₐff, fast and kₐff, slow) exponential decay model. All data shown represent means ± s.e.m. of at least three independent experiments performed in duplicate. An unpaired, two-tailed Student’s t-test was used to compare differences in pIC₅₀ as well as differences in kinetic parameters. Differences in binding enhancement (%Binding) in the absence (set at 100%) or presence of BMS-681 were analysed using a one-way analysis of variance with Dunnett’s post-hoc test. Significant differences are denoted as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

**Data availability.** The atomic coordinates and structure factors for the CCR2–BMS-681–CCR2-RA–[R] complex have been deposited in the Protein Data Bank under accession number 5T1A. The structure of free BMS-681 is deposited in the Cambridge Crystallographic Data Centre (http://www.ccdc.cam.ac.uk/) under accession number 1479580. All other data are available from the corresponding authors upon reasonable request.
Extended Data Figure 1 | CCR2-T4L crystals and crystal packing. 

a–c, Crystal packing of CCR2-T4L. CCR2 is a blue ribbon with ECL2 coloured red and T4L yellow. The unit cell is shown as a green box. CCR2-T4L molecules are arranged in a type I packing with hydrophilic stacking mediated by T4L and T4L–ECL2 interactions along axis c. a, Crystal packing in the ac plane. CCR2 makes abundant hydrophobic contacts with its neighbour via an interface mediated by antiparallel helix IV–helix VI interactions related by a screw axis along axis a. b, Crystal packing in the bc plane. Contacts between receptors and T4L involve ECL2 and the intracellular surface of CCR2 including helix VIII. Direct contacts between T4L are along axis b. One layer of CCR2-T4L molecules at the very top of the stacking column is omitted for clarity. c, Crystal packing in the ab plane. There are no direct interactions between T4L along axis a. d, Crystals of CCR2-T4L in the LCP bolus. Average crystals grew to $60\,\mu m \times 10\,\mu m \times 10\,\mu m$ before harvesting.
Extended Data Figure 2 | BMS-681 binding may disrupt a chemokine-recognizing conformation of the CCR2 N terminus and helix I.

a, Model of CCR2–CCL2 built by homology from the structure of CXCR4–vMIP-II suggests that a productive chemokine-compatible conformation of the receptor requires re-orientation of the N terminus from almost parallel to almost perpendicular to the membrane plane, and formation of an extra helical turn in helix I to bring it closer to helix VII and ECL3.

b, Binding of BMS-681 may disrupt this chemokine-compatible conformation by inserting between helices I and VII.
Extended Data Figure 3 | CCR2-RA-[R] directly binds to CCR2 residues that are homologous to those involved in G-protein coupling in other GPCRs. Partial alignment of intracellular regions of CCR2 and homologous regions in bovine Rho (bRho) and β2 adrenergic receptor (β2AR), alongside profile of contacts that CCR2-RA-[R], the Gαt C-terminal peptide21, and Gαs C terminus22 make with the three respective receptors. Contacts are shown by circles above and below the alignment, with circle area indicative of contact strength. Backbone and side-chain contacts are grey and black, respectively. Assuming structural homology between the CCR2–G-protein interface and at least one of the bRho–Gαt and β2AR–Gαs interfaces, several residue positions seem to be involved in binding both CCR2-RA-[R] and the C terminus of the G protein.
Extended Data Figure 4 | Equilibrium binding and binding kinetics of BMS-681 and CCR2-RA-[R] with WT CCR2 and CCR2-T4L.

a, b, Displacement of [3H]INCB-3344 (5 nM, a) and [3H]CCR2-RA-[R] (3 nM, b) from WT CCR2 and CCR2-T4L in CHO cells by increasing concentrations of unlabelled INCB-3344, CCR2-RA-[R] and BMS-681.

c, d, Association and (e, f) dissociation of 7 nM [3H]CCR2-RA from CHO cell membranes transiently expressing WT CCR2 (c, e) or CCR2-T4L (d, f) at 25 °C, in the absence or presence of 1 μM BMS-681. Figures represent normalized and combined data from three independent experiments performed in duplicate, with results presented as mean ± s.e.m. percentage of specific [3H]CCR2-RA binding.
Extended Data Figure 5 | A Zn^{2+} binding site was identified by X-ray fluorescence emission analysis of the CCR2-T4L–BMS-681–CCR2-RA-[R] crystals. a, View of the Zn^{2+} ion at an interface formed by CCR2 helices III and VI and the N terminus of T4L. The Zn^{2+} ion is coordinated by side chains of H144 3.56 (from WT receptor), E238 6.30 (from the engineered part of the receptor), and E1005 (from T4L) as well as a structured water. b, Background fluorescence signal of an empty MiTeGen micromount is low, indicating the absence of metal ion. Excitation at 12 keV results in a peak at 11.7 keV (owing to the incidence beam). c, X-ray fluorescence emission signal from a wide fluorescence scan of the CCR2-T4L crystal. The fluorescence peaks at 8.60 keV and 9.53 keV correspond to X-ray emission lines $K\alpha$ (8.64 keV) and $K\beta$ (9.57 keV) and indicate the presence of Zn^{2+} bound to CCR2-T4L. d, A zoomed-in view of the X-ray fluorescence emission signal from c.
## Extended Data Table 1 | Data collection and refinement statistics (molecular replacement)

|                          | **CCR2-T4L–BMS-681–CCR2-RA-[R]†** |
|--------------------------|-----------------------------------|
| Data collection† wavelength (Å) | 1.03319                           |
| Space group              | P212121                            |
| Unit cell parameters $a, b, c$ (Å) | 59.19 64.69 169.90               |
| Number of reflections measured | 82,111                             |
| Number of unique reflections | 15,550                            |
| Resolution (Å)           | 48-2.8 (2.95-2.8)                  |
| $R_{merge}$ (%)          | 22.5(101)                          |
| $R_{pim}$ (%)            | 12.8(88.4)                         |
| Mean $I/\sigma(I)$       | 6.9(0.8)                           |
| Completeness (%)         | 93.1(66.6)                         |
| Redundancy               | 5.3(1.8)                           |

**Refinement**

| Resolution (Å)                          | 25-2.81 (3.0, 3.0, 2.81) |
| Number of reflections (test set)        | 14515 (746)            |
| $R_{work}/R_{free}$                     | 0.233/0.274 (0.319/0.392) |
| Number of atoms                         | 3,580                  |
| CCR2                                    | 2,215                  |
| T4L                                     | 1,243                  |
| BMS-681                                  | 35                     |
| CCR2-RA-[R]                             | 24                     |
| Monoolein                                | 25                     |
| Sulfate                                  | 20                     |
| Water                                    | 17                     |
| Zn                                       | 1                      |
| Mean overall B value (Å$^2$)             | 41.4                   |
| Wilson B                                 | 40.4                   |
| Protein                                  | 41.5                   |
| Ligands                                  | 41.3                   |
| Water                                    | 22.9                   |

**Root mean square deviation**

| Bond lengths (Å)                          | 0.003                  |
| Bond angles (°)                           | 0.85                   |

**Ramanchandran plot statistics§ (%)**

| Favored regions                          | 97.1                   |
| Allowed regions                          | 2.9                    |
| Disallowed regions                       | 0                      |

†Diffraction data from 17 crystals were merged into a complete data set.
‡Highest resolution shell statistics are shown in parentheses.
§As defined in MolProbity$^{42}$.

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Extended Data Table 2  |  Small-molecule (BMS-681) X-ray data collection and refinement

| Empirical formula | C26 H36 F3 N5 O3.58 |
|-------------------|----------------------|
| Formula weight    | 532.80               |
| Temperature       | 173(2) K             |
| Wavelength        | 1.54178 Å            |
| Crystal system    | Tetragonal           |
| Space group       | P4_32               |
| Unit cell dimensions | a = 20.4436(4) Å  α= 90°,  
                       | b = 20.4436(4) Å  β= 90°,  
                       | c = 28.9325(7) Å  γ = 90°.  |
| Volume            | 12092.1(4) Å³       |
| Z                 | 16                   |
| Density (calculated) | 1.171 Mg/m³        |
| Absorption coefficient | 0.768 mm⁻¹       |
| F(000)            | 4522                 |
| Crystal size      | 0.46 x 0.18 x 0.16 mm³ |
| Theta range for data collection | 2.65 to 58.78°.  |
| Resolution range  | 16.7 to 0.9 Å        |
| Index ranges      | -22 ≤ h ≤ 22, -21 ≤ k ≤ 22, -31 ≤ l ≤ 14 |
| Reflections collected | 108743            |
| Independent reflections | 8518 [R(int) = 0.1259] |
| Completeness to theta = 58.78° | 98.6 %         |
| Absorption correction | None              |
| Refinement method | Full-matrix least-squares on F² |
| Data / restraints / parameters | 8518 / 22 / 713 |
| Goodness-of-fit on F² | 1.058             |
| Final R indices [I>2sigma(I)] | R1 = 0.0770, wR2 = 0.2087 |
| R indices (all data) | R1 = 0.0860, wR2 = 0.2178 |
| Absolute structure parameter; Flack(x) | 0.1(2)          |
| Absolute structure parameter; Hooft(y), P3true | 0.03(5), 1.000 |
| Largest diff. peak and hole | 0.543 and -0.405 e.Å⁻³ |
Extended Data Table 3 | Displacement of specific $[^3]H$INCB-3344 (5 nM) and $[^3]H$CCR2-RA (3 nM) binding from CCR2 constructs transiently expressed on CHO cells

| Construct       | $[^3]H$-INCB-3344 displacement by INCB-3344 | $[^3]H$-INCB-3344 displacement by BMS-681 | $[^3]H$-CCR2-RA displacement by CCR2-RA-[R] | $[^3]H$-CCR2-RA enhancement by BMS-681 |
|-----------------|---------------------------------------------|-------------------------------------------|---------------------------------------------|----------------------------------------|
| WT CCR2         | 7.8 ± 0.0 (17)                              | 8.1 ± 0.0 (8)                             | 7.9 ± 0.0 (13)                              | 134 ± 3%†***                           |
| CCR2-T4L        | 8.1 ± 0.1* (8)                              | 8.6 ± 0.1** (3)                           | 8.2 ± 0.0** (6)                             | 157 ± 13%†****                         |

Values represent mean ± s.e.m. of at least three independent experiments performed in duplicate.

†Percentage of $[^3]H$CCR2-RA (3 nM) binding in presence of BMS-681 (1 μM). Values higher than 100% represent binding enhancement compared with the 100% control without BMS-681.

Differences in pIC$_{50}$ values between constructs were analysed using a Student’s t-test, with significant differences noted as follows: *P < 0.05, **P < 0.01.

Differences in percentage binding in the absence (100%) and presence of BMS-681 were analysed using a one-way analysis of variance with Dunnett’s post-hoc test, with significant differences noted as follows: *P < 0.01, ****P < 0.0001.
Extended Data Table 4 | Observed association and dissociation rate constants of [3H]CCR2-RA (7 nM) on membranes from CHO cells transiently expressing WT CCR2 and CCR2-T4L, in the absence or presence of 1µM BMS-681

|                  | CHO-CCR2 |                  | CHO-CCR2-T4L |                  |
|------------------|----------|------------------|--------------|------------------|
|                  | Control  | + 1 µM BMS-681   | Control      | + 1 µM BMS-681   |
| k_{obs} (min⁻¹)  | 0.031 ± 0.002 | 0.038 ± 0.003*   | 0.015 ± 0.003 | 0.015 ± 0.001   |
| % B/B_{control}† | 100 ± 0.0 | 135 ± 2.0****    | 100 ± 0.0    | 162 ± 8.4**     |
| k_{off,fast} (min⁻¹) | 0.089 ± 0.015 | 0.069 ± 0.012*   | 0.077 ± 0.013 | 0.049 ± 0.003‡  |
| k_{off,slow} (min⁻¹) | 0.016 ± 0.005 | 0.012 ± 0.004    | 0.010 ± 0.003 | 0.010 ± 0.003   |
| %fast            | 70 ± 10  | 71 ± 11          | 69 ± 8       | N/A‡            |

Values represent mean ± s.e.m. of three independent experiments performed in duplicate.

†The percentage of maximum binding in the absence (B_{control}) or presence (B) of BMS-681 (1µM).

‡For CHO-CCR2-T4L only, dissociation kinetics of [3H]CCR2-RA (7 nM) in the presence of BMS-681 (1µM) fitted best with a monophasic exponential decay model, resulting in a single k_{off} value, as shown in the Table. Thus, for CHO-CCR2-T4L, the statistical significance between k_{off} measurements with and without BMS-681 could not be calculated.

Statistical significance was analysed using a Student’s t-test, with significant differences versus control noted as follows: *P < 0.05, **P < 0.01, ****P < 0.0001.