Determination of the binding mode for the cyclopentapeptide CXCR4 antagonist FC131 using a dual approach of ligand modifications and receptor mutagenesis.

Running title: Determination of FC131 binding in CXCR4.

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Abstract

Background and purpose. The cyclopentapeptide FC131 (cyclo(-L-Arg[1]-L-Arg[2]-L-2-Nal[3]-Gly[4]-D-Tyr[5]-)) is an antagonist for CXC-chemokine receptor 4 (CXCR4), which plays a role in HIV-infection, cancer and stem cell recruitment. Binding modes for FC131 in CXCR4 have previously been suggested based on molecular docking guided by structure-activity relationship (SAR) data; however, none of these have been verified by *in vitro* experiments.

Experimental approach. Heterologous ^{125}I-12G5-competition binding and functional assays (inhibition of CXCL12-mediated activation) of FC131 and three analogues were performed on WT CXCR4 and 25 receptor mutants. Computational modelling was used to rationalize the experimental data.

Key results. The Arg^{2} and 2-Nal^{3} side chains of FC131 interact with residues in TM-3 (His^{113}, Asp^{171}) and TM-5 (hydrophobic pocket), respectively. Arg^{1} forms charge-charge interactions with Asp^{187} in ECL-2, while D-Tyr^{5} points to the extracellular side of CXCR4. Furthermore, the backbone of FC131 interacts with the chemokine receptor-conserved Glu^{288} via two water molecules. Intriguingly, Tyr^{116} and Glu^{288} form a H-bond in CXCR4 crystal structures and mutation of either residue to Ala abolishes CXCR4 activity.

Conclusions and implications. Ligand modification, receptor mutagenesis and computational modelling approaches were used to identify the binding mode of FC131 in CXCR4, which was in agreement with binding modes suggested from previous SAR studies. Furthermore, insights into the mechanism for CXCR4 activation by CXCL12 were gained. The combined findings will facilitate future design of novel CXCR4 antagonists.

Keywords. CXCR4, FC131, binding mode, receptor mutagenesis, SAR

Abbreviations. 2-Nal, 3-(2-naphthyl)alanine; 7TM, 7 transmembrane helix; Aib, 2-aminoisobutyric acid; Cit, citrulline; CXCR4, CXC-chemokine receptor 4; G-CSF, granulocyte colony-stimulating factor; HIV, human immunodeficiency virus; SAR, structure-activity relationship; WT, wildtype

Introduction

The chemokine receptor CXCR4 belongs to class A 7 transmembrane helix (7TM) receptors, also known as G protein-coupled receptors (GPCRs). It plays a role in HIV infection by being a cell-entry co-factor for T-cell tropic HIV strains (Feng, 1996; Berson, 1996) and is together with its endogenous agonist CXCL12 central for stem cell recruitment and cancer development, progression and metastasis (Murphy, 2000; Balkwill, 2004; Bachelerie, 2014). These implications have facilitated the development of drug candidates targeting CXCR4 (Choi, 2012). One of these, the bicyclam compound AMD3100 (plerixafor), was approved in 2008 for hematopoietic stem cell mobilization in patients with multiple myeloma and non-Hodgkin´s lymphoma (DiPersio, 2009a; DiPersio, 2009b; Micallef, 2009), although the initial indication was as an anti-HIV compound (De Clercq, 1994; Steen, 2009).

The polyphemusin II-derived peptides are a large class of CXCR4 antagonists that include the 14-mer T140 (Tamamura, 1998) and analogues, e.g. CVX15 (Wu, 2010), as well as the cyclic pentapeptide FC131 (Figure 1A) and analogues (Fujii, 2003). The cyclopentapeptide CXCR4 antagonists were designed by combining the four most important residues of T140 (Arg^2, 2-Nal^3, D-Tyr^5, and Arg^{14}) with a Gly residue to give a cyclopentapeptide library (Tamamura, 2000; Fujii, 2003). The optimal combination of sequence and stereochemistry was shown to be cyclo(-L-Arg^1-L-Arg^2-L-2-Nal^3-Gly^4-D-Tyr^5-), i.e. FC131, which displays nanomolar affinity and potency at CXCR4 (Fujii, 2003) and serves as lead compound for development of more drug-like peptidomimetic CXCR4 antagonists. SAR studies of FC131 have shown that although a positive charge is preferred at position 1, substitution of Arg^1 with the uncharged citrulline (Cit), or even less structurally related amino acids, is tolerated (Tamamura, 2005a; Demmer, 2011; Mungalpara 2012). In contrast, Arg^2 is a crucial functionality and minor modifications abolish activity (Tamamura, 2005b; Demmer, 2011; Mungalpara 2012). The aromatic residues in position 3 (2-Nal^3) and 5 (D-Tyr^5) are also important for proper function; importantly, position 3 requires conservation as 2-Nal (Tamamura, 2005b; Mungalpara, 2013) while position 5 allows for some modifications (Tamamura, 2005b; Tanaka, 2009; Mungalpara, 2013).

Several computational models for binding of peptide antagonists to CXCR4 have been suggested. The first models for T140 (Trent, 2003) and FC131 (Våbenø, 2006a; Kawatkar, 2011) were based on the crystal structure of bovine rhodopsin (Palczewski, 2000); however, the subsequent publication of the crystal structures of CXCR4 (Wu, 2010) revealed significant structural differences between rhodopsin and CXCR4. The co-crystal complex between CXCR4 and CVX15, a 16-mer analogue of T140, also showed that peptide CXCR4 antagonists bind differently than previously suggested. Based on molecular docking to this crystal structure, guided by SAR data, more reliable binding models have since emerged for the cyclopentapeptide antagonists (Demmer, 2011; Yoshikawa, 2012; Kobayashi, 2012; Mungalpara, 2012; Mungalpara, 2013). These models collectively suggest an interaction between Arg^1 of FC131 and Asp^{187} (in ECL-2) and Asp^{97} in TM-2, while Arg^2 interacts with His^{113} (TM-3) and Asp^{171} (TM-4). (The position of residues according to the Baldwin/Schwartz- (Schwartz, 1994; Baldwin, 1997) and the Ballesteros/Weinstein-numbering system (Ballesteros, 1995) is given in the tables.) Furthermore, the 2-Nal^3 side chain is located in a hydrophobic pocket facing TM-5, while D-Tyr^5 is proposed to interact with either Glu^{32} in the N-terminus, Tyr^{45} in TM-1, or aromatic residues in ECL-2. Moreover, the chemokine-receptor conserved Glu^{288}, a residue often involved in binding of positively charged small-molecule ligands (Rosenkilde, 2006), is suggested to interact indirectly with FC131 via water molecules (Yoshikawa, 2012; Mungalpara, 2012). Thus, in line with reported SAR for FC131, the crucial Arg^2 and 2-Nal^3 side chains bind deep in the receptor main binding crevice, while the less important Arg^1 and D-Tyr^5 side chains experience a larger degree of conformational flexibility and are partly solvent-exposed, facing the extracellular surface of the receptor (Mungalpara, 2013). However, none of these computational models has been accompanied by *in vitro* experiments that verify the suggested binding modes.

To determine the binding mode for the lead cyclopentapeptide CXCR4 antagonist FC131, we here report experimental studies that involve modifications of both receptor and ligand. Thus, FC131 and the three analogues [Cit^1]FC131 (substitution of the positively charged L-Arg in position 1 with the neutral L-citrulline), [Aib^1]FC131 (substitution of Arg^1 with the small hydrophobic 2-aminoisobutyric acid) and [D-Arg^1]FC131 (opposite stereochemistry in position 1) (Figure 1B), were tested in a library of 25 CXCR4 mutations including Ala-, Asn- or Trp-substitutions of residues in TM-1 to -7 and ECL-2 (Figure 1C) in ^{125}I-12G5-binding and receptor-activation assays. This combined approach is the first of its kind to directly investigate the binding mode for FC131 in CXCR4 with *in vitro* experiments. Interestingly, the receptor-mutagenesis also revealed residues important for CXCL12-induced receptor activation. The combined findings provide new experimental insight into the molecular mechanisms of CXCR4 antagonism and will facilitate future design of novel CXCR4 antagonists.

Methods

Materials - All reagents and solvents were purchased and used as received without further purification. The human chemokine CXCL12 was purchased from Peprotech (NJ, USA). Human CXCR4 receptor cDNA was kindly provided by Timothy NC Wells (GSK, UK). [^{3}H]-myo-Inositol (PT6-271), SPA beads and ^{125}I-Bolton-Hunter reagent were purchased from Perkin Elmer (MA, USA). 12G5 antibody was kindly provided by Jim Hoxie (University of Pennsylvania, Philadelphia, PA, USA) and was iodinated in house as described previously (Rosenkilde, 2004). cDNA for the promiscuous chimeric G protein $G_{\Delta 6qi4myr}$ (abbreviated G_{qi4myr}) was kindly provided by Evi Kostenis (University of Bonn, Germany) (Kostenis, 1998). Primers for mutations were bought from TAG Copenhagen (Copenhagen, Denmark). The stock solution and dilutions of peptide antagonists were made in water. Stock solution and all dilutions of CXCL12 were made in buffer (1 mM acetic acid + 0.1% BSA).

Compounds - Complete details of the synthesis and characterization of the cyclopentapeptide ligands FC131, [Cit^1]FC131, [Aib^1]FC131 and [D-Arg^1]FC131 have been reported earlier (Mungalpara, 2012).

Site-directed mutagenesis - Receptor mutations were introduced by the polymerase chain reaction overlap extension technique or the QuikChange technique (Agilent Technologies, CA, USA) using WT CXCR4 as template. All reactions were carried out using Pfu polymerase (Stratagene, CA, USA) under conditions recommended by the manufacturer. The mutations were cloned into the eukaryotic expression vector pcDNA3.1+ (Invitrogen, UK) and verified by restriction endonuclease digestion and

DNA sequencing (Eurofins MWG Operon, Germany).

Transfections and tissue culture - COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS), 180 units/ml penicillin and 45 µg/ml streptomycin (PenStrep) at 37 °C in a 10% CO_2/90% air-humidified atmosphere. Transfection of cells was carried out by the calcium phosphate precipitation method (Rosenkilde, 1994; Kissow, 2012). Briefly, plasmid DNA (20 µg of receptor cDNA and 30 µg of the chimeric G protein $G\alpha_{qi4myr}$ for IP-assays or 40 µg receptor cDNA for ^{125}I-12G5-binding assays) were mixed with TE-buffer (10 mM Tris-HCl, 2 mM EDTA-Na_2, pH 7.5) and 30 µl calcium chloride (2 M) to a total volume of 480 µl, and was then added to the same amount of HEPES buffered saline (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.2). Precipitation was allowed for 45 min at room temperature, after which the precipitate together with 300 µl chloroquine (2 mg/ml) in 10 ml culture media was added to the 6×10^6 COS-7 cells seeded the day before. Transfection was stopped after 5 h by replacing media and cells were incubated overnight.

Functional assay - The potency was measured using inositol-phosphate (IP) accumulation assays. In brief, one day after transfection COS-7 cells (1.5×10^5 cells/well) were incubated for 24 h with 2 µCi of [^{3}H]-myo-inositol in 0.3 ml of growth medium per well in a 24-well plate. The following day, cells were washed twice in PBS and were incubated in 0.2 ml of Hank's balanced salt solution (Invitrogen, U.K.) supplemented with 10 mM LiCl at 37 °C in the presence of various concentrations of ligands for 90 min. All mutations were tested for their ability to become activated by CXCL12 using concentrations from 10 pM to 0.1 μM. The IC_{50} of the antagonists were determined in cells activated by CXCL12 to approximately 80% of CXCL12 E_{max}. The antagonists (in a range from 1 nM to 100 μM) were added 10 min prior to addition of CXCL12, and co-incubated with CXCL12 for 90 min. Assay-medium was then removed, and 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 [^{3}H]inositol-phosphates were purified on an AG 1-X8 anion exchange resin. After addition of multipurpose liquid scintillation cocktail (Gold Star, Triskem-International, France), radiation was counted in a Beckman Coulter counter LS6500. As an alternative assay measuring IP accumulation, the scintillation proximity assay (SPA)-IP was sometimes used. In brief, one day after transfection, COS-7 cells (35.000 cells/well) were incubated with [^{3}H]myo-inositol (5 μl/ml, 2 μCi/ml) in 0.1 ml of media overnight in a 96-well plate. The next day, cells were treated as mentioned above with volumes adjusted as follows: 100 μl of reaction solution with LiCl and 50 μl ice cold formic acid (10 mM, 50 μl/well). The [^{3}H]inositol-phosphates in the formic acid cell lysates were thereafter quantified by Ysi-poly-D-Lys coated SPA beads. Briefly, 20 μl of cell extract was mixed with 80 μl of SPA bead suspension in H_2O (12.5 μg/μl) to give a final volume of 100 μl in a PicoPlate-96 white plate. Plates were sealed, agitated for at least 30 min and centrifuged (5 min, 1500 rpm). SPA beads were allowed to settle and react with the extract for 8 h before radioactivity was determined using a Packard Top Count NXT™ scintillation counter (PerkinElmer, MA, USA). All determinations were made in duplicate. These overall readouts have earlier been used effectively for CXCR4 and other chemokine receptors and were found to give comparable results (Brandish, 2003; Thiele, 2012; Mungalpara, 2012).

Binding experiments - Cells were transfected as described above. The number of cells seeded per well was determined by the apparent receptor expression efficiency 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 ^{125}I-12G5 plus unlabelled ligand in 0.2 ml (in 24-well-plates, up to 150.000 cells/well) or 0.1 ml (96-well plates, up to 35.000 cells/well) of 50 mM HEPES buffer, pH 7.4, supplemented with 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% (w/v) bovine serum albumin in 24-well plates. The binding of ^{125}I-12G5 was competed for with increasing concentrations of the unlabelled ligand ranging from 10 pM to 100 nM (12G5) or from 1 nM to 100 μM (FC131, [Cit^1]FC131, [Aib^1]FC131 or [D-Arg^1]FC131). After incubation, cells were washed quickly two times in 4 °C binding buffer supplemented with 0.5 M NaCl. Cells were lysed by addition of 0.5 ml carbamide solution (18% acetic acid, 8 M urea, 2% v/v P-40) and radioactivity was counted in a WALLAC Wizard Gamma Counter. Nonspecific binding was determined in the presence of 0.1 μM unlabelled 12G5. Determinations were made in duplicate.

Enzyme-linked immunosorbent assay (ELISA) - COS-7 cells were seeded in 96-well plates (6×10^3 cells/well) and transfected with 12.5 ng/well N-terminally FLAG-tagged receptor DNA using lipofectamine transfection according to manufacturers instructions (Invitrogen, CA, USA). Two days after transfection, cells were washed in Tris-buffered saline (TBS; 0.05 M Tris Base, 0.9% NaCl, pH7.6), fixed in 3.7% formaldehyde for 15 min at room temperature, washed three times in TBS and incubated in TBS with 2% BSA for 30 min. The cells were then incubated for 2 h with anti-FLAG M1-antibody (Sigma-Aldrich, MO, USA) at 2 µg/ml in TBS with 1 mM $CaCl_2$ and 1% BSA. After three washes with TBS supplemented with 1 mM $CaCl_2$, the cells were incubated with goat anti-mouse HRP-conjugated antibody at 0.8 µg/ml (Thermo Fisher Scientific, IL, USA) for 1 h. The immunoreactivity was revealed by addition of TMB Plus substrate (Kem-En-Tec, Taastrup, Denmark) after three additional washes, and the reaction was stopped with 0.2 M H_2SO_4. Absorbance was measured at 450 nm on a Wallac Envision 2104 Multilabel Reader (PerkinElmer, MA, USA).

Molecular docking - Docking of the cyclopentapeptide ligands to the CXCR4 receptor was performed as described earlier (Mungalpara, 2012). Briefly, the X-ray structure of human CXCR4 (bound to CVX15, PDB code 3OE0) (Wu, 2010) was prepared with the Protein Preparation Wizard workflow (Schrödinger Suite 2011 Protein Preparation Wizard; Epik version 2.2, Schrödinger, LLC, New York, NY, 2011; Impact version 5.7, Schrödinger, LLC, New York, NY, 2011; Prime version 2.3, Schrödinger, LLC, New York, NY, 2011), and our previously reported bioactive backbone conformation for FC131 (Våbenø, 2006b) was used to build the structure of the cyclopentapeptide ligands. The ligands were docked using Schrödingers induced-fit docking workflow (Schrödinger Suite 2012 Induced Fit Docking protocol; Glide version 5.8, Schrödinger, LLC, New York, NY, 2012; Prime version 3.1, Schrödinger, LLC, New York, NY, 2012), which takes the conformational flexibility of both ligand and receptor residues into account. Asp^{187} was used as centroid for the docking box (a cube with 26 Å length) and a H-bond constraint was applied to the carboxylate oxygen atoms of Asp^{171}. As the side chain of Arg^{188} partly restricted access to Asp^{171}, the “trim” option was used for Arg^{188}, i.e. the side chain is replaced with a methyl group (alanine) in the initial docking step and then placed back in the final redocking step. For all four ligands, 100 initial poses were generated, and the top 10 optimized poses were retrieved.

Data analysis and statistics - Statistical analyses were performed using the GraphPad Prism 5 software (GraphPad Software, San Diego, CA). The EC_{50} and IC_{50} values represent the mean of at least three independent experiments (except for [Cit^1]FC131 and [Aib^1]FC131 in 125G-binding to Y116A) performed in duplicates (for exact number of experiments, see tables). In cases of incomplete sigmoidal curves (plateau not reached), the curves were extrapolated to baselines (see below) to predict an EC_{50} or IC_{50}. If this seemed unjustified, $\log EC_{50}/IC_{50}$ values were indicated as >-4 or >-7 in the tables. P values were calculated using unpaired two-tailed t-test with 95% confidence intervals. Dose-response curves represent averaged, normalized curves. The normalizations were done as follows: (1) In the functional assay, cells were activated to approximately 80% by addition of an appropriate concentration of CXCL12 (see section on functional assay). This activation was set to 100% in each individual experiment, while the background-response observed for transfected cells in absence of ligand was set to 0%. The average of these normalized curves for each assay ("row means" in Prism 5) was then calculated. (2) In the ^{125}I-12G5 binding assays, 100% equals maximum ^{125}I-12G5 binding to receptor-expressing cells in the absence of unlabelled ligand, while 0% equals the unspecific binding observed with 0.1 μM 12G5.

Results

Expression and functionality of mutant receptors.

A library of 25 CXCR4 mutations with Ala-, Asn- and steric hindrance substitutions of residues located in TM-1 to -7 and ECL-2 (Figure 1C) was created based on previously suggested binding modes of FC131 (Demmer, 2011; Yoshikawa, 2012; Mungalpara, 2012) and the ability of the residues to engage in H-bond, charge-charge, and hydrophobic interactions. Thus, these residues constituted likely interaction sites for the main functionalities of FC131, i.e. the positively charged side chains of Arg^1 and Arg^2, the aromatic side chains of $2\text{-}Nal^3$ and $D\text{-}Tyr^5$, and the peptide backbone. Initially, WT CXCR4 and all mutant receptors were tested for their surface expression using ELISA, and for their functional response towards the endogenous chemokine CXCL12 using COS-7 cells transiently transfected with receptor and the $G\alpha_i$- to $G\alpha_q$-signal-converting chimeric G_α subunit G_{qi4myr}, thus measuring accumulation of intracellular inositol-phosphate (Table 1, Figure 1D). The majority of receptors displayed expression levels from 47-111% of WT. R183A, I259W and W94A ranged at the lower end of the scale with 18%, 34% and 39% of WT expression, respectively, while two receptors (R188A and H281A) showed expression levels higher than 130% compared to WT (Table 1). I259A and Q200A displayed the lowest expression with 4.7% and 3.0% of WT-level; however, both receptors showed good responses towards CXCL12, demonstrating that they were functional and correctly folded. Likewise, the majority of the receptors showed good responses towards CXCL12 (Table 1, Figure 1D). Only W94A, D97A and D187A resulted in 8.6- to 14-fold decreased potencies compared to WT CXCR4 and no response was observed for Y116A and E288A (discussed below), despite good surface expression (Table 1, Figure 1D).

Nine mutations were also assessed in ^{125}I-12G5 competition binding experiments in transiently transfected COS-7 cells (Table 2). This assay has earlier been shown to correlate better with HIV-1 antiviral potency of CXCR4 antagonists than functional assays measuring CXCR4 signalling, and also displays a larger dynamic range (Gerlach, 2003; Rosenkilde, 2007). These selected receptors were able to bind 12G5 with WT-like affinities (1.9 to 16 nM) (Table 2). The B_{max} values were slightly, yet significantly, reduced for H113A, D171N, H281A and E288A (Table 2), which however did not correlate to their WT-like surface expression (Table 1).

While secondary/global effects of the mutations on receptor structure and function cannot be excluded, the created set of receptor mutants was deemed suitable for mapping the binding site of FC131 by assessing its ability to inhibit CXCL12-mediated activation or to displace ^{125}I-12G5.

FC131-mediated inhibition of CXCL12-induced receptor activation.

The entire mutant library was tested in a functional assay determining the ability of the cyclopentapeptide antagonist FC131 to inhibit CXCL12-induced accumulation of intracellular inositol-phosphate. H113A, D171N and D262N in the major binding pocket resulted in 12- to 119-fold reduced FC131 potencies (Figure 2A), while no effects were observed for mutations in ECL-2 (D187A) and the top of TM-7 (H281A) (Figure 2B). Ala-substitution of TM-2 residues Trp^{94} and Asp^{97}, pointing towards the minor binding pocket (defined by TM-1, -2, -3, -7), improved the potency of FC131 (Figure 2C). CXCL12-induced activity was highly impaired in Y116A and E288A, both pointing into the major binding pocket (delimited by TM-3 to -7, Figure 1C), and FC131 was consequently not tested further here. A large number of mutations in TM-3 (Thr^{117}), ECL-2 (Arg^{183}, Arg^{188}, Phe^{189}, and Tyr^{190}), TM-5 (Val^{196}, Phe^{199}, Gln^{200}, and His^{203}), TM-6 (Trp^{252}, Tyr^{255}, and Ile^{259}), and TM-7 (Ile^{284}) did not impair the antagonistic potency of FC131 (Table 1). However, a small decrease (4.1-fold) was observed for Ala-substitution of Tyr^{45} in TM-1.

The binding site of FC131 is located in the major binding pocket of CXCR4.

The impact of the nine selected mutations on the affinity of FC131 was assessed in the ^{125}I-12G5 heterologous competition binding assay (Table 2). Here, similar results were obtained, yet with the expected generally larger changes in affinity (Table 2) as compared to changes in potency (Table 1). Thus, FC131 displayed high affinity to WT CXCR4 (IC_{50} of 0.74 µM), whereas the H113A, Y116A, D171N and D262N mutants resulted in 63- to >260-fold decreased affinities (Figure 2D). H281A and D187A resulted in a lower, though significant decrease (18- and 10-fold, respectively). A minor decrease in affinity was also observed for E288A (5.5-fold) (Figure 2E). Finally, in analogy to the functional assay results, W94A and D97A led to 25- and 4.6-fold *increased* affinities, respectively (Figure 2F).

Molecular docking of FC131 in CXCR4.

Next, FC131 was docked to the X-ray crystal structure of CXCR4 (PDB code 3OE0 (Wu, 2010)) using the induced-fit docking protocol developed by Schrödinger (see Methods). As the binding and functional studies (Tables 1 and 2) both showed a dependence on the spatially close residues His^{113} (TM-3) and Asp^{171} (TM-4), a H-bond constraint was set on the carboxylate group of Asp^{171}. The following binding mode, which was among the top 10 optimized poses and supports the experimental data outlined above, is suggested (Figure 3A-C): Arg^{1} of FC131 interacts with Asp^{187}, while Arg^{2} interacts with His^{113}/Asp^{171}; although also a direct interaction of Asp^{97}

with Arg^{1} in FC131 is observed in this docking pose (not shown) and in earlier computational studies (Demmer, 2011; Yoshikawa, 2012; Mungalpara, 2012), the observation that the D97A mutation led to an increased affinity and potency of FC131 argues for a different role of Asp^{97} (Figure 2C,F). The aromatic 2-Nal^{3} side chain is positioned in a tight hydrophobic pocket facing TM-5, and sandwiched between Arg^{188} (cation-π-interactions) and His^{203} (π-π-interactions). In most poses, D-Tyr^{5} of FC131 points towards Glu^{32} in the receptor N-terminus, while in some poses an interaction with Asp^{262} was observed (not shown). Finally, Glu^{288} interacts with the backbone of the ligand via a water-mediated hydrogen-bond network. Thus, FC131 binds in the major binding pocket of CXCR4, with the Arg^{2} and 2-Nal^{3} side chains buried deeply, while the Arg^{1} and D-Tyr^{5} side chains point outward.

Collectively, it was found that His^{113}, Asp^{171}, Asp^{187}, and Glu^{288} are part of the binding site (Glu^{288} via water molecules), confirming recently suggested binding modes for FC131 (Demmer, 2011; Yoshikawa, 2012; Mungalpara, 2012), while Tyr^{45}, Tyr^{116}, and His^{281} are not directly interacting with FC131, but nevertheless influence its binding and activity to varying extents. A direct interaction of D-Tyr^{5} of FC131 with Asp^{262} is only seen in a few poses and is not likely to account for the large impact of the D262N mutation (96- and 12-fold decrease in affinity and potency, respectively, of FC131). However, Asp^{262} is a central residue in a H-bond network involving Gln^{200} (TM-5), His^{281} (TM-7) and Arg^{30} (N-terminus) (not shown) and removal of the charge in Asp^{262} may disturb this network and thereby indirectly affect FC131 binding and function.

[Cit^{1}]FC131 without a positively charged side chain in position 1 loses dependency on Asp^{187} in ECL-2. Previous SAR studies of FC131 (Figure 4A) have shown that

Arg^1 (but not Arg^2) can be replaced with the uncharged L-citrulline residue (Figure 4B) (Mungalpara, 2012). In order to confirm the suggested binding mode of FC131 (Figure 3), we subjected the [Cit^1]FC131 analogue to the same mutational analysis in ^{125}I-12G5-binding and CXCL12-functional studies. Consistent with previous data (Mungalpara, 2012), [Cit^1]FC131 displayed 6- to 8-fold lower affinity and potency as compared to FC131 (Tables 1 and 2). However, the effect of most mutations on [Cit^1]FC131 was similar to that observed for FC131 (Figure 4D). Thus, mutation of residues facing the major binding pocket (H113A, Y116A, D171N, D262N) resulted in 9.6- to >20-fold decreases in affinity (Figure 4E and Table 2). H281A, in the top of TM-7, led to a 6.3-fold decrease, while E288A resulted in a partial displacement with unchanged affinity. Furthermore, as for FC131, mutations in TM-2 of the minor binding pocket (W94A and D97A) led to increased affinities (Table 2). However, contrary to what was observed for FC131 (Figure 4D), D187A in ECL-2 did not affect the binding of [Cit^1]FC131 (Figure 4E). Thus, the affinity of FC131 on D187A (IC_{50} of 7.7 μM) is similar to the affinity of [Cit^1]FC131 on WT CXCR4 (IC_{50} of 4.9 μM), pointing towards an interaction of side chain 1 with Asp^{187}.

The effect of receptor mutants on the ability of [Cit^1]FC131 to inhibit CXCL12-mediated activation confirmed the picture observed in ^{125}I-12G5-binding. Mutation of residues in the major binding pocket, including those located deeply and those located more superficially, resulted in strongly (H113A) or moderately (I284A, D171N) decreased, or unchanged (D262N, H281A) potency of [Cit^1]FC131 (Table 1). The lack of effect for D262N could be due to the generally smaller dynamic window in functional studies compared to ^{125}I-12G5-binding and the smaller effect observed in binding for [Cit^1]FC131 (12-fold) vs. FC131 (96-fold). Furthermore, the potency of [Cit^1]FC131 was decreased 5.7-fold for Y45A, while mutation of residues in TM-2 led to increased potencies (W94A, D97A) (Table 1). As expected from ^{125}I-12G5-binding experiments, D187A did not impair the antagonistic potency of [Cit^1]FC131 (Figure 4H). Analysis of neighbouring residues in ECL-2 (Figure 4G,H) revealed that Ala-substitution of Arg^{188} led to a 15-fold reduced potency of [Cit^1]FC131 (Figure 4H), while having no effect on FC131 (Figure 4G). None of the other aromatic residues in ECL-2 (Phe^{189}, Tyr^{190}) affected the potency of either [Cit^1]FC131 or FC131 (Figure 4G,H). This highlights the impact of the cation-π-interaction between 2-Nal^3 and Arg^{188} in the absence of Arg^1. Molecular docking of [Cit^1]FC131 into CXCR4 also reveals that Asp^{187} is pointing away from side chain Cit^1 (Figure 4K,L). Of the remaining mutations (Thr^{117}, Val^{196}, Phe^{199}, Gln^{200}, His^{203}, Trp^{252}, Tyr^{255}, Ile^{259}) only W252A lead to 4.3-fold impaired potency (Table 1).

[Aib^1]FC131, which lacks a side chain functionality in position 1, displays the same binding mode as FC131 and [Cit^1]FC131. As described above, FC131 tolerates removal of the positive charge from the side chain in position 1. It also tolerates truncation of this side chain to the backbone stabilizing di-substituted residue Aib, i.e. [Aib^1]FC131 (Figure 4C) (Mungalpara, 2012), which has the same affinity and potency as [Cit^1]FC131 (Tables 1 and 2). The mutagenesis study of this compound in ^{125}I-12G5 binding revealed a stronger dependence on residues in the major binding pocket (H113, Y116, D171, D262, H281) than for [Cit^1]FC131. However, in analogy with [Cit^1]FC131, no impact was observed for D187A or E288A, while W94A and D97A led to similar increases in affinity (Table 2, Figure 4F). [Aib^1]FC131 also mirrored [Cit^1]FC131 in the functional studies, with a few exceptions (Table 1): compared to [Cit^1]FC131 it showed decreased dependency on Asp^{171} and Arg^{188} in TM-IV and ECL-2, respectively. In contrast, it depended to a higher degree on His^{203},

as its potency was decreased 6.8-fold by H203A (Figure 4I), while that of [Cit^{1}]FC131 was only impaired 2.3-fold (Figure 4H). Thus, the importance of Arg^{188} and His^{203} in sandwiching the 2-Nal^{3} side chain, as discussed above, seems to swap from Arg^{188} for [Cit^{1}]FC131 to His^{203} for [Aib^{1}]FC131. Furthermore, Y45A resulted in 16-fold decreased potency of [Aib^{1}]FC131, compared to the smaller impact of 5.7-fold for [Cit^{1}]FC131. Finally, computational modelling confirms a binding mode that overlaps with those of FC131 and [Cit^{1}]FC131 for side chains L-Arg^{2}, 2-Nal^{3}, Gly^{4} and D-Tyr^{5} (Figure 4K,L). This emphasizes that side chain 1 is not required for achieving this binding mode of cyclopentapeptides in CXCR4, yet plays a role for high potency and affinity, as described earlier (Tamamura, 2005a; Demmer, 2011; Mungalpara, 2012).

The close analogue [D-Arg^{1}]FC131 behaves differently than FC131 in its ability to displace ^{125}I-12G5. [D-Arg^{1}]FC131 differs from FC131 only in the stereochemistry in position 1 and displays similar potency and 2-fold higher affinity; however, this compound interacted differently with CXCR4. In ^{125}I-12G5-binding experiments, a stronger dependency was observed on His^{113}, Tyr^{116}, Asp^{171}, Asp^{187}, Asp^{262}, His^{281} and Glu^{288} for [D-Arg^{1}]FC131 than for FC131, while in functional assays both compounds behaved largely similar on all mutants (Figure 5A, Tables 1 and 2). Interestingly, [D-Arg^{1}]FC131 acted differently on mutations in TM-2 than FC131; while W94A consistently lead to increased affinities and potencies, D97A abrogated the ability of [D-Arg^{1}]FC131 to displace ^{125}I-12G5, while it, as for FC131, increased its antagonistic potency (Figure 5B). Molecular docking of [D-Arg^{1}]FC131 to CXCR4 revealed a larger flexibility of the exteriorly located part of the molecule as compared to FC131. While D-Arg^{1} still mainly interacted with Asp^{187}, D-Tyr^{5}

displayed a larger degree of conformational freedom and pointed everywhere from TM-2 and the N-terminus to TM-6 (Figure 5C); however, the crucial ligand side chains Arg^{2} and 2-Nal^{3} bound to His^{113}/Asp^{171} and the hydrophobic pocket around TM-5 in the same way as in FC131.

A H-bond between Tyr^{116} and Glu^{288} plays a role in the activation of CXCR4. In the crystal structure of the complex between CXCR4 and the peptide antagonist CVX15 (Figure 6A) (Wu, 2010) and in our models of the receptor-bound cyclopentapeptide ligands (Figure 3C) a H-bond is observed between Tyr^{116} in TM-3 and Glu^{288} in TM-7. *In vitro* experiments showed that Ala-substitution of Tyr^{116} or Glu^{288} abolished the agonistic action of CXCL12 (Figure 6B), despite surface expression levels of 69% and 77% of WT, respectively (Table 1). Both mutant receptors bound ^{125}I-12G5 with WT-like affinities suggesting proper folding of the receptors (Figure 6C). Furthermore, all four cyclopentapeptide ligands were unable to displace ^{125}I-12G5 from Y116A-CXCR4, while only [D-Arg^{1}]FC131 was affected by E288A (Figure 6D, Table 2). Although Tyr^{116} was not revealed as a direct interaction partner for the cyclopentapeptide ligands in the docking studies, these mutagenesis data point towards a role of Tyr^{116} for the ability of the ligands to displace ^{125}I-12G5. Furthermore, the H-bond between Tyr^{116} and Glu^{288} seems crucial for the activation of CXCR4 by CXCL12. Further studies are needed to fully understand the functional importance of the link between Tyr^{116} and Glu^{288} in CXCR4.

Discussion and Conclusions

We have used a dual approach combining receptor mutational analysis with ligand modifications to determine the binding mode of FC131 in CXCR4: Arg^1, Arg^2, 2-Nal^3 and D-Tyr^5 of FC131 interact with ECL-2 (Asp^{187}), TM-3/-4 (His^{113}, Asp^{171}), TM-5 and the exterior receptor part (Glu^{32}), respectively. The orientation of FC131 in the pocket was confirmed by [Cit^1]FC131 and [Aib^1]FC131 that both lack the positive charge at position 1 and at the same time do not depend on Asp^{187}. Overall, our data are consistent with earlier proposed binding modes predicting Arg^1 and D-Tyr^5 to point outwards, while Arg^2 and 2-Nal^3 interact with residues deep in the major binding pocket (Figure 3) (Demmer, 2011; Yoshikawa, 2012; Mungalpara, 2013).

Comparison of the binding modes of FC131, CVX15 and AMD-compounds.

The recent crystal structure of CXCR4 with the small-molecule IT1t or the peptide CVX15 (Wu, 2010) gave first-hand insights into antagonist interaction with CXCR4. Surprisingly, IT1t interacted with residues Glu^{288}, Asp^{187} and Asp^{97} in the *minor binding pocket*, while the peptide ligand CVX15, a 16-mer analogue of the 14-mer T140 that FC131 was developed from, was located in the *major binding pocket* and in extracellular receptor regions. Specifically, Arg^1 of CVX15 interacted with Asp^{187}, Arg^2 with His^{113}/Asp^{171}, and Arg^{14} with Asp^{262} (Wu, 2010). We find that FC131 mimics the binding of CVX15 as it interacts with Asp^{187} via Arg^1, and His^{113}/Asp^{171} via Arg^2. Thus, the two arginine residues in FC131 correspond to Arg^1 and Arg^2 of CVX15, and not to Arg^2 and Arg^{14} as originally intended (Figure 7). Asp^{262} was not found to interact with Arg^1 or Arg^2 of FC131 in any docking pose. Alternatively, the impact of D262N might be attributed to a central role of this residue in a H-bond network involving Arg^{30}, Gln^{200} and His^{281}, as mentioned above. Furthermore, a link between ECL-2 (carrying Asp^{187}) and Asp^{262} is established via Gln^{200} in TM-5, which is directly linked to ECL-2. Removing a conformational constraint between Asp^{262} in TM-6 and Gln^{200} in TM-5 might therefore alter the position of ECL-2. Such a scenario would also explain the weakened effect of D262N on analogues [Cit^{1}]FC131 and [Aib^{1}]FC131, lacking the positive charge at position 1 and dependency on Asp^{187} in ECL-2.

Finally, 2-Nal^{3} of FC131 and the corresponding 1-Nal^{3} of CVX15 bind in a hydrophobic sub-pocket at TM-5; however, as previously suggested by comparing SAR data for the cyclopentapeptides and the larger peptide antagonists, the naphthyl groups do not completely overlap (Mungalpara, 2013). Clearly, the 2-Nal^{3} side chain of FC131 goes deeper into the hydrophobic sub-pocket, and presumably contributes more to activity than the 1-Nal^{3} side chain of CVX15. The tyrosine residue in position 5 of both ligands takes up different positions. While Tyr^{5} of CVX15 faces the upper part of the hydrophobic pocket around TM-5 (Wu, 2010), rotation of D-Tyr^{5} to Glu^{32} (N-terminus) was observed in the FC131-CXCR4 complex, again in agreement with SAR studies on this position suggesting a solvent-exposed, freely rotatable position in CXCR4 (Mungalpara, 2013).

The well-described non-peptide AMD-compound series (the bicyclam AMD3100, the monocyclam AMD3465 and the non-cyclam AMD11070) was earlier shown to depend on Asp^{262}/Glu^{288} in TM-6/-7 on one side, and Asp^{171} in TM-4 on the opposite side of the major binding pocket (Gerlach, 2003; Rosenkilde, 2004; Rosenkilde, 2007). Furthermore, mutation of residues in the minor binding pocket were found to impair their action and multiple binding modes were subsequently suggested (Gerlach, 2003; Rosenkilde, 2004; Hatse, 2005; Rosenkilde, 2007; Wong, 2008;

Gudmundsson, 2009a; Gudmundsson, 2009b; Catalano, 2010; Miller, 2010; Skerlj, 2011). In the present study, FC131 was found to only indirectly interact with Glu^{288} via a water-mediated H-bond network, and therefore behaves somewhat differently from these reference CXCR4 antagonists and from most other small-molecule antagonists where the chemokine-receptor conserved Glu in position VII:06/7.39 seems to function as a general anchor point for positively charged nitrogens (Rosenkilde, 2006).

The role of ECL-2 in the binding of cyclic pentapeptides in CXCR4.

ECL-2 connects TM-4 with TM-5 and is covalently linked to the top of TM-3 via a conserved disulphide bond. Thereby, the C-terminal part of ECL-2 (also called ECL-2b) is being held close to the extracellular surface of the main binding crevice of CXCR4. Asp^{187} is located in position Cys+1 in ECL-2b and the D187A mutation resulted in decreased affinities of FC131, but not [Cit^{1}]FC131, pointing towards an interaction of Asp^{187} with Arg^{1} of the cyclopentapeptides. The adjacent Arg^{188} was earlier suggested to interact with the aromatic 2-Nal^{3} side chain of FC131 by engaging in a cation-π-interaction. This is also observed in our studies (Figure 4H), yet we do not see an effect of R188A on the potency of FC131, whereas the potency of [Cit^{1}]FC131 decreases by 15-fold and that of [Aib^{1}]FC131 by 3.5-fold (however, for [Aib^{1}]FC131 a role of the second suggested interaction partner for 2-Nal^{3}, His^{203} in TM-V, becomes visible) (Table 1). Thus, Asp^{187} seems to be the most important residue for FC131 in ECL-2, yet in the absence of the interaction between Arg^{1} in FC131 and Asp^{187} (in [Cit^{1}] and [Aib^{1}]FC131), the impact of Arg^{188} becomes visible. Alternatively, Arg^{188} of CXCR4 and Arg^{1} of FC131 might repel each other. Thus, the R188A mutation would remove the favourable interaction with 2-Nal^{3}, but also the unfavourable electrostatic repulsion of Arg^{1}, leading to a zero net effect of R188A on FC131. Importantly, a similar direct role of ECL-2b was found for the CCR5 antagonist aplaviroc (Maeda, 2006; Thiele, 2011). In a broader perspective, binding of small-molecule compounds to extracellular chemokine receptor domains has the potential of overlapping with the binding sites of chemokines, which due to their large size interact with the exterior parts of their receptors (Allen, 2007; Scholten, 2012). Small-molecule antagonists, although by default considered allosteric, may therefore become partially overlapping, resulting in competitive behaviour.

The role of Tyr^{116} for the function of CXCR4 antagonists.

According to the two-step model of chemokine receptor activation, the interaction between CXCR4 and CXCL12 involves distinct receptor and chemokine domains in binding and activation (Crump, 1997; Gupta, 2001). In CXCR4, the initial high-affinity binding is mainly mediated by sulpho-tyrosines in the receptor N-terminus that interact with positively charged residues of CXCL12. In a second step, N-terminal CXCL12 residues interact with transmembrane receptor residues, and presumably also ECL-2 to induce receptor activation (Crump, 1997; Ludeman, 2013). In agreement with this model, and consistent with the data presented here, Ala substitution of transmembrane residues Asp^{97}, Tyr^{116}, Glu^{288} affect the signalling properties (Table 1, Figure 6), but not the binding, of CXCL12 (Rosenkilde, 2007; Wong, 2008). Thus, it can be speculated that the observed H-bond between Tyr^{116} and Glu^{288} (Wu, 2010) is crucial for CXCL12-mediated receptor activation. Furthermore, the function of all tested cyclopentapeptides depended on Tyr^{116} (Table 2, Figure 6D), probably via an indirect mechanism, as no direct interaction with the ligand was observed (Figure 3C). Interestingly, the function of AMD3100 and AMD3465 has also been shown to depend on Tyr^{116} (Wong, 2008); however, it remains to be determined whether this effect is direct or indirect. Finally, as described above, Glu^{288} was found to be an indirect interaction partner for FC131, but mutation only resulted in minor effects for most cyclopentapeptide ligands, except binding of [D-Arg^{1}]FC131 (Table 2). Thus, the Tyr^{116}-Glu^{288} H-bond at the bottom of the major binding pocket is central not only for the activation but also for the inhibition of CXCR4, and consequently for the activity states of CXCR4.

The entrance to the binding crevice in CXCR4 is covered by H-bond and Cys-bridge. While mutations in TM-2 (W94A, D97N) impair the affinity of the AMD-compounds (Wong, 2008), we observed that W94A and D97A increased the potencies and affinities of the cyclopentapeptide antagonists. In the crystal structure of CXCR4, Asp^{97} forms a salt bridge with Arg^{183} in ECL-2, which together with the chemokine-receptor conserved disulphide bridge between the N-terminus and top of TM-7 results in a partly covered major binding pocket (Wu, 2010). This is not seen in the newly released structure of CCR5, which lacks Asp^{97} (or an equivalent thereof) and has a more open entrance to its binding pocket (Tan, 2013). Although speculative, it could therefore be argued that breaking the Asp^{97}/Arg^{183}-salt bridge by Ala-substitution of Asp^{97} releases the closed extracellular conformation of CXCR4 and gives FC131 easier access to its binding pocket. Mutation R183A does however not lead to increased potency of FC131 (Table 1); yet in the absence of the Arg^{183} side chain it can be speculated that another residue takes over its function in the salt bridge to Asp^{97}; a H-bond is indeed observed between Asp^{97} and the backbone of Cys^{186}. W94A may have a similar effect by providing more space for Asp^{97}, thereby breaking the H-bond with Arg^{183}, or simply by creating more room for the ligand.

In conclusion, by combining receptor mutagenesis with ligand modifications we determined the binding site of FC131 in CXCR4. In addition, our studies suggest a H-bond in the center of the receptor between Tyr^{116} and Glu^{288} to be essential for the activation state of CXCR4. Finally, consistent with other studies of class A 7TM receptors, hereunder EBI2 (GPR183), CCR5, and CCR2, where a central role of the top of TM-2 is identified for the activity state (Alvarez Arias, 2003; Benned-Jensen, 2008; Rosenkilde, 2010), a possible gating function of the top of TM-2 for the entrance into the main binding crevice of CXCR4 is suggested, implying that improved CXCR4 antagonists could be obtained by creating smaller molecules that can easily migrate into the main binding crevice of CXCR4.

Acknowledgments

Financial support for this project was obtained from the Research Council of Norway (Grant 190728/V30) (J.M. and J.V.) and from the Danish Council for Independent Research/Medical Sciences, the NovoNordisk Foundation, the Lundbeck Foundation, the European Community's Sixth Framework program (INNOCHEM: Grant LSHBCT-2005-518167) (S.T., A.S. and M.M.R). We also thank Inger Smith Simonsen, Randi Thøgersen and Maibritt Sigvardt Baggesen for outstanding technical assistance with the biological assays.

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Tables

Table 1. Functional analysis of the interaction between CXCR4 WT and mutants with CXCL12, FC131 and analogues. PI turnover was measured in COS-7 cells co-transfected with CXCR4 receptor constructs and the promiscuous G protein G_{qi4myr}. Residue positions are given according to the numbering systems of Baldwin/Schwartz and Ballesteros/Weinstein. The number of experiments is shown in parentheses, and F_{mut} indicates the fold-difference (ratio) between the potency on WT CXCR4 compared to mutant CXCR4 with colour codes as follows: red > 50; orange > 15, yellow > 5, green < 0.2. $^{\#}$ Mutant also tested in binding assay (Table 2). Significance: *** P<0.001, ** P<0.01, * P<0.05.

Receptor			**Surface expression**		**CXCL12**					**FC131**				**[Cit^1]FC131**				**[Aib^1]FC131**				**[D-Arg^1]FC131**			
Helix	Position	**Mutation**	% ± SEM	(n)	EC_{50} ± SEM *(log)*	EC_{50} *(nM)*	F_{mut}	response at 0.1µM % of WT ± SEM	(n)	EC_{50} ± SEM *(log)*	EC_{50} *(µM)*	F_{mut}	(n)	EC_{50} ± SEM *(log)*	EC_{50} *(µM)*	F_{mut}	(n)	EC_{50} ± SEM *(log)*	EC_{50} *(µM)*	F_{mut}	(n)	EC_{50} ± SEM *(log)*	EC_{50} *(µM)*	F_{mut}	(n)
wt	wt	**wt**$^{\#}$	100 ± 0.0	(5)	-8.8 ± 0.04	1.5	1.0	94 ± 1.3	(69)	-6.4 ± 0.04	0.40	1.0	(31)	-5.5 ± 0.11	3.0	1.0	(22)	-5.5 ± 0.09	2.9	1.0	(24)	-6.3 ± 0.09	0.52	1.0	(19)
TM-1	I:07/1.39	**Y45A**	90 ± 7.0	(3)	-8.5 ± 0.20	3.4	2.3 **	40 ± 5.5	(10)	-5.8 ± 0.09	1.6	4.1 ***	(6)	-4.8 ± 0.12	17	5.7 **	(4)	-4.3 ± 0.07	46	16 ***	(3)	-5.8 ± 0.10	1.6	3.0 *	(4)
TM-2	II:20/2.60	**W94A**$^{\#}$	38 ± 6.4	(3)	-7.7 ± 0.10	18	13 ***	31 ± 11	(17)	-8.0 ± 0.30	0.01	0.03 ***	(8)	-6.8 ± 0.41	0.17	0.06 ***	(9)	-7.7 ± 0.14	0.02	0.01 ***	(3)	-7.3 ± 0.67	0.05	0.09 **	(3)
	II:23/2.63	**D97A**$^{\#}$	99 ± 6.3	(4)	-7.7 ± 0.07	20	14 ***	34 ± 3.5	(10)	-7.4 ± 0.09	0.04	0.11 ***	(5)	-6.4 ± 0.02	0.44	0.15 *	(3)	-6.5 ± 0.07	0.35	0.12 **	(3)	-7.2 ± 0.05	0.06	0.12 **	(3)
TM-3	III:05/3.29	**H113A**$^{\#}$	80 ± 9.0	(3)	-9.1 ± 0.08	0.84	0.58 **	83 ± 8.1	(21)	-4.3 ± 0.10	48	119 ***	(11)	> -4	> 100	> 33	(10)	> -4	> 100	> 35	(5)	> -4	> 100	> 170	(3)
	III:08/3.32	**Y116A**$^{\#}$	69 ± 12	(3)	no activation		🟥	-0.8 ± 5.9	(1)	not determined				not determined				not determined				not determined			
	III:09/3.33	**T117A**	74 ± 5.5	(3)	-8.8 ± 0.09	1.7	1.2	57 ± 13	(4)	-6.9 ± 0.05	0.14	0.34 **	(3)	-5.4 ± 0.10	4.2	1.4	(3)	-6 ± 0.14	1.0	0.34	(3)	-6.1 ± 0.12	0.74	1.4	(3)
TM-4	IV:20/4.60	**D171N**$^{\#}$	67 ± 11	(3)	-8.5 ± 0.08	3.2	2.2 ***	38 ± 4.7	(25)	-5.3 ± 0.12	4.6	12 ***	(13)	-4.6 ± 0.12	25	8.3 ***	(12)	-5.2 ± 0.18	6.2	2.2	(5)	-4.5 ± 0.27	28	54 ***	(4)
ECL-2	ECL-2/Cys-3	**R183A**	17 ± 1.8	(3)	-10 ± 0.20	0.10	0.07 ***	24 ± 1.4	(3)	-6.3 ± 0.18	0.49	1.2	(3)	not determined				not determined				not determined			
	ECL-2 / Cys+1	**D187A**$^{\#}$	49 ± 8.7	(4)	-7.9 ± 0.06	13	8.6 ***	41 ± 5.2	(3)	-6.9 ± 0.02	0.14	0.35 ***	(3)	-6.1 ± 0.03	0.76	0.25	(3)	-6.3 ± 0.12	0.49	0.17 **	(3)	-6.5 ± 0.15	0.35	0.66	(3)
	ECL-2 / Cys+2	**R188A**	174 ± 21	(3)	-9.3 ± 0.08	0.53	0.36 **	44 ± 3.5	(4)	-6.1 ± 0.17	0.72	1.8	(3)	-4.4 ± 0.09	44	15 ***	(3)	-5.0 ± 0.25	10	3.5	(3)	-5.7 ± 0.10	2.2	4.2 *	(3)
	ECL-2 / Cys+3	**F189A**	97 ± 8.7	(3)	-8.7 ± 0.11	1.8	1.2	73 ± 9.5	(9)	-7.0 ± 0.20	0.10	0.25 ***	(6)	-6.1 ± 0.19	0.79	0.27 *	(5)	-6.5 ± 0.30	0.31	0.11 ***	(6)	-6.8 ± 0.02	0.15	0.28 *	(4)
	ECL-2 / Cys+4	**Y190A**	105 ± 19	(3)	-8.9 ± 0.18	1.2	0.82	69 ± 7.0	(8)	-6.3 ± 0.24	0.47	1.2	(5)	-5.6 ± 0.54	2.6	0.87	(4)	-5.4 ± 0.13	4.0	1.4	(6)	-6.2 ± 0.24	0.66	1.3	(3)
TM-5	V:01/5.35	**V196A**	101 ± 13	(3)	-8.9 ± 0.17	1.4	0.96	67 ± 15	(8)	-6.3 ± 0.04	0.47	1.2	(4)	-5.4 ± 0.22	3.6	1.2	(3)	-5.3 ± 0.27	4.6	1.6	(3)	-5.8 ± 0.27	1.6	3.0	(3)
	V:04/5.38	**F199A**	79 ± 7.2	(3)	-8.8 ± 0.06	1.4	0.98	76 ± 16	(4)	-6.7 ± 0.11	0.21	0.52 *	(4)	-5.6 ± 0.17	2.3	0.77	(3)	-5.5 ± 0.06	2.9	1.0	(3)	-6.5 ± 0.16	0.31	0.59	(3)
	V:05/5.39	**Q200A**	4.7 ± 2.8	(3)	-8.9 ± 0.07	1.4	0.95	71 ± 5.1	(10)	-6.4 ± 0.13	0.36	0.89	(5)	-6.2 ± 0.12	0.61	0.20 *	(3)	-5.2 ± 0.19	6.9	2.4	(3)	-6.2 ± 0.05	0.57	1.1	(3)
	V:05/5.39	**Q200W**	75 ± 5.2	(3)	-8.7 ± 0.08	1.8	1.2	34 ± 3.4	(11)	-6.5 ± 0.19	0.33	0.83	(4)	-5.6 ± 0.15	2.7	0.89	(3)	-5.5 ± 0.16	2.8	1.0	(4)	-6.1 ± 0.24	0.86	1.6	(3)
	V:08/5.42	**H203A**	111 ± 4.6	(3)	-8.9 ± 0.17	1.4	1.0	108 ± 24	(5)	-6.2 ± 0.08	0.58	1.4	(3)	-5.1 ± 0.24	8.0	2.7	(3)	-4.7 ± 0.12	20	6.8 **	(3)	-6.0 ± 0.15	0.98	1.9	(3)
TM-6	VI:13/6.48	**W252A**	51 ± 4.3	(3)	-9.1 ± 0.06	0.78	0.53 **	75 ± 6.2	(11)	-6.1 ± 0.11	0.71	1.8 *	(5)	-4.9 ± 0.19	13	4.3 *	(5)	-4.8 ± 0.18	16	5.8 **	(5)	-5.5 ± 0.01	3.1	6.0 **	(3)
	VI:16/6.51	**Y255A**	47 ± 3.5	(3)	-8.9 ± 0.11	1.1	0.77	32 ± 9.7	(7)	-6.6 ± 0.36	0.27	0.68	(5)	-5.5 ± 0.24	3.2	1.1	(4)	-5.1 ± 0.30	8.5	3.0	(3)	-6.1 ± 0.55	0.8	1.6	(3)
	VI:20/6.55	**I259A**	3.0 ± 0.3	(3)	-8.7 ± 0.09	2.1	1.4	59 ± 4.6	(7)	-7.1 ± 0.16	0.08	0.21 ***	(5)	-6.4 ± 0.49	0.41	0.14 *	(3)	-6.3 ± 0.32	0.53	0.18 *	(3)	-6.4 ± 0.53	0.4	0.84	(3)
	VI:20/6.55	**I259W**	34 ± 4.3	(3)	-8.9 ± 0.06	1.3	0.91	28 ± 4.2	(6)	-6.8 ± 0.20	0.15	0.38 **	(5)	-5.7 ± 0.23	2.2	0.74	(3)	-6.2 ± 0.19	0.68	0.24 *	(3)	-6.2 ± 0.18	0.66	1.3	(3)
	VI:23/6.58	**D262N**$^{\#}$	54 ± 4.1	(3)	-8.2 ± 0.04	5.8	4.0 ***	63 ± 7.0	(23)	-5.2 ± 0.09	6.1	15 ***	(11)	-5.3 ± 0.13	5.5	1.8	(12)	-5.4 ± 0.13	4.3	1.5	(6)	-5.0 ± 0.18	10	20 ***	(4)
TM-7	VII:-02/7.32	**H281A**$^{\#}$	169 ± 29	(3)	-8.7 ± 0.13	1.8	1.2	33 ± 9.5	(18)	-6.1 ± 0.19	0.80	2.0 *	(12)	-5.3 ± 0.17	5.2	1.7	(7)	-6.4 ± 0.53	0.36	0.12 **	(4)	-6.2 ± 0.29	0.60	1.1	(4)
	VII:02/7.35	**I284A**	56 ± 9.7	(4)	-8.6 ± 0.05	2.3	1.6 *	38 ± 4.3	(13)	-6.6 ± 0.10	0.27	0.68	(5)	-4.8 ± 0.42	15	4.9 *	(3)	-5.4 ± 0.13	3.8	1.3	(4)	-5.6 ± 0.16	2.6	5.0 **	(4)
	VII:06/7.39	**E288A**$^{\#}$	77 ± 16	(5)	> -7	> 100	> 68	11 ± 4.0	(10)	not determined				not determined				not determined				not determined			

Table 2. Affinity of 12G5, FC131, [Cit^1]FC131, [Aib^1]FC131 and [D-Arg^1]FC131 for WT CXCR4 and various CXCR4 mutations. The data were obtained from competition binding with ^{125}I-labeled antibody 12G5 as radioligand on transiently transfected COS-7 cells. Values in parentheses represent number of experiments (n), and F_{mut} indicates the fold-difference (ratio) between the affinities on mutant receptor compared to WT receptor with colour codes as follows: red > 100 or no displacement at all, orange > 25, yellow > 5, green < 0.2. Significance: *** P<0.001, ** P<0.01, * P<0.05. Residue nomenclature is given as in Table 1.

Receptor			**12G5**								**FC131**						**[Cit^1]FC131**						**[Aib^1]FC131**						**[D-Arg^1]FC131**					
Helix	Position	**Mutation**	IC$_{50}$ *(log)*	± SEM	IC$_{50}$ *(nM)*	F$_{mut}$	B$_{Max}$	± SEM	(n)	P (B$_{max}$)	IC$_{50}$ *(log)*	± SEM	IC$_{50}$ *(µM)*	F$_{mut}$	P	(n)	IC$_{50}$ *(log)*	± SEM	IC$_{50}$ *(µM)*	F$_{mut}$	P	(n)	IC$_{50}$ *(log)*	± SEM	IC$_{50}$ *(µM)*	F$_{mut}$	P	(n)	IC$_{50}$ *(log)*	± SEM	IC$_{50}$ *(µM)*	F$_{mut}$	P	(n)
wt	wt	**wt**	-8.3	± 0.13	4.7	1.0	0.096	± 0.018	(12)		-6.1	± 0.09	0.76	1.0		(12)	-5.3	± 0.12	4.9	1.0		(11)	-5.5	± 0.16	2.9	1.0		(5)	-6.4	± 0.14	0.38	1.0		(6)
TM-2	II:20/2.60	**W94A**	-8.7	± 0.15	1.9	0.40	0.053	± 0.022	(8)		-7.5	± 0.07	0.03	0.04	***	(9)	-6.6	± 0.16	0.27	0.06	***	(9)	-6.9	± 0.33	0.14	0.05	**	(3)	-7.5	± 0.09	0.03	0.08	**	(3)
	II:23/2.63	**D97A**	-8.0	± 0.12	9.4	2.0	0.086	± 0.013	(3)		-6.8	± 0.15	0.17	0.22	**	(3)	-6.1	± 0.05	0.87	0.18	**	(3)	-5.9	± 0.09	1.2	0.40	ns	(3)	No displacement			🟥		(3)
TM-3	III:05/3.29	**H113A**	-8.6	± 0.15	2.7	0.6	0.037	± 0.006	(7)	*	-4.3	± 0.18	48	63	***	(8)	> -4		> 100	20	***	(8)	No displacement			🟥		(3)	No displacement			🟥		(3)
	III:08/3.32	**Y116A**	-8.1	± 0.08	8.7	1.9	0.036	± 0.016	(5)		> -4		> 100	132		(3)	> -4		> 100	20	***	(2)	No displacement			🟥		(2)	> -4		> 100	> 261		(3)
TM-4	IV:20/4.60	**D171N**	-8.7	± 0.18	2.2	0.47	0.034	± 0.017	(7)	*	-4.3	± 0.12	55	72	***	(8)	-4.3	± 0.11	47	9.6	***	(8)	> -4		> 100	34	***	(3)	> -4		> 100	> 261		(3)
ECL-2	ECL-2 / Cys+1	**D187A**	-7.8	± 0.04	16	3.4	0.161	± 0.012	(3)		-5.1	± 0.18	7.7	10	***	(3)	-5.4	± 0.20	4.3	0.87	ns	(3)	-5.6	± 0.19	2.6	0.9	ns	(3)	-4.9	± 0.17	12	31	***	(3)
TM-6	VI:23/6.58	**D262N**	-8.4	± 0.15	3.7	0.8	0.101	± 0.020	(8)		-4.1	± 0.18	73	96	***	(9)	-4.2	± 0.10	61	12	***	(9)	> -4		> 100	34	***	(3)	> -4		> 100	> 261		(3)
TM-7	VII:-02/7.32	**H281A**	-8.6	± 0.15	2.4	0.52	0.028	± 0.008	(7)	*	-4.9	± 0.11	13	18	***	(8)	-4.5	± 0.17	31	6.3	***	(8)	-4.0	± 0.10	91	31	***	(3)	-4.0	± 0.21	107	280	***	(3)
	VII:06/7.39	**E288A**	-8.7	± 0.16	2.1	0.45	0.035	± 0.011	(7)	*	-5.4	± 0.12	4.1	5.5	***	(8)	-5.7	± 0.19	2.1	0.44	ns	(6)	-5.4	± 0.10	4.2	1.4	ns	(3)	> -4		> 100	> 261		(3)

Figure legends

Figure 1. Compounds and mutations included in this study. (A) Structure of FC131 and (B) analogues [Cit^1]FC131, [Aib^1]FC131 and [D-Arg^1]FC131, for which only the structure of the modified side chain 1 is shown. (C) Helical wheel diagram of CXCR4 as seen from the extracellular side showing the upper halves of the TMs and parts of ECL-2. Residues with black background are conserved among class A GPCRs and residues on grey background were mutated in this study. (D) Surface expression and response to 0.1 μM CXCL12 in functional assay of each mutant.

Figure 2. Mutational analysis of FC131 in CXCL12-inhibition and ^{125}I-12G5-binding studies. The ability of FC131 to inhibit CXCL12-mediated activation (A-C) or to displace ^{125}I-12G5 (D-F) from WT CXCR4 (stippled line) or mutants in the TM-area (H113A, Y116A, D171N, D262N) (A, D), the exterior receptor parts (H281A, D187A) and E288A (B, E) or the minor binding pocket (W94A, D97A) (C, F) was assessed (see methods for details). H113A, white square; Y116A, black square; D171N, black circle; D262N, white circle; D187A, white triangle/tip up; H281A, white triangle/tip down, E288A, black triangle/tip down, W94A, black triangle/tip up; D97A, white diamond. Y116A and E288A were not activated by CXCL12 and could therefore not be assessed in functional studies of FC131 (A, B); $n \geq 3$.

Figure 3. The binding mode of FC131 in CXCR4. (A) 2D representation of the FC131 binding site in CXCR4. Residue colours: red, negative; purple, positive; cyan, polar; green, hydrophobic. Interactions: pink full and stippled arrows, H-bond with main and side-chain, respectively; green line, π-π stacking; red line, cation-π

interaction; grey cloud, solvent exposed atom. 3D model of FC131 binding in CXCR4 as seen from top (B) or the side (C). TM-5 and -6 have been removed for clarity.

Figure 4. [Cit^{1}]FC131 and [Aib^{1}]FC131 confirm the orientation of FC131 in CXCR4, and highlight the role of Arg^{188} and His^{203} for sandwiching the 2-Nal^{3} side chain. The structures of the side chains in position 1 of FC131 (A), [Cit^{1}]FC131 (B), and [Aib^{1}]FC131 (C) are given. (D-F) ^{125}I-12G5 binding assays on WT CXCR4 and mutant receptors (H113A, D171N and D187N, symbols as in Figure 2). (G-I) Impact of mutations D187A, R188A, F189A, Y190A (ECL-2) and H203A (TM-5) in binding (D187A) or functional assays (other mutants) shown as fold-decreases. (K, L) Molecular docking of the analogues.

Figure 5. Mutational analysis and computational modelling of [D-Arg^{1}]FC131 binding in CXCR4. (A) Fold-decreases of FC131 (grey) and [D-Arg^{1}]FC131 (white) affinity (upper diagram) and antagonistic potency (lower diagram) observed for a range of mutants in comparison to WT CXCR4. (B) The effect of D97A (triangle, tip down) in comparison to WT (circles, stippled line) on the affinity (upper part) and potency (lower part) of FC131 (grey) and [D-Arg^{1}]FC131 (white). (C) Molecular docking of [D-Arg^{1}]FC131 (white structures) showing multiple binding poses in overlay with the FC131 binding pose (green structure).

Figure 6. Mutation of the H-bond forming residues Tyr^{116} and Glu^{288} abolished CXCL12-induced receptor activation. (A) Tyr^{116} and Glu^{288} form a H-bond in the crystal structure of CXCR4 (here bound to CVX15, PDB code 3OE0). (B) Ability of CXCL12 to activate WT CXCR4 (white circles) and mutants Y116A (white

diamonds) and E288A (black diamonds). (C) Homologous ^{125}I-12G5 competition binding experiments on WT, Y116A and E288A (symbols as in A) and the B_{max} of 12G5 in fmol/1 × 10^6 cells for each receptor (inset). (D) Ability of FC131 to displace ^{125}I-12G5 from WT, Y116A, and E288A (symbols as in A).

Figure 7. The binding of FC131 compared to CVX15. Overlay of the binding modes for FC131 (green) identified in the present study and CVX15 (yellow) from the co-crystal structure of CXCR4 and CVX15 (PDB code: 3OE0).

Statement of conflicts of interest

None

Supporting Information

None