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Non-competitive antagonism and inverse agonism as mechanism of action of non-peptidergic antagonists at primate and rodent CXCR3 chemokine receptors

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Abstract

The chemokine receptor CXCR3 is involved in various inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, psoriasis and allograft rejection in transplantation patients. The CXCR3 ligands CXCL9, CXCL10 and CXCL11 are expressed at sites of inflammation and attract CXCR3-expressing lymphocytes, thus contributing to the inflammatory process. Here, we characterize 5 non-peptidergic compounds of different chemical classes that block the action of CXCL10 and CXCL11 at the human CXCR3, i.e. VUF10472/NBI-74330, VUF10085/AMG-487, VUF5834, VUF10132 and TAK-779. In order to understand the action of these CXCR3 antagonists in various animal models of disease, the compounds were also tested at rat and mouse CXCR3, as well as at CXCR3 from rhesus macaque, cloned and characterized for the first time in this study. Except for TAK-779, all compounds show slightly lower affinity for rodent CXCR3 than for primate CXCR3. Additionally, we have characterized the molecular mechanism of action of the various antagonists at the human CXCR3 receptor. All tested compounds act as noncompetitive antagonists at CXCR3. Moreover, this non-competitive behavior is accompanied by inverse agonistic properties of all 5 compounds as determined on an identified constitutively active mutant of CXCR3, CXCR3 N3.35A. Interestingly, all compounds except TAK-779 act as full inverse agonists at CXCR3 N3.35A. TAK-779 shows weak partial inverse agonism at CXCR3 N3.35A, and likely has a different mode of interaction with CXCR3 than the other three classes of small molecule inverse agonists.

Chemokines are secreted peptides that are important mediators in inflammation. They are classified into four families based on the number and position of conserved Nterminal

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¹GPCR numbering according to Ballesteros and Weinstein, where the first digit refers to the transmembrane helix and the second number indicates the position of the residue relative to the most conserved amino acid at position 50 in that helix (i.e. N1.50, D2.50, R3.50, W4.50, P5.50, P6.50 and P7.50) (Ballesteros and Weinstein, 1995).

cysteine residues, i.e. CC, CXC, CX₃C and XC chemokines (Murphy et al., 2000). Chemokines bind to a subset of class A G-protein coupled receptors (GPCRs), which are named based on their specific chemokine preferences (Murphy et al., 2000). The chemokine receptor CXCR3 is mainly expressed on activated Th1 cells, but also on B cells and natural killer cells (Qin et al., 1998). CXCR3 is activated by the INF- γ -inducible chemokines CXCL9, CXCL10 and CXCL11, with CXCL11 having the highest affinity (Loetscher et al., 1996; Cole et al., 1998). Upon activation, CXCR3 activates pertussis toxin-sensitive G-proteins of the G α_i class and mediates e.g. chemotaxis, calcium flux and activation of kinases such as p44/p42 MAPK and Akt (Smit et al., 2003).

CXCR3 and its ligands are upregulated in a wide variety of inflammatory diseases, implying a role for CXCR3 in e.g. rheumatoid arthritis (Qin et al., 1998), multiple sclerosis (Sorensen et al., 1999), transplant rejection (Hancock et al., 2000), atherosclerosis (Mach et al., 1999) and inflammatory skin diseases (Flier et al., 2001). The role of CXCR3 in cancer is two-fold: on one hand CXCR3 may be involved in the metastasis of CXCR3-expressing cancer cells (Walser et al., 2006), while on the other hand expression of CXCL10 (Luster and Leder, 1993) or CXCL11 (Hensbergen et al., 2005) at tumor sites may attract CXCR3-expressing immune cells, that help control tumor growth and metastasis.

Several animal models have been developed for CXCR3, among which a murine model of metastatic breast cancer (Walser et al., 2006), a murine model of renal cell carcinoma (RENCA) (Pan et al., 2006) and an arthritis model in Lewis rats (Salomon et al., 2002). In a mouse rheumatoid arthritis model TAK-779, a small molecule antagonist with affinity for CCR5, CCR2b and CXCR3, inhibits the development of arthritis by downregulating T cell migration, indicating that targeting chemokine receptors in models of inflammation is feasible and effective (Baba et al., 1999; Yang et al., 2002; Gao et al., 2003).

Several classes of small molecule compounds targeting CXCR3 have recently been described, including 4-*N*-aryl-[1,4]diazepane ureas (Cole et al., 2006), 1-aryl-3-piperidin-4-yl-urea derivatives (Allen et al., 2007), quinazolin-4-one and 3*H*-pyrido[2,3-*d*]pyrimidin-4-one derivatives (Heise et al., 2005; Storelli et al., 2005; Johnson et al., 2007; Storelli et al., 2007) and the above mentioned quarternary ammonium anilide TAK-779 (Gao et al., 2003). So far, not detailed information is available on the molecular mechanism of action of these small molecule antagonists at the CXCR3 receptor, despite the general notion that small molecule ligands most likely will not have overlapping binding sites with the chemokines, which are supposed to bind mainly to the N-terminus and extracellular receptor loops (Murphy et al. 2000). Moreover, although most compounds have been tested on human CXCR3 using *in vitro* assays, little or no information on their affinity for CXCR3 of other species is available. Especially in view of rodent models of inflammatory diseases it is important to know the relative affinities of the compounds for the receptors of different species.

Here, we report on the molecular characterization of the 3*H*-pyrido[2,3-*d*]pyrimidin-4-one derivatives VUF10472 (NBI-74330) (Heise et al., 2005; Storelli et al., 2007) and VUF10085 (AMG-487) (Johnson et al., 2007; Storelli et al., 2007), the quinazolin-4-one VUF5834 (Storelli et al., 2005; Johnson et al., 2007) the imidazolium compound VUF10132 (Axtén et al., 2003) and the quarternary ammonium anilide TAK-779 (Baba et al., 1999) at CXCR3 of human (Loetscher et al., 1996), rat (Wang et al., 2000) and mouse (Lu et al., 1999). Additionally, CXCR3 from rhesus macaque was cloned, characterized and subjected to a detailed pharmacological analysis using the non-peptidergic compounds. Moreover, we constructed and characterized a constitutively active mutant (CAM) of CXCR3, which was used to further determine the inverse agonistic properties of the small molecule compounds.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and trypsin were purchased from PAA Laboratories GmbH (Paschen, Austria), RPMI 1640 with glutamax-I and 25 mM HEPES, non essential amino acids, sodium pyruvate and 2-mercaptoethanol were from Sigma-Aldrich, penicillin and streptomycin were obtained from Cambrex, fetal bovine serum (FBS) was purchased from Integro B.V. (Dieren, The Netherlands), certified FBS was from Invitrogen. *myo*-[2-³H]inositol (10–20 Ci/mmol) was bought from GE Healthcare, ¹²⁵I-CXCL10 (2200 Ci/mmol) and ¹²⁵I-CXCL11 (2000 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences. Chemokines were obtained from PeproTech (Rocky Hill, N.J.). TAK-779 was obtained from the NIH AIDS research and reference reagent program.

DNA constructs

The cDNA of human CXCR3 inserted in pcDNA₃ (Loetscher et al., 1996) was amplified by PCR and inserted into pcDEF₃ (a gift from Dr. Langer).

A cDNA containing the rhesus macaque (*Macaca mulatta*) CXCR3 open-reading frame was obtained from rhesus macaque peripheral blood mononuclear cells (PBMCs) stimulated overnight with phytohemagglutinin-P plus phorbol myristate acetate. Total RNA was extracted using Trizol (Life Technologies) and cDNA was generated by reverse transcription (RT) with avian myeloblastosis virus RT (Promega) and oligo(dT) primer. PCR was then performed with Taq polymerase (Promega) and primers TRCXCR3F2 (5'-AGCCCAGCCATGGTCCTTG-3') and TRCXCR3R2 (5'-CCTCACAAGCCCGAGTAGGA-3'). The resulting PCR product was spin-column purified (Qiagen) and ligated to pGEM-T vector (Promega). Subsequently the cDNA was subcloned into pcDEF₃.

A cDNA containing the *rattus norvegicus* CXCR3 open-reading frame was cloned from an F344 heart allograft that was transplanted into a Lewis rat at 28 days post-transplantation (Streblow et al., 2003). Total RNA was prepared from 0.25 g of rat tissue using the Trizol method. First strand cDNA was synthesized from 2 µg of total RNA using 1.0 µM oligo dT-T7 primer (GGCCAGTGAATTGTAATACGACTCACTATAGGG(A)₂₄) and 200 U Superscript III reverse transcriptase (Invitrogen). Doubled-stranded cDNA was generated by the addition of second strand buffer (Invitrogen) according to the manufacturer's protocol and purified by phenol/chloroform extraction. PCR was performed with Taq DNA polymerase and primers ratCXCR3Fwd:

ATAGGATTCATGTACCTTGAGGTCAGTGAACGTCA and ratCXCR3Rev: ATAGAATTCTTACAAGCCCAAGTAGGAGGCCTCAGT. The resulting PCR fragment was cloned into pGEM-Teasy (Promega), and subcloned into pcDEF₃. The cDNA of mouse CXCR3 was a kind gift from Dr. Luster (Lu et al., 1999) and was subcloned into pcDEF₃. The chimeric G-protein Gα_{qi5} (pcDNA₁-HA-mGα_{qi5}) was a gift from Dr. Conklin (Coward et al., 1999).

Synthesis of small molecule compounds

Synthesis of VUF5834 (Storelli et al., 2005), VUF10472 and VUF10085 (Storelli et al., 2007) has been described previously. Synthesis of VUF10132 was adapted from the procedure described by Axten et al (Axten et al, 2003). Shortly, 1-(3,4-Dichloro-phenyl)-2-imidazol-1-yl-ethanone (0.19 g, 0.75 mmol). and 2-Bromo-1-(3,4-dichloro-phenyl)-ethanone (0.20 g, 0.75 mmol) were stirred in acetonitrile (50 mL) overnight at rt. The mixture was diluted with ether, the solid product filtered, washed with ether and dried to afford

VUF10132 as white solid. Yield: 0.31 g (78%). ^1H NMR (DMSO) δ : 6.20 (s, 4H), 7.67–7.80 (m, 4H), 7.88–8.12 (m, 3H), 8.23–8.25 (m, 2H), 9.12 (s, 1H).

Cell culture and transfection

HEK293T cells were grown at 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin and streptomycin. HEK293T cells were transfected with 2.5 μg cDNA encoding CXCR3 supplemented with 2.5 μg pcDNA₁-HA-mG α_{q15} (for PLC activation experiments and ELISA) or 2.5 μg pcDEF₃ using linear polyethyleneimine (PEI) (MW 25,000; Polysciences Inc, Warrington, PA, USA). The total amount of DNA in gene-dosing experiments was kept constant at 5 μg by addition of pcDEF₃. Briefly, a total of 5 μg DNA was diluted in 250 μl 150 mM NaCl. Subsequently 30 μg PEI in 250 μl 150 mM NaCl was added to the DNA solution and incubated for 10 min at RT. The mixture was added to adherent HEK293T cells in 100 mM tissue culture dishes. Next day, cells were trypsinized, resuspended into culture medium and plated in the appropriate poly-L-lysine (Sigma) coated assay plates. For membrane preparation, cells were harvested 48 h after transfection from the tissue culture dishes in which they were transfected.

Murine pre-B L1.2 cells were grown in RPMI 1640 medium with glutamax-I and 25 mM HEPES, supplemented with 10% heat-inactivated certified FBS, penicillin, streptomycin, glutamine, non-essential amino acids, 2-mercaptoethanol and sodium pyruvate. L1.2 cells were transfected with 10 μg per 20 million cells using a BioRad Gene Pulser II and grown in culture medium supplemented with 10 mM sodium butyrate.

Chemokine binding

Cell membrane fractions from transiently transfected HEK293T cells were prepared as follows. Cells were washed with cold PBS, detached using cold PBS containing 1 mM EDTA and centrifuged at 1500 g for 10 min at 4°C. Subsequently, cells were washed with PBS and centrifuged at 1500 g for 10 min at 4°C. The pellet was resuspended in cold membrane buffer (15 mM Tris pH 7.5, 1 mM EGTA, 0.3 mM EDTA, 2 mM MgCl_2) and homogenized by 10 strokes at 1100–1200 rpm using a teflon-glass homogenizer and rotor. The membranes were subjected to two freeze thaw cycles using liquid nitrogen and centrifuged at 40,000 g for 25 min at 4°C. The pellet was rinsed with cold Tris-sucrose buffer (20 mM Tris pH 7.4, 250 mM sucrose), resuspended in the same buffer and frozen in liquid nitrogen. Protein concentration was determined using a Bio-Rad protein assay. Membranes were incubated in 96 well plates in binding buffer (50 mM HEPES pH 7.4, 1 mM CaCl_2 , 5 mM MgCl_2 , 100 mM NaCl, 0.5% BSA with approximately 50 pM ^{125}I -CXCL10 or ^{125}I -CXCL11 and increasing amounts of displacer for 2 h at RT. Subsequently, membranes were harvested via filtration through Unifilter GF/C plates (PerkinElmer Life and Analytical Sciences) pretreated with 1% polyethylenimine and washed three times with wash buffer (50 mM HEPES pH 7.4, 1 mM CaCl_2 , 5 mM MgCl_2 , 500 mM NaCl). Radioactivity was measured using a MicroBeta (PerkinElmer Life and Analytical Sciences).

Phospholipase C activation

Twenty-four h after transfection, HEK293T cells were labeled overnight in Earle's inositol-free minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin and streptomycin and *myo*-[2- ^3H]inositol (1 $\mu\text{Ci}/\text{ml}$). Cells were washed with InsP assay buffer (20 mM HEPES pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM CaCl_2 , 10 mM glucose and 0.05% (w/v) BSA) and incubated for 2 h in InsP assay buffer supplemented with 10 mM LiCl in the presence or absence of indicated ligands. The incubation was stopped by aspiration of the assay buffer and addition of ice-cold 10 mM

formic acid. After incubation for 90 min at 4°C, ³H-InsP were isolated by anion-exchange chromatography (Dowex AG1-X8 columns, Bio-Rad) and counted by liquid scintillation.

Chemotaxis

Twenty-four h after transfection, chemotaxis of L1.2 cells towards CXCL10 (10 nM) was determined in the presence or absence of small molecule compounds, using 5 µM pore ChemoTx 96 well plates (Neuroprobe Inc, MD, USA). Briefly, ChemoTx plates were blocked using RPMI 1640 with glutamax-I and 25 mM Hepes supplemented with 1% (w/v) BSA. Chemokine and compound dilutions were made in RPMI 1640 with glutamax-I and 25 mM Hepes supplemented with 0.1% (w/v) BSA and added to the wells. L1.2 cells were added on top of the membrane and incubated for 5 h in a humidified chamber at 37 °C. The number of migrated cells per well was determined using a haemocytometer.

ELISA

Forty-eight hours after transfection, HEK293T cells were washed with TBS and fixed with 4% paraformaldehyde in PBS. After blocking with 1% skim milk in 0.1 M NaHCO₃ (pH 8.6), cells were incubated with mouse monoclonal anti-CXCR3 antibody (MAB160, R&D Systems, Inc) in TBS containing 0.1% BSA, washed three times with TBS and incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Subsequently, cells were incubated with substrate buffer containing 2mM *o*-phenylenediamine (Sigma, St. Louis, Mo), 35 mM citric acid, 66 mM Na₂HPO₄, 0.015% H₂O₂ (pH 5.6). The reaction was stopped with 1 M H₂SO₄ and absorption at 490 nm was determined.

Data analysis

Nonlinear regression analysis of the data and calculation of K_d and K_i values was performed using Prism 4.03. The pK_b values in the PLC activation assay were calculated using the Cheng-Prusoff equation $pK_b = IC_{50} / (1 + [agonist]/EC_{50})$ (Cheng and Prusoff, 1973).

Results

Characterization of small non-peptidergic antagonists at human CXCR3

A selection of CXCR3-targeting small molecule antagonists from four different structural classes was synthesized as previously described and subjected to detailed pharmacological analysis, including the quinazolinone-derived 3*H*-pyrido[2,3-*d*]pyrimidin-4-one compounds VUF10472 (NBI-74330) (Heise et al., 2005; Storelli et al., 2007) and VUF10085 (AMG 487) (Johnson et al., 2007; Storelli et al., 2007), the quinazolin-4-one VUF5834 (Storelli et al., 2005; Johnson et al., 2007), and the imidazolium compound VUF10132 (Axten et al., 2003) (Fig. 1). The well described CCR5 antagonist TAK-779 (Baba et al., 1999) has been reported to show affinity for mouse CXCR3 (Gao et al., 2003) and was therefore included in our set of small molecule compounds as well (Fig. 1).

Binding studies were performed with radiolabeled CXCL10 and CXCL11 on membranes prepared from HEK293T cells transiently transfected with cDNA encoding human CXCR3. Homologous displacement of the radioligands with unlabeled chemokines resulted in pK_d values ± SEM of 9.8 ± 0.1 for CXCL10 and 10.6 ± 0.1 for CXCL11 (Fig. 2A, Table 1), in accordance with the values and rank-order for these chemokines reported in literature (Cole et al., 1998; Heise et al., 2005).

In HEK293 cells human CXCR3 does not couple efficiently to the phospholipase C (PLC) - inositolphosphates (InsP) pathway (Wang et al., 2000). Therefore, CXCR3 was transiently co-transfected with cDNA encoding the chimeric G-protein Gα_{q15} (Coward et al., 1999),

after which robust PLC activation could be observed upon stimulation with CXCL10 and CXCL11. Using this functional assay, pEC_{50} values of 7.5 ± 0.1 and 8.5 ± 0.1 were obtained for respectively CXCL10 and CXCL11 (Fig. 2B, Table 1). CXCL10 acted as a partial agonist, with an intrinsic activity of 0.48 ± 0.8 compared to the full agonists CXCL11 (Fig. 2B, $n=5$).

The panel of small molecule antagonists inhibited ^{125}I -CXCL10 binding to CXCR3 with K_i values ranging between 4 nM for VUF10472 and 1.3 μ M for TAK-779 (Fig. 3A and Table 2). VUF10085, VUF5834 and VUF10132 showed intermediate K_i values of 20 nM, 158 nM and 251 nM respectively (Fig. 3A and Table 2). Subsequently, the ability of the small molecules to inhibit CXCL10-induced PLC activation was determined. All compounds reduced CXCL10-mediated (10–50 nM) PLC activation with K_b values ranging from 6 nM for VUF10472 to 800 nM for TAK-779 (Fig. 3B, Table 2). A good correlation ($r^2 = 0.997$) was observed between the measured pK_i values and pK_b values determined against CXCL10 (Fig. 3C). For the most potent compound, VUF10472, inhibition of CXCL11-induced (5 nM) PLC activation was also determined, resulting in a K_b value of 12.6 nM (Fig. 3D). This value corresponds well to the value obtained against CXCL10 (Fig. 3D and Table 2). Finally, the ability of the small molecule compounds to inhibit chemotaxis was investigated. The murine pre-B cell line L1.2 was transiently transfected with cDNA encoding human CXCR3 and migration of cells towards CXCL10 in the presence or absence of VUF10085 and VUF5834 was determined. CXCR3-transfected L1.2 cells did not migrate in the absence of CXCL10, whereas 10.3 ± 0.8 % ($n=3$) of the cells migrated to CXCL10 (10 nM) in the absence of inhibitors (Fig. 3E, inset). Both compounds completely inhibited CXCL10-induced (10 nM) migration at a concentration of 10 μ M, with pIC_{50} values of 6.8 ± 0.2 ($n=2$) and 5.8 ± 0.1 ($n=3$) for VUF10085 and VUF5834 respectively (Fig. 3E).

In order to determine whether the antagonists show competitive or non-competitive behavior, a Schild analysis was performed. HEK293T expressing CXCR3 and $G_{\alpha_{q15}}$ were incubated with increasing concentrations of CXCL10 (Fig. 4A) or CXCL11 (Fig. 4B) in the absence or presence of different concentrations of VUF10472. A decreased maximal effect combined with a rightward shift of the curves was observed in the presence of VUF10472, indicating non-competitive antagonist behavior. The mechanism of antagonism of the other classes of small molecule compounds was investigated using only CXCL11. VUF5834 (Fig. 4C), VUF10132 (Fig. 4D) and TAK-779 (Fig. 4E) all showed noncompetitive antagonistic behavior similar to that of VUF10472.

Characterization of small non-peptidergic antagonists at a constitutively active mutant of CXCR3

Inverse agonism has recently been shown to be an important molecular mechanism of action of small molecule antagonists at a variety of GPCR family members (Parra and Bond, 2007). Human chemokine receptors generally do not show high levels of constitutive activity. However, constitutively active chemokine receptor mutants have been described that signal in the absence of ligands. Examples are CXCR2 D3.49V (Burger et al., 1999), mutation of N3.35 of CXCR4 (Zhang et al., 2002) and mutation of T2.56 in CCR2 and CCR5 (Arias et al., 2003). To determine if the studied CXCR3 antagonists act as neutral antagonists or inverse agonists at CXCR3, analogous CAMs of CXCR3 were constructed (Fig. 6). Upon expression of the CXCR3 mutants N3.35A, N3.35S and T2.56P significant constitutive activity was shown, whereas no constitutive activity was found for the CXCR3 D3.49V mutant (data not shown). In view of the signal to noise ratio we choose CXCR3 N3.35A for further characterization. Upon transfection of HEK293T cells with increasing amounts of cDNA encoding CXCR3 WT or CXCR3 N3.35A an increase in receptor expression was detected in ELISA experiments (Fig. 5A). When similar amounts of cDNA were transfected, CXCR3 WT was expressed at higher levels than CXCR3 N3.35A (Fig.

5A). As expected, CXCR3 WT did not activate PLC in the absence of ligands (Fig. 5B). Even though CXCR3 N3.35A was expressed at lower levels than CXCR3 WT, CXCR3 N3.35A showed a marked increase of PLC activation upon transfection of increasing amounts of cDNA (Fig. 5B).

The affinities of CXCL10 (pK_d 10.2 ± 0.2) and CXCL11 (pK_d 11.3 ± 0.3) for CXCR3 N3.35A were slightly higher than for CXCR3 WT (Table 1). CXCL10 and CXCL11 activated CXCR3 N3.35A over basal levels in a similar manner as CXCR3 WT, with pEC_{50} values of 7.5 ± 0.0 ($n=2$) and 8.7 ± 0.1 ($n=4$) respectively (Fig. 5C, Table 1). Subsequently, the affinity of the small molecule antagonists at CXCR3 N3.35A was determined using ^{125}I -CXCL10. The affinity of all compounds, except TAK-779, was slightly reduced for CXCR3 N3.35A compared to CXCR3 WT (Table 2). Finally, the effect of the small molecule antagonists on the constitutive activation of PLC was investigated. VUF10472, VUF10085, VUF5834 and VUF10132 all acted as full inverse agonists, with IC_{50} values ranging between 8 nM for VUF10472 and 251 nM for VUF10132 (Fig. 5D, Table 2). In contrast, TAK-779 acted as a partial inverse agonist with an intrinsic activity of -0.42 ± 0.0 and an IC_{50} of 1.3 μ M ($n=3$) (Fig. 5D, Table 2).

Characterization of small non-peptidergic antagonists acting at CXCR3 of rhesus macaque, rat and mouse

Since the affinities of small molecule compounds targeting chemokine receptors may differ among species, and significant differences between the amino acid sequences of primate and rodent CXCR3 are apparent (Fig. 5B), we set out to characterize the behavior of small non-peptidergic compounds at human, rhesus macaque, mouse and rat CXCR3. Human (Loetscher et al., 1996), mouse (Lu et al., 1999) and rat (Wang et al., 2000) CXCR3 have previously been cloned and described. Here, we report the cloning and characterization of rhesus macaque CXCR3 from PBMCs (see experimental procedures). The deduced amino acid sequence of rhesus macaque CXCR3 was found to be 99% identical to human CXCR3. The Thr⁸³ residue in the first intracellular loop of human CXCR3 is Ala⁸³ in rhesus macaque CXCR3, and Val²⁵⁹ at position 6.39 in TM6 of human CXCR3 is Ala²⁵⁹ in rhesus macaque CXCR3 (Fig. 6).

After expression in HEK293T cells, CXCR3 from rhesus, rat and mouse showed similar binding affinities for CXCL10 and CXCL11 as human CXCR3 (Table 1). B_{max} values of rodent CXCR3 were increased compared to human CXCR3, approximately 2-fold higher for CXCL10 and 3 to 4-fold higher for CXCL11 (Table 1) when equal amounts of cDNA were transfected. The B_{max} for CXCL10 of rhesus CXCR3 was 2-fold lower than for human CXCR3, whereas human and rhesus CXCR3 had comparable B_{max} values for CXCL11 (Table 1). Rhesus CXCR3 stimulated PLC with similar maximum effect (E_{max}) and similar EC_{50} values for CXCL10 and CXCL11 as human CXCR3 (Fig. 7A and 7B, Table 1). In contrast, the rodent CXCR3 species had approximately 10-fold higher potencies and showed increased E_{max} for CXCL10 compared to human CXCR3 (Fig. 7A and 7B, Table 1). The E_{max} for CXCL11 at rat CXCR3 was also increased compared to the effect of CXCL11 at CXCR3 of the other species (Fig. 7B).

Next, the affinity of the small molecule compounds for CXCR3 of the different species was determined using ^{125}I -CXCL10. The pK_i values of the compounds tested at rhesus CXCR3 were identical to the values found for human CXCR3 (Table 2). Similarly, the affinities of the compounds tested at rat and mouse CXCR3 were identical to each other. However, VUF10472, VUF10085, VUF5834 and VUF10132 showed an approximately 4-fold lower affinity for the rodent CXCR3 (mouse and rat) compared to the primate CXCR3 (human and rhesus). Only for TAK-779 no difference in affinity between CXCR3 from the different species was observed.

Discussion

CXCR3 has attracted considerable attention as a new potential drug target due to its involvement in a variety of serious disorders, including cancer (Walser et al., 2006), atherosclerosis (Mach et al., 1999), inflammatory disorders like rheumatoid arthritis (Qin et al., 1998), skin diseases (Flier et al., 2001) and transplant rejection (Hancock et al., 2000). Following the recognition of CXCR3 as a potential attractive drug target, several small molecule CXCR3 antagonists have recently been identified (Gao et al., 2003; Heise et al., 2005; Storelli et al., 2005; Cole et al., 2006; Allen et al., 2007; Johnson et al., 2007; Storelli et al., 2007). In this study, we have selected five non-peptidergic antagonists from four different structural classes and studied their mechanism of action at the human CXCR3. In addition, the interaction of these antagonists with human, rhesus macaque, rat and mouse CXCR3 was investigated.

All tested non-peptidergic antagonists inhibited CXCL10-induced activation of PLC with pK_b values that correlated very well with their affinity, with a rank order VUF10472 > VUF10085 > VUF5834 > VUF10132 > TAK-779 (Table 2). Subsequently, the mechanism of action of the antagonists was explored using Schild analysis. The dose-response curves for CXCL10 and CXCL11 did not reach the maximal response in the presence of VUF10472 (Fig. 4), as has been previously shown by others for human and mouse CXCR3 (Heise et al., 2005; Jopling et al., 2007). Antagonists from other structural classes also decreased the maximum response of CXCL11, in a manner that follows the rankorder of their affinities. This indicates that all tested non-peptidergic antagonists behave as non-competitive antagonists for the PLC activation by the endogenous agonists of CXCR3.

Antagonists of GPCRs can be classified as neutral antagonists or (partial) inverse agonists (Kenakin, 1996; Parra and Bond, 2007). Although both antagonist classes are able to block an agonist-induced response by occupying the receptor, there is an ongoing debate about the exact clinical benefits or disadvantages of each class (Parra and Bond, 2007). Treatment with inverse agonists may be beneficial when a constitutively active receptor underlies the pathogenesis. Yet, long-term exposure to inverse agonists may result in undesired receptor upregulation (Smit et al., 1996). To date, there are no reports about constitutive activity of CXCR3 and there are only few examples of constitutive activity of chemokine receptors. Constitutive activity is a function of the relative stoichiometry of receptors and G-proteins (Kenakin, 1996), and chemokine receptors, including CXCR3, are often upregulated under inflammatory conditions (Rabin et al., 1999; Murphy et al., 2000). Hence, under pathophysiological conditions constitutive activity of chemokine receptors might become apparent and therefore the use of inverse agonists beneficial. In order to study the relative efficacy of the non-peptidergic antagonists, we generated mutants of CXCR3, i.e. CXCR3 N3.35A, N3.35S, D3.39V and T2.56P, based on CAMs of chemokine receptors reported in literature (Burger et al., 1999; Zhang et al., 2002; Arias et al., 2003). All generated mutants, except for CXCR3 D3.49V with a mutation in the conserved DRY motif at the cytoplasmatic end of TM3, displayed basal signaling. Mutation of the DRY motif of CXCR2 to VRY confers constitutive activity to CXCR2 (Burger et al., 1999), while the same mutation for CXCR1 (Burger et al., 1999) or CCR5 (Lagane et al., 2005) did not result in constitutive activity. Conversely, mutation of the VRY motif to DRY in the highly constitutively active chemokine receptor ORF74 encoded by Kaposi's sarcoma-associated herpesvirus did not diminish its constitutive activity but even increased it (Ho et al., 2001). Therefore, mutation of D3.49 to Val does not appear to be a universal switch for constitutive activity in chemokine receptors. In the same way, mutation of T2.56 in the conserved TXP motif resulted in a CAM for CXCR3 (this study), CCR5 and CCR2 but not for CCR1, CCR3, CCR4, CXCR2 and CXCR4 (Arias et al., 2003). Mutation of N3.35 in the N(L/F)Y motif in TM3 of CXC chemokine receptors resulted in CAMs for both CXCR3 (this study)

and CXCR4 (Zhang et al., 2002). The non-peptidergic compounds acted as full inverse agonists at CXCR3 N3.35A, except for TAK-779, which only partially inhibited constitutive signaling at the highest concentration used (Fig. 4D). Since VUF10132 and TAK-779 have similar affinities for CXCR3 N3.35A (Table 2), it appears that TAK-779 lacks certain structural features needed for full inverse agonism at CXCR3. Interestingly, TAK-779 acts as a full inverse agonist at CCR5 (Lagane et al., 2005). As expected for a receptor in the active state (Kenakin, 1996), the affinities of the agonists CXCL10 and CXCL11 for CXCR3 N3.35A were increased compared to CXCR3 WT (Table 1). The full inverse agonists VUF10472, VUF10085, VUF5834 and VUF10132, which are predicted to have a higher affinity for an inactive receptor conformation (Kenakin, 1996), all showed reduced affinity for CXCR3 N3.35A compared to CXCR3 WT (Table 2). In contrast, the affinity of the weak partial inverse agonist TAK-779 did not change, indicating a different mode of interaction with CXCR3 compared to the other structural classes of compounds.

If antagonists are to be tested in animal models, detailed information on the interaction of the compound with the receptor of that specific species is required, since species differences may occur. We therefore tested the non-peptidergic antagonists on rhesus macaque CXCR3, which was cloned in this study, as well as on rat and mouse CXCR3. Human and rhesus macaque show 99% amino acid identity (Fig. 6). Consistent with this high homology, no differences in affinity or potency of CXCL10 and CXCL11, or in the affinity of the non-peptidergic antagonists for the two receptors were found. Similarly, the protein sequences of rat and mouse CXCR3 are 96% identical and no significant differences in affinity of the endogenous agonists or the non-peptidergic compounds are observed between the CXCR3 of these rodent species. However, lower sequence identity is found when human and rhesus macaque CXCR3 are compared with rat and mouse CXCR3. Approximately 85% identity exists between the primate (human and rhesus macaque) and rodent (rat and mouse) species (Fig. 6). Nevertheless, the affinities of CXCL10 and CXCL11 found for rodent CXCR3 were comparable to the affinities found for primate CXCR3 (Table 1). While the affinities of CXCL10 and CXCL11 were comparable, the affinities of VUF10472, VUF10084, VUF5834 and VUF101032 were about 4-fold lower for rodent CXCR3 than for primate CXCR3 (Table 2). In contrast, the affinity of TAK-779 was equal for CXCR3 of all tested species, again indicating that TAK-779 interacts with CXCR3 in another manner than the other small molecule antagonists. TAK-779, developed as a CCR5 antagonist, has been thoroughly investigated because of its potential use as a HIV-entry inhibitor. TAK-779 shows high affinity for human CCR5 and to a lesser extend for human CCR2b (Baba et al., 1999). Furthermore, TAK-779 was reported to bind mouse CCR5, as well as mouse CXCR3 (Gao et al., 2003). Remarkably, TAK-779 shows a more than 100-fold higher affinity for human CCR5 than for mouse CCR5 (Baba et al., 1999; Gao et al., 2003). This species selectivity of TAK-779 for CCR5 is not observed for human and mouse CXCR3, which both bind TAK-779 with affinities around 1 μ M (Table 2). It appears that TAK-779 has nanomolar affinity for human CCR5 and micromolar affinity for human CXCR3, whereas it has only micromolar affinity for mouse CCR5 and mouse CXCR3. This should be kept in mind when analyzing and extrapolating data from rodent models, since at a certain effective concentration of TAK-779 different receptors will be blocked in humans and mice. While in mice the observed effects would likely be mediated by a combination of blockage of CCR5 and CXCR3, in humans the effects of TAK-779 would be mostly through inhibition of CCR5 with low nanomolar affinity. The non-peptidergic CXCR3 antagonists investigated here only showed a 3 to 4-fold species selectivity for CXCR3 of primates versus rodents, which is favorable for their use in rodent models.

In summary, we characterized four classes of small, non-peptidergic CXCR3 noncompetitive antagonists at CXCR3 of four different species. The relatively small difference in affinity observed between human and rodent CXCR3 implies that these

compounds are useful in rodent models of CXCR3-mediated pathogenesis. It was found that the compounds act as inverse agonists at a constitutively active CXCR3 mutant, with VUF10472 being the most potent compound at human, rhesus, rat and mouse CXCR3. The compounds all non-competitively antagonized chemokine-induced signaling at human CXCR3.

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....Others...

Abbreviations

BSA	bovine serum albumin
CAM	constitutively active mutant
GPCR	G protein-coupled receptor
InsP	inositol phosphates
PMBC	peripheral blood mononuclear cell
PLC	phospholipase C

References

- Allen DR, Bolt A, Chapman GA, Knight RL, Meissner JW, Owen DA, Watson RJ. Identification and structure-activity relationships of 1-aryl-3-piperidin-4-ylurea derivatives as CXCR3 receptor antagonists. *Bioorg Med Chem Lett*. 2007; 17:697–701. [PubMed: 17097877]
- Arias DA, Navenot JM, Zhang WB, Broach J, Peiper SC. Constitutive activation of CCR5 and CCR2 induced by conformational changes in the conserved TXP motif in transmembrane helix 2. *J Biol Chem*. 2003; 278:36513–36521. [PubMed: 12837756]
- Axten JM, J FJ, D KW, Sarau HM. Imidazolium CXCR3 inhibitors (Smithkline Beecham Corporation) WO 03/101970 A1, in. 2003
- Baba M, Nishimura O, Kanzaki N, Okamoto M, Sawada H, Iizawa Y, Shiraishi M, Aramaki Y, Okonogi K, Ogawa Y, Meguro K, Fujino M. A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. *Proc Natl Acad Sci U S A*. 1999; 96:5698–5703. [PubMed: 10318947]
- Ballesteros JA, Weinstein H. Integrated Methods for the Construction of Three Dimensional Models and Computational Probing of Structure-Function Relations in G-Protein Coupled Receptors. *Methods in Neuroscience*. 1995; 25:366–428.
- Burger M, Burger JA, Hoch RC, Oades Z, Takamori H, Schraufstatter IU. Point mutation causing constitutive signaling of CXCR2 leads to transforming activity similar to Kaposi's sarcoma herpesvirus-G protein-coupled receptor. *J Immunol*. 1999; 163:2017–2022. [PubMed: 10438939]
- Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol*. 1973; 22:3099–3108. [PubMed: 4202581]
- Cole AG, Stroke IL, Brescia MR, Simhadri S, Zhang JJ, Hussain Z, Snider M, Haskell C, Ribeiro S, Appell KC, Henderson I, Webb ML. Identification and initial evaluation of 4-N-aryl-[1,4]diazepane ureas as potent CXCR3 antagonists. *Bioorg Med Chem Lett*. 2006; 16:200–203. [PubMed: 16213722]
- Cole KE, Strick CA, Paradis TJ, Ogborne KT, Loetscher M, Gladue RP, Lin W, Boyd JG, Moser B, Wood DE, Sahagan BG, Neote K. Interferon-inducible T cell alpha chemoattractant (I-TAC): a

- novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J Exp Med*. 1998; 187:2009–2021. [PubMed: 9625760]
- Coward P, Chan SD, Wada HG, Humphries GM, Conklin BR. Chimeric G proteins allow a high-throughput signaling assay of Gi-coupled receptors. *Anal Biochem*. 1999; 270:242–248. [PubMed: 10334841]
- Flier J, Boorsma DM, van Beek PJ, Nieboer C, Stoof TJ, Willemze R, Tensen CP. Differential expression of CXCR3 targeting chemokines CXCL10, CXCL9, and CXCL11 in different types of skin inflammation. *J Pathol*. 2001; 194:398–405. [PubMed: 11523046]
- Gao P, Zhou XY, Yashiro-Ohtani Y, Yang YF, Sugimoto N, Ono S, Nakanishi T, Obika S, Imanishi T, Egawa T, Nagasawa T, Fujiwara H, Hamaoka T. The unique target specificity of a nonpeptide chemokine receptor antagonist: selective blockade of two Th1 chemokine receptors CCR5 and CXCR3. *J Leukoc Biol*. 2003; 73:273–280. [PubMed: 12554804]
- Hancock WW, Lu B, Gao W, Csizmadia V, Faia K, King JA, Smiley ST, Ling M, Gerard NP, Gerard C. Requirement of the chemokine receptor CXCR3 for acute allograft rejection. *J Exp Med*. 2000; 192:1515–1520. [PubMed: 11085753]
- Heise CE, Pahuja A, Hudson SC, Mistry MS, Putnam AL, Gross MM, Gottlieb PA, Wade WS, Kiankarimi M, Schwarz D, Crowe P, Zlotnik A, Alleva DG. Pharmacological characterization of CXC chemokine receptor 3 ligands and a small molecule antagonist. *J Pharmacol Exp Ther*. 2005; 313:1263–1271. [PubMed: 15761110]
- Hensbergen PJ, Wijnands PG, Schreurs MW, Scheper RJ, Willemze R, Tensen CP. The CXCR3 targeting chemokine CXCL11 has potent antitumor activity in vivo involving attraction of CD8+ T lymphocytes but not inhibition of angiogenesis. *J Immunother* (1997). 2005; 28:343–351.
- Ho HH, Ganeshalingam N, Rosenhouse-Dantsker A, Osman R, Gershengorn MC. Charged residues at the intracellular boundary of transmembrane helices 2 and 3 independently affect constitutive activity of Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor. *J Biol Chem*. 2001; 276:1376–1382. [PubMed: 11031271]
- Johnson M, Li AR, Liu J, Fu Z, Zhu L, Miao S, Wang X, Xu Q, Huang A, Marcus A, Xu F, Ebsworth K, Sablan E, Danao J, Kumer J, Dairaghi D, Lawrence C, Sullivan T, Tonn G, Schall T, Collins T, Medina J. Discovery and optimization of a series of quinazolinone-derived antagonists of CXCR3. *Bioorg Med Chem Lett*. 2007; 17:3339–3343. [PubMed: 17448658]
- Jopling LA, Watt GF, Fisher S, Birch H, Coggon S, Christie MI. Analysis of the pharmacokinetic/pharmacodynamic relationship of a small molecule CXCR3 antagonist, NBI-74330, using a murine CXCR3 internalization assay. *Br J Pharmacol*. 2007
- Kenakin T. The classification of seven transmembrane receptors in recombinant expression systems. *Pharmacol Rev*. 1996; 48:413–463. [PubMed: 8888308]
- Lagane B, Ballet S, Planchenault T, Balabanian K, Le Poul E, Blanpain C, Percherancier Y, Staropoli I, Vassart G, Oppermann M, Parmentier M, Bachelier F. Mutation of the DRY motif reveals different structural requirements for the CC chemokine receptor 5-mediated signaling and receptor endocytosis. *Mol Pharmacol*. 2005; 67:1966–1976. [PubMed: 15761117]
- Loetscher M, Gerber B, Loetscher P, Jones SA, Piali L, Clark-Lewis I, Baggiolini M, Moser B. Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. *J Exp Med*. 1996; 184:963–969. [PubMed: 9064356]
- Lu B, Humbles A, Bota D, Gerard C, Moser B, Soler D, Luster AD, Gerard NP. Structure and function of the murine chemokine receptor CXCR3. *Eur J Immunol*. 1999; 29:3804–3812. [PubMed: 10556837]
- Luster AD, Leder P. IP-10, a C-X-C-chemokine, elicits a potent thymus-dependent antitumor response in vivo. *J Exp Med*. 1993; 178:1057–1065. [PubMed: 8350046]
- Mach F, Sauty A, Iarossi AS, Sukhova GK, Neote K, Libby P, Luster AD. Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. *J Clin Invest*. 1999; 104:1041–1050. [PubMed: 10525042]
- Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev*. 2000; 52:145–176. [PubMed: 10699158]

- Pan J, Burdick MD, Belperio JA, Xue YY, Gerard C, Sharma S, Dubinett SM, Strieter RM. CXCR3/CXCR3 ligand biological axis impairs RENCA tumor growth by a mechanism of immunoangiostasis. *J Immunol.* 2006; 176:1456–1464. [PubMed: 16424173]
- Parra S, Bond RA. Inverse agonism: from curiosity to accepted dogma, but is it clinically relevant? *Curr Opin Pharmacol.* 2007; 7:146–150. [PubMed: 17284360]
- Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J Clin Invest.* 1998; 101:746–754. [PubMed: 9466968]
- Rabin RL, Park MK, Liao F, Swofford R, Stephany D, Farber JM. Chemokine receptor responses on T cells are achieved through regulation of both receptor expression and signaling. *J Immunol.* 1999; 162:3840–3850. [PubMed: 10201901]
- Salomon I, Netzer N, Wildbaum G, Schiff-Zuck S, Maor G, Karin N. Targeting the function of IFN-gamma-inducible protein 10 suppresses ongoing adjuvant arthritis. *J Immunol.* 2002; 169:2685–2693. [PubMed: 12193742]
- Smit MJ, Leurs R, Alewijnse AE, Blauw J, Van Nieuw Amerongen GP, Van De Vrede Y, Roovers E, Timmerman H. Inverse agonism of histamine H2 antagonist accounts for upregulation of spontaneously active histamine H2 receptors. *Proc Natl Acad Sci U S A.* 1996; 93:6802–6807. [PubMed: 8692899]
- Smit MJ, Verdijk P, van der Raaij-Helmer EM, Navis M, Hensbergen PJ, Leurs R, Tensen CP. CXCR3-mediated chemotaxis of human T cells is regulated by a Gi-and phospholipase C-dependent pathway and not via activation of MEK/p44/p42 MAPK nor Akt/PI-3 kinase. *Blood.* 2003; 102:1959–1965. [PubMed: 12750173]
- Sorensen TL, Tani M, Jensen J, Pierce V, Lucchinetti C, Folcik VA, Qin S, Rottman J, Sellebjerg F, Strieter RM, Frederiksen JL, Ransohoff RM. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J Clin Invest.* 1999; 103:807–815. [PubMed: 10079101]
- Storelli S, Verdijk P, Verzijl D, Timmerman H, van de Stolpe AC, Tensen CP, Smit MJ, De Esch IJ, Leurs R. Synthesis and structure-activity relationship of 3-phenyl-3H-quinazolin-4-one derivatives as CXCR3 chemokine receptor antagonists. *Bioorg Med Chem Lett.* 2005; 15:2910–2913. [PubMed: 15911279]
- Storelli S, Verzijl D, Al-Badie J, Elders N, Bosch L, Timmerman H, Smit MJ, De Esch IJ, Leurs R. Synthesis and Structure-Activity Relationships of 3H-Quinazolin-4-ones and 3H-Pyrido[2,3-d]pyrimidin-4-ones as CXCR3 receptor antagonists. *Arch Pharm (Weinheim).* 2007; 340:281–291. [PubMed: 17562560]
- Streblow DN, Kreklywich C, Yin Q, De La Melena VT, Corless CL, Smith PA, Brakebill C, Cook JW, Vink C, Bruggeman CA, Nelson JA, Orloff SL. Cytomegalovirus-mediated upregulation of chemokine expression correlates with the acceleration of chronic rejection in rat heart transplants. *J Virol.* 2003; 77:2182–2194. [PubMed: 12525653]
- Walser TC, Rifat S, Ma X, Kundu N, Ward C, Goloubeva O, Johnson MG, Medina JC, Collins TL, Fulton AM. Antagonism of CXCR3 inhibits lung metastasis in a murine model of metastatic breast cancer. *Cancer Res.* 2006; 66:7701–7707. [PubMed: 16885372]
- Wang X, Li X, Schmidt DB, Foley JJ, Barone FC, Ames RS, Sarau HM. Identification and molecular characterization of rat CXCR3: receptor expression and interferon-inducible protein-10 binding are increased in focal stroke. *Mol Pharmacol.* 2000; 57:1190–1198. [PubMed: 10825390]
- Yang YF, Mukai T, Gao P, Yamaguchi N, Ono S, Iwaki H, Obika S, Imanishi T, Tsujimura T, Hamaoka T, Fujiwara H. A non-peptide CCR5 antagonist inhibits collagen-induced arthritis by modulating T cell migration without affecting anti-collagen T cell responses. *Eur J Immunol.* 2002; 32:2124–2132. [PubMed: 12209624]
- Zhang WB, Navenot JM, Haribabu B, Tamamura H, Hiramatsu K, Omagari A, Pei G, Manfredi JP, Fujii N, Broach JR, Peiper SC. A point mutation that confers constitutive activity to CXCR4 reveals that T140 is an inverse agonist and that AMD3100 and ALX40-4C are weak partial agonists. *J Biol Chem.* 2002; 277:24515–24521. [PubMed: 11923301]

Fig. 1. Structures of small non-peptidergic compounds targeting CXCR3

The structures of VUF10474 (NBI-74330; *N*-1*R*-[3-(4-ethoxy-phenyl)-4-oxo-3,4-dihydropyrido[2,3-*d*]pyrimidin-2-yl]-ethyl-*N*-pyridin-3-ylmethyl-2-(4-fluoro-3-trifluoromethyl-phenyl)-acetamide), VUF10085 (AMG-487; *N*-1*R*-[3-(4-ethoxy-phenyl)-4-oxo-3,4-dihydropyrido[2,3-*d*]pyrimidin-2-yl]-ethyl-*N*-pyridin-3-ylmethyl-2-(4-trifluoromethoxy-phenyl)-acetamide), VUF5834 (decanoic acid {1-[3-(4-cyano-phenyl)-4-oxo-3,4-dihydro-quinazolin-2-yl]-ethyl}-(2-dimethylamino-ethyl)-amide), VUF10132 (1,3-Bis-[2-(3,4-dichloro-phenyl)-2-oxo-ethyl]-3*H*-imidazol-1-ium bromide) and TAK-779 (*N,N*-dimethyl-*N*-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5*H*-benzocyclohepten-8-yl]carbonyl]amino]benzyl] tetrahydro-2*H*-pyran-4-aminium chloride) are shown.

Fig. 2. Characterization of human CXCR3

A, Homologous displacement binding at human CXCR3. Binding experiments were performed with approximately 50 pM ^{125}I -CXCL10 (○) or ^{125}I -CXCL11 (●) and increasing concentrations of homologous unlabeled chemokine on membranes from HEK293T cells transfected with cDNA encoding human CXCR3. Data are presented as percentage of total binding. pK_d values for CXCL10 and CXCL11 were 9.8 ± 0.1 (n=11) and 10.6 ± 0.1 (n=7) respectively.

B, Activation of PLC by human CXCR3. HEK293T cells were transfected with cDNA encoding human CXCR3 and $\text{G}\alpha_{\text{qi5}}$. After 48 h, ^3H -InsP accumulation was determined in the presence of increasing concentrations of CXCL10 (○) or CXCL11 (●). Data are presented as percentage of the maximal response obtained with CXCL11 (100 nM). pEC_{50} values for CXCL10 and CXCL11 were 7.5 ± 0.1 (n=8) and 8.5 ± 0.1 (n=12) respectively.

Fig. 3. Characterization of small non-peptidergic compounds at human CXCR3

A, Displacement of ^{125}I -CXCL10. Binding experiments were performed with approximately 50 pM ^{125}I -CXCL10 and increasing concentrations of VUF10472 (■), VUF10085 (□), VUF5834 (●), VUF10132 (○) or TAK-779 (▲) on membranes from HEK293T cells transfected with cDNA encoding human CXCR3. Data are presented as percentage of total binding. pK_i values were 8.4 ± 0.1 (VUF10472, $n=7$), 7.7 ± 0.0 (VUF10085, $n=18$), 6.8 ± 0.1 (VUF5834, $n=3$), 6.6 ± 0.0 (VUF10132, $n=4$) and 5.9 ± 0.0 ($n=6$).

B, Inhibition of CXCL10-induced PLC activation. HEK293T cells were transfected with cDNA encoding human CXCR3 and $\text{G}\alpha_{q/15}$. After 48 h, CXCL10-induced (10–50 nM) ^3H -InsP accumulation was determined in the presence of increasing concentrations of

VUF10472 (■), VUF10085 (□), VUF5834 (●), VUF10132 (○) or TAK-779 (▲). pK_b values were 8.2 ± 0.1 (VUF10472, $n=5$), 7.7 ± 0.1 (VUF10085, $n=9$), 7.0 ± 0.1 (VUF5834, $n=5$), 6.7 ± 0.1 (VUF10132, $n=5$) and 6.1 ± 0.1 (TAK-779, $n=4$).

C, Relationship between pK_i and pK_b of small molecule compounds. pK_b values obtained using CXCL10 were plotted against pK_i values obtained against ^{125}I -CXCL10 ($r^2 = 0.997$).

D, Inhibition of CXCL11-induced PLC activation by VUF10472. HEK293T cells were transfected with cDNA encoding human CXCR3 and $G_{\alpha_{q15}}$. After 48 h, CXCL11-induced (5 nM) ^3H -InsP accumulation was determined in the presence of increasing concentrations of VUF10472 (■). The pK_b value of VUF10472 was 7.9 ± 0.0 ($n=3$). For comparison, inhibition of CXCL10-induced (50 nM) PLC activation by VUF10472 is shown as well (□). Data are represented as percentage stimulation in the absence of antagonist. pK_b values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

E, Inhibition of CXCL10-induced chemotaxis. L1.2 cells were transfected with cDNA encoding human CXCR3. After 24 h, inhibition of CXCL10-induced (10 nM) chemotaxis was determined. pIC_{50} values were 6.8 ± 0.2 (VUF10085, $n=2$) and 5.8 ± 0.1 (VUF5834, $n=3$). Data are presented as percentage of migrated cells in the absence of antagonist. Inset, CXCL10-induced chemotaxis of L1.2 cells. Data are presented as percentage of total L1.2 cells that migrated to CXCL10 (10 nM) in the absence of antagonist ($n=3$).

Fig. 4. Non-competitive behavior of non-peptidergic compounds at human CXCR3

A, Schild analysis of VUF10472 using CXCL10 as agonist. HEK293T cells were transfected with cDNA encoding human CXCR3 and $\text{G}\alpha_{\text{qi5}}$. After 48 h, CXCL10-induced $^3\text{H-InsP}$ accumulation was determined in the absence (■) or in the presence of 10 nM (□) or 30 nM (●) VUF10472 ($n=2-3$).

B-E, Schild analysis using CXCL11 as agonist. HEK293T cells were transfected with cDNA encoding human CXCR3 and $\text{G}\alpha_{\text{qi5}}$. After 48 h, CXCL11-induced $^3\text{H-InsP}$ accumulation was determined in the absence (■) or in the presence of 10 nM (□), 30 nM (●), 100 nM (○), 1 μM (▲), 10 μM (△) or 100 μM (◆) VUF10472 (Fig. 3F, $n=2-3$), VUF5834 (Fig. 3G, $n=2$), VUF10132 (Fig. 3H, $n=2$) or TAK-779 (Fig. 3I, $n=3$). Data are presented as

percentage of chemokine-induced (100 nM) stimulation in the absence of small molecule compound.

Fig. 5. Characterization of the constitutively active mutant CXCR3 N3.35A

A, Expression of CXCR3 WT and CXCR3 N3.35A. HEK293T cells were transfected with cDNA encoding $G\alpha_{qi5}$ and increasing amounts of cDNA encoding human CXCR3 WT or CXCR3 N3.35A. Mock cells were transfected with cDNA encoding $G\alpha_{qi5}$ and pcDEF₃ empty vector. After 48 h, CXCR3 expression was determined in an ELISA assay. Data are presented as percentage signal obtained in mock-transfected cells ($n=2-3$).

B, Constitutive activity of CXCR3 N3.35A. HEK293T cells were transfected with cDNA encoding $G\alpha_{qi5}$ and increasing amounts of cDNA encoding human CXCR3 WT or CXCR3 N3.35A. Mock cells were transfected with cDNA encoding $G\alpha_{qi5}$ and pcDEF₃ empty vector. After 48 h, ³H-InsP accumulation was determined in the absence of chemokines. Data are presented as percentage ³H-InsP in mock-transfected cells ($n=3$).

C, Chemokine-induced PLC activation by CXCR3 N3.35A. HEK293T cells were transfected with cDNA encoding CXCR3 N3.35A and $G\alpha_{qi5}$. After 48 h, ³H-InsP accumulation was determined in the presence of increasing concentrations CXCL10 or CXCL11. pEC₅₀ values for CXCL10 and CXCL11 were 7.5 ± 0.0 ($n=2$) and 8.7 ± 0.1 ($n=4$) respectively. Data are presented as percentage of the maximal response obtained with CXCL11 (100 nM).

D, Inverse agonism at CXCR3 N3.35A. HEK293T cells were transfected with cDNA encoding CXCR3 N3.35A and $G\alpha_{qi5}$. After 48 h, ³H-InsP accumulation was determined in the presence of increasing concentrations VUF10472 (■), VUF10085 (□), VUF5834 (●), VUF10132 (○) or TAK-779 (▲). pIC₅₀ values ($n=3$) were 8.1 ± 0.1 (VUF10472), 8.0 ± 0.1

(VUF10085), 7.3 ± 0.0 (VUF5834), 6.6 ± 0.1 (VUF10132) and 5.9 ± 0.1 (TAK-779). Data are presented as percentage of CXCR3 N3.35A-mediated constitutive ^3H -InsP accumulation.

Fig. 6. Snakeplot of CXCR3

Amino acids that were mutated in order to generate constitutively active mutants (T2.56, N3.35 and D3.49) are indicated as bold circled residues. Amino acids that differ between human and rhesus macaque CXCR3 (T⁸³ in intracellular loop 1 and V6.39) are both Ala in rhesus macaque CXCR3) are shown as grey circles. Residues which are different between primate and rodent (i.e. rat and/or mouse) CXCR3 are indicated as black circles. Human and rhesus macaque CXCR3 have an additional Cys (C³⁷) in their amino-terminus compared to rat and mouse CXCR3.

Fig. 7. Activation of PLC by primate and rodent CXCR3

A, CXCL10-induced activation of PLC. HEK293T cells were transfected with cDNA encoding human, rhesus, rat or mouse CXCR3 and $G_{\alpha_{q15}}$. After 48 h, ^3H -InsP accumulation was determined in the presence of increasing concentrations CXCL10. pEC_{50} values for CXCL10 were 7.5 ± 0.1 (human CXCR3, $n=8$), 7.4 ± 0.1 (rhesus CXCR3, $n=3$), 8.8 ± 0.1 (rat CXCR3, $n=3$) and 8.3 ± 0.1 (mouse CXCR3, $n=5$).

B, CXCL11-induced activation of PLC. HEK293T cells were transfected with cDNA encoding human, rhesus, rat or mouse CXCR3 and $G_{\alpha_{q15}}$. After 48 h, ^3H -InsP accumulation was determined in the presence of increasing concentrations CXCL11. pEC_{50} values for CXCL11 were 8.5 ± 0.1 (human CXCR3, $n=12$), 8.5 ± 0.1 (rhesus CXCR3, $n=3$), 9.5 ± 0.1 (rat CXCR3, $n=3$) and 9.2 ± 0.2 (mouse CXCR3, $n=3$). Data are presented per receptor as percentage of ^3H -InsP accumulation in the absence of chemokine.

Properties of small non-peptidergic compounds at CXCR3

Table 2

pK_i values $\pm$ SEM were generated in heterologous radioligand binding experiments using ^{125}I -CXCL10. pK_b values were obtained at human CXCR3 with CXCL10 as agonist in the PLC activation assay and pIC_{50} values were generated using CXCR3 N3.35A in the PLC activation assay.

compound	pK _i										pK _b		pIC ₅₀	
	human	n	N3.35A	n	rhesus	n	rat	n	mouse	n	human	n	N3.35A	n
VUF10472	8.4 ± 0.1	7	7.9 ± 0.2	3	8.4 ± 0.1	3	7.8 ± 0.0	3	7.9 ± 0.1	3	8.2 ± 0.1	5	8.1 ± 0.1	3
VUF10085	7.7 ± 0.0	18	7.3 ± 0.1	3	7.5 ± 0.0	3	6.9 ± 0.1	10	7.0 ± 0.1	3	7.7 ± 0.1	9	8.0 ± 0.1	3
VUF5834	6.8 ± 0.1	3	6.4 ± 0.0	3	6.8 ± 0.0	3	6.2 ± 0.1	6	6.2 ± 0.1	3	7.0 ± 0.1	5	7.3 ± 0.0	3
VUF10132	6.6 ± 0.0	4	5.9 ± 0.1	3	6.6 ± 0.1	3	6.0 ± 0.1	6	6.0 ± 0.1	3	6.7 ± 0.1	5	6.6 ± 0.1	3
TAK-779	5.9 ± 0.0	6	5.9 ± 0.0	3	5.9 ± 0.1	3	5.8 ± 0.0	6	5.9 ± 0.0	3	6.1 ± 0.1	4	5.9 ± 0.1	3