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Title Page

Pharmacological Characterization of [^{3}H]-VUF11211, a Novel Radiolabeled Small-Molecule Inverse Agonist for the Chemokine Receptor CXCR3

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Running Title Page

[^{3}H]-VUF11211 as allosteric radioligand for CXCR3

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Nonstandard abbreviations:

AMG487, (R)-N-(1-(3-(4-ethoxyphenyl)-4-oxo-3,4-dihydropyrido[2,3-d]pyrimidin-2-yl)ethyl)-N-(pyridin-3-ylmethyl)-2-(4-(trifluoromethoxy)phenyl)acetamide; BSA, bovine serum albumin; CCR, CC chemokine receptor; CXCL, CXC chemokine ligand; CXCR, CXC chemokine receptor; GPCR, G protein-coupled receptor; DMEM, Dulbecco's modified Eagle medium; EL, extracellular loop; FBS, fetal bovine serum; GTPγS, guanosine 5γ-O-(3-thio)triphosphate; HEK293T, human embryonic kidney 293T cells; maraviroc, 4,4-difluoro-cyclohexanecarboxylic acid {(S)-3 [(1S,3S,5R)-3-(3-isopropyl- 5-methyl-[1,2,4]triazol-4-yl)-8-aza-bicyclo[3.2.1]oct-8-yl]-1-phenyl-propyl}-amide; NBI-74330, (R)-N-(1-(3-(4-ethoxyphenyl)-4-oxo-3,4-dihydropyrido[2,3-d]pyrimidin-2-yl)ethyl)-2-(4-fluoro-3-(trifluoromethyl)phenyl)-N-(pyridin-3-ylmethyl)acetamide; PBS, phosphate-buffered saline; PEI, polyethylene immine; RT, residence time; SAR, structure-activity relationship; SCH-527123, (R)-2-Hydroxy-N,N-dimethyl-3-((2-((1-(5-methylfuran-2-yl)propyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)benzamide; TM, transmembrane; TMS, transmembrane site; VUF11211, (S)-5-Chloro-6-(4-(1-(4-chlorobenzyl)piperidin-4-yl)-3-ethylpiperazin-1-yl)-N-ethylnicotinamide; WT, wild type;

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Abstract

Chemokine receptor CXCR3 has attracted much attention, as it is thought to be associated with a wide range of immune-related diseases. As such, several small molecules with different chemical structures targeting CXCR3 have been discovered. Despite limited clinical success so far, these compounds serve as interesting tools to interrogate receptor activation and antagonism. Accumulating evidence suggests that many of these compounds are allosteric modulators for CXCR3. One feature of allosteric ligands is that the magnitude of the mediated allosteric effect is dependent on the used orthosteric probe. Consequently, there is a risk for incorrect assessment of affinity for allosteric modulators with orthosteric radioligands, which has so far been the most applied approach for chemokine receptors. Therefore, we aimed to use a small-molecule allosteric ligand from the piperazinyl-piperidine class, also known as VUF11211. VUF11211 acts as an inverse agonist at a constitutively active mutant of CXCR3. Radiolabeling of VUF11211 gave [^{3}H]-VUF11211, which in radioligand binding studies shows high affinity for CXCR3 (K_d = 0.65 nM), and reasonably fast association (k_{on}= 0.03 min^{-1}nM^{-1}) and dissociation kinetics (k_{off}= 0.02 min^{-1}). The application of the [^{3}H]-VUF11211 to assess CXCR3 pharmacology was validated with diverse classes of CXCR3 compounds, including both antagonists and agonists as well as VUF11211 analogs. Interestingly, VUF11211 seems to bind to a different population of CXCR3 conformations compared to the CXCR3 agonists CXCL11, VUF11418, and VUF10661. Altogether, this allosteric inverse agonist radioligand for CXCR3 may facilitate the discovery, characterization, and optimization of allosteric modulators for the chemokine receptor CXCR3.

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Introduction

Discovery of small-molecule ligands targeting chemokine receptors, belonging to the family of G protein-coupled receptors (GPCRs), has been the focus of many laboratories in the past decade, resulting in a variety of chemokine receptor agonists and, mostly, antagonists (Wijtmans *et al.*, 2012a). Such molecules tend to bind in an allosteric fashion (Scholten *et al.*, 2012a) and to modulate the binding of the large peptide chemokines to their respective GPCRs via interaction with a transmembrane (TM) domain binding pocket. A key example of such a molecule is the CCR5 antagonist maraviroc, which is currently being marketed as Selzentry® and used in combination treatment of HIV-1 infection. This molecule shows non-competitive allosteric behavior towards CCR5 chemokines in binding experiments (Watson *et al.*, 2005). Maraviroc binds to the TM domains (Tan *et al.*, 2013), whereas chemokines bind predominantly to the external parts of the receptor, including extracellular loops (ELs) and the CCR5 N-terminus (Garcia-Perez et al., 2011; Grunbeck et al., 2012; Tan et al., 2013).

For many other chemokine receptors the quest for small-molecule binders has also been successful. In view of the potential therapeutic interest in CXCR3 blockade in diseases like rheumatoid arthritis and allograft rejection (Wijtmans et al., 2011), many different drug discovery programs have yielded several distinct chemical classes of small-molecule compounds, including antagonists as well as a few agonists (Wijtmans et al., 2008; 2011). These include the 8-azaquinazolinone compounds from Amgen (AMG487) and Neurocrine Biosciences (NBI-74330), which bind to CXCR3 with affinities in the nanomolar range (Heise et al., 2005; Johnson et al., 2007; Verzijl et al., 2008). Moreover, a piperazinyl-piperidine compound class containing ligands with nanomolar CXCR3 affinities was reported by Schering Plough (now Merck Sharp & Dohme) (Mcguinness et al., 2009; Shao et al., 2011; Kim et al., 2011; Nair et al., 2014).

Despite the interest in small-molecule ligands for CXCR3, information regarding their specific interaction with the receptor at a molecular level remains limited. Fortunately, understanding of these interactions has started to emerge in the past years (Scholten et al., 2012b; Bernat et al., 2012; Nedjai et al., 2012; Scholten et al., 2014). In a recent study (Scholten et al., 2014) we reported on the binding mode of two CXCR3 ligands originating from the 8-azaquinazolinone class (NBI-74330) and the piperazinyl-piperidine class (VUF11211) using site-directed mutagenesis in conjunction with *in silico*

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modeling of CXCR3. We showed that NBI-74330 mainly binds to Transmembrane Site 1 (TMS1), between TM domains 2, 3, and 7, whereas VUF11211 binds both TMS1 and TMS2 (TM domains 3-7). With the availability of highly potent CXCR3 antagonists, there is an increasing opportunity to develop small-molecule radioligands. Radiolabeled chemokines are still predominantly used for receptor-ligand interaction studies. Since these radioligands are orthosteric ligands, they potentially limit the full pharmacological characterization of allosteric small molecules, and might also result in failure to discover other allosteric ligands. A radiolabeled allosteric ligand would facilitate not only more accurate measurements of affinity for allosteric ligands, but also the discovery of other classes of ligands by screening compound libraries (May et al., 2007). Recently, Bernat and colleagues have shown with racemic [^{3}H]-RAMX3 that a small-molecule radioligand for CXCR3 can be developed for use in radioligand binding assays (Bernat et al., 2012). In our studies we report on a novel CXCR3 radioligand ([^{3}H]-VUF11211, figure 1) of a different chemotype (piperazinyl piperidine) and characterize this ligand in various types of radioligand binding assays. Moreover, we also evaluated the pharmacological profile of unlabeled VUF11211 in more detail.

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Materials and methods

Materials. Dulbecco's modified Eagle's medium and trypsin were purchased from Sigma Aldrich (St. Louis, MO, USA), penicillin and streptomycin were obtained from Lonza (Verviers, Belgium), fetal bovine serum (FBS) was purchased from PAA Laboratories GmbH (Pasching, Austria), [^{125}I]-CXCL11 (±1000 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). Unlabeled chemokines were purchased from PeproTech (Rocky Hill, NJ, USA). Polyethyleneimmine linear 25 kDa for transfection was obtained from Polysciences (Warrington, PA, USA). Unless stated otherwise, all other chemicals were obtained from Sigma Aldrich.

CXCR3 ligands. VUF11211 (compound **18i** in (Shao et al., 2011)) was prepared in enantiopure form in our laboratory as described previously (Scholten et al., 2014). [^{3}H]-VUF11211 was prepared from VUF11211 (Supplementary Information Fig. S1) and analyzed (Supplementary Information Fig. S3, S4, S5) by PerkinElmer Health Sciences (38.4 Ci/mmol). VUF14479, VUF14263 and VUF13948 were synthesized (Supplementary Information Fig. S2) and analyzed (Supplementary Information Fig. S6-S14) in our laboratory.

DNA Constructs. The cyclic AMP response element-luciferase (CRE-Luc) reporter gene construct was a generous gift from Dr. W. Born (National Jewish Medical and Research Center, Denver, CO., USA). The DNA coding for human CXCR3 in pcDEF$_3$ was described in a previous report (Verzijl et al., 2008). β-arrestin2-enhanced yellow fluorescent protein (eYFP) and CXCR3-Renilla luciferase (RLuc) fusion constructs have been described previously (Scholten et al., 2012b).

Cell Culture and Transfection. HEK293 cells stably expressing CXCR3 were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin, streptomycin, and 400 µg/ml G418. HEK293T cells were grown at the same conditions, with the exception of G418 selection. Transfection was performed by polyethyleneimmine (PEI) as DNA carrier agent. 8 µg of DNA per 4*10^6 cells was combined with 48 µg PEI in a total volume of 500 µl 150 mM NaCl and incubated for 10 min at room temperature. Subsequently, the DNA/PEI mix was added to a suspension of HEK293T cells in full growth medium (0.5*10^6 cells/ml) and subsequently plated out in a 96-well plate and cultured over night at standard culture conditions.

CRE-Luciferase reportergene assay. pcDEF$_3$-CXCR3, CRE-Luc reportergene DNA, and empty plasmid DNA were transfected with a weight ratio of 1:3:4. 24 hrs after transfection the ligands were added to the cells (homogenous format) and incubated for 6 hrs at standard growth conditions.

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Subsequently, the medium was replaced by 25 µl substrate buffer (39mM Tris-H_3PO_4 pH 7.8, 39% glycerol, 2.6% Triton X-100, 0.9 mM dithiothreitol, 18 mM $MgCl_2$, 0.8 mM ATP, 77 µM disodium pyrophosphate, 230 µg/mL beetle luciferin) and luminescence was measured after 30 min with a Victor^3 plate reader (PerkinElmer, Boston, MA, USA).

β-arrestin recruitment assay. pcDEF$_3$-CXCR3-Rluc and pcDEF$_3$-β-arrestin-eYFP were transfected in a ratio of 1:4. The next day, medium was aspirated and cells were washed with Hank's Balanced Salt Solution (HBSS). Next, cells were incubated in HBSS with or without antagonist 30 min prior to agonist addition. To assess BRET, agonists were added 10 min after incubation with coelenterazine-h, and incubated for an additional 10 min before measurements on the Victor3. BRET ratios were calculated and net BRET signals were determined as described previously (Scholten et al., 2012b).

Membrane Preparation. Membrane preparation of HEK293 cells stably expressing CXCR3 receptors was performed as described previously (Verzijl et al., 2008). In brief, the cells were washed with ice-cold PBS and collected in tubes and centrifuged at 1500*g* for 10 min. The cells were resuspended in ice-cold membrane buffer (15 mM Tris, pH 7.5, 1 mM EGTA, 0.3 mM EDTA, and 2 mM $MgCl_2$), and subsequently homogenized with a Teflon-glass homogenizer and rotor. Next, the membranes were subjected to two freeze-thaw cycles using liquid nitrogen and centrifuged at 40,000 *g* for 25 min. The resulting pellet was resuspended in Tris-sucrose buffer (20 mM Tris, 250 mM sucrose, pH 7.4) and stored at -80°C for future radioligand binding experiments.

[^{125}I]-CXCL11 binding assay. 2 µg of HEK293/CXCR3 membranes were used per well in 96-well clear plates (Greiner Bio One, Alphen a/d Rijn, the Netherlands). For displacement binding experiments, membranes were incubated in chemokine binding buffer (50 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 100 mM NaCl, and 0.5% (w/v) BSA fraction V) with approximately 70 pM of [^{125}I]-CXCL11 and a concentration range of cold CXCL11, VUF11211 or NBI-74330 for 2 h at room temperature. Next, the membranes were harvested by filtration through Unifilter 96-well GF/C plates (Perkin–Elmer) presoaked with 0.5% PEI, using ice-cold wash buffer (chemokine binding buffer supplemented with 0.5M of NaCl). Bound radioactivity was determined with a MicroBeta scintillation counter using a ^{125}I protocol (PerkinElmer).

[^{3}H]-VUF11211 binding assay. 2 µg of HEK293/CXCR3 membranes were used per well in 96-well clear plates and incubated with varying amounts of [^{3}H]-VUF11211 depending on the assay type (see respective figures and legends). All ligands and membranes were prepared in binding buffer (50 mM

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Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% (w/v) Tween80, 0.1% (w/v) BSA fraction V. In competition binding experiments four different concentrations of radioligand were each combined with a concentration series of displacer ligand. The affinity value for the cold ligand was determined using global fitting of the four different curves. In the heterologous competition binding assays 1 nM of [^{3}H]-VUF11211 was coincubated with a concentration series of different cold ligands. In saturation binding experiments, increasing concentrations of [^{3}H]-VUF11211 were used and NBI-74330 was used to determine non-specific binding levels. All equilibrium binding assay incubations were performed for 1 hr. Subsequently, the membranes were collected by rapid filtration over 96-well GF/C filter plates presoaked in 0.5% w/v BSA/H_2O, and subsequent washing with ice-cold wash buffer (50 mM Tris-HCl, pH 7.4, 0.5 M NaCl). Bound radioactivity was determined with the MicroBeta scintillation counter using a tritium protocol.

Data analysis. Prism v6.0b from GraphPad software was used to plot and analyze the data. Curve fitting was performed using non-linear regression in the software. The following equations were used to fit and analyze the data. Association kinetics with global fitting of data using three different concentrations of radioligand: $Y=([L]/([L]+K_d)*Bmax)*(1-e^{(-1*(Kon*[L]+Koff)*X)})$ where [L] is the concentration of radioligand in nM. Dissociation kinetics with one-phase exponential decay: $Y=(Y0-NS)*e^{(-K*X)} + NS$. Y0 is the Y value at t=0, NS is the minimum binding plateau reached after longer incubations, in units of Y. K is the rate constant of dissociation (inverse units of X). Saturation binding: $Y=B_{max}*X/(K_d + X)$, where X depicts increasing concentrations of [^{3}H]-VUF11211. K_d is the equilibrium dissociation constant in units of X. B_{max} is maximum binding in units of Y. One site homologous competition binding: $Y = (B_{max}*[L])/([L]+10^{(X+9)}+10^{(logKd+9)}) + NS*[L]$, where [L] is the radioligand concentration in nM and NS is nonspecific binding in units of Y. Heterologous competition binding: $Y=Bottom+(Top-Bottom)/(1+10^{(X-log(10^{logKi*(1+[L]/Kd)}))})$, where [L] is the radioligand concentration in nM and K_d is the affinity value for the radioligand in nM (determined using homologous competition binding). Top and bottom are both in units of Y. Although distinct classes of allosteric ligands might bind differently to CXCR3 compared to the used radioligands, the observation that these ligands completely displace the radioligand indicates strong negative cooperativity. Therefore, using competitive analysis for affinity determination is justified.

For statistical analysis, one-way ANOVA with a Bonferroni post-test, included in the GraphPad Prism software, was used with a confidence interval of 99%.

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Results

VUF11211 pharmacology

First, we used functional readouts to further characterize the CXCR3 antagonist VUF11211, reported by us to bind the TM region of the CXCR3 receptor and to traverse the TMS1 and TMS2 binding pockets (Scholten et al., 2014). A cAMP response element (CRE)-luciferase reporter gene assay was employed to measure CXCR3 receptor activity (Wijtmans et al., 2012b). As expected, CXCL11 acted as an agonist in this assay with a pEC_{50} of 8.9 ± 0.1 (figure 2a). Next, an EC_{80} concentration of CXCL11 (3 nM) and a concentration series of VUF11211 were co-incubated, resulting in dose-dependent antagonism of CXCL11-induced receptor activity by VUF11211 (pIC_{50} = 7.5 ± 0.1) (figure 2a). Interestingly, high concentrations of VUF11211 (>1 μM) even decreased receptor activity below basal levels, which might suggest inverse agonism and constitutive activity of CXCR3 wildtype (WT) in this assay. Hence, a concentration series of VUF11211 was applied to CXCR3 WT and to the constitutively active mutant CXCR3-N3.35A (Verzijl et al., 2008). From this experiment it follows that VUF11211 inhibited basal receptor signaling at the N3.35A mutant (pIC_{50} = 8.4 ± 0.2), and to a much lesser extent at CXCR3 WT (figure 2b). To further probe the mechanism of action of VUF11211, the compound was investigated in a Schild Analysis approach. A concentration range of CXCL11 was co-incubated with different constant concentrations of VUF11211. Increasing concentrations of VUF11211 insurmountably suppressed the maximal response elicited by CXCL11 in this assay, and concomitantly decreased its potency (figure 2c).

VUF11211 was also studied in the context of β-arrestin recruitment towards CXCR3 (figure 2d). While CXCL11 was an agonist in this assay (pEC_{50} = 8.0 ± 0.1), as reported before (Scholten et al., 2012b), VUF11211 was able to inhibit the CXCL11-mediated β-arrestin recruitment to CXCR3 with high potency (pIC_{50} = 8.3 ± 0.1). Moreover, VUF11211 did not induce β-arrestin recruitment on its own (figure 2d).

Radiolabeling of VUF11211

As VUF11211 had proven a reliable and high-affinity tool compound (McGuinness et al., 2009; Scholten et al., 2014) and showed good potency in antagonizing CXCL11 function (confirmed by the CRE assays), we decided to explore its use as radiolabel. Several synthetic approaches in which the tritium was introduced during the existing synthesis of enantiopure VUF11211 (McGuinness et al.,

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2009; Scholten et al., 2014) were considered and some subjected to initial unlabeled trials, but all were eventually not deemed attractive due to lack of required ^{3}H-reagents or because the conceived labeling reaction could in theory erode the enantiomeric excess. Eventually, we settled on outsourcing the radiolabeling of enantiopure VUF11211 itself to PerkinElmer Health Sciences. Its proprietary technology delivered [^{3}H]-VUF11211 with a specific activity of 38.4 Ci/mmol and 99.6% radiochemical purity (Supplementary Information Fig. S1, S3-S5). Mass spectrometry (MS) analysis revealed the incorporation of, on average, two ^{3}H-atoms per molecule VUF11211.

Assay development for [^{3}H]-VUF11211 binding to human CXCR3

First we investigated whether CXCR3 could be detected by the radiolabeled VUF11211. Significantly more radioligand binding was observed when HEK293/CXCR3 membranes were incubated with the radioligand compared with membranes lacking CXCR3 expression (figure 3a). Moreover, co-incubation of 1 nM of [^{3}H]-VUF11211 with a saturating concentration of NBI-74330 (10 µM) led to radioligand binding levels comparable to non-specific binding as detected in membranes without CXCR3 receptors (figure 3a). As expected, specific [^{3}H]-VUF11211 binding was proportional to the amount of membranes, whereas non-specific [^{3}H]-VUF11211 binding remained largely unchanged when membrane amounts were increased, suggesting that non-specific binding is mostly dependent on constituents other than membranes. 3 µg/well of HEK293/CXCR3 membranes was selected for further [^{3}H]-VUF11211 binding experiments.

Preliminary experiments (not shown) revealed lower [^{3}H]-VUF11211 concentrations in the assay than would be expected from calculations based on the properties of the radioligand. This suggests loss of radioligand, by binding to e.g. plastics. Therefore, different agents were considered to decrease these potential non-specific binding issues (figure 3b). As can be seen in figure 3b, a combination of 0.1% w/v bovine serum albumin (BSA) and 0.1% w/v Tween80 resulted in the highest specific binding window (± 6-fold over non-specific binding), hence this combination was selected as condition for future binding experiments with [^{3}H]-VUF11211. Under these conditions the bound radioligand fraction did not exceed 10%, thereby avoiding ligand depletion. In general, the filterplate used to harvest the membranes is pre-incubated with polyethyleneimmine (PEI), to counteract non-specific binding to glass fibers. In our study either 0.5% w/v PEI or BSA were compared, and the latter resulted in significantly lower non-specific binding counts (roughly 50% lower) for a range of radioligand

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concentrations (figure 3c). Consequently, BSA was selected as an agent to counteract non-specific binding in future experiments.

Characterization of [^{3}H]-VUF11211 binding to human CXCR3

With the optimal conditions at hand, the radioligand was subjected to several types of experiments to determine its pharmacological properties. Firstly, an association kinetic experiment was performed to establish how fast the radioligand binding to CXCR3 would reach an equilibrium state (on-rate; k_{on}). The association of a radioligand is determined by both its on- and off-rate (k_{on} and k_{off}) but also by its concentration, and is described by the following equation: $k_{obs} = k_{on}[L] + k_{off}$, where k_{obs} is the observed association constant k, and [L] is the radioligand concentration. By using different [^{3}H]-VUF11211 concentrations, the kinetics of binding to CXCR3 could be fitted using global fitting, resulting in a k_{on} of 0.034 ± 0.002 min^{-1} nM^{-1}, and k_{off} of 0.022 ± 0.004 min^{-1} (figure 4a and Table 1). Since the equilibrium affinity constant K_d is defined as k_{off}/k_{on}, the affinity of [^{3}H]-VUF11211 for CXCR3 binding could be calculated with the obtained kinetic parameters and is 0.69 ± 0.10 nM. Next, the k_{off} of [^{3}H]-VUF11211 binding was also determined by a dissociation kinetic binding assay. HEK293/CXCR3 membranes were incubated with 1 nM of [^{3}H]-VUF11211 for 1 hr, allowing it to reach equilibrium, and subsequently an excess of unlabeled VUF11211 (10 µM) was added at different time points to compete with the radioligand for CXCR3 binding, preventing dissociated [^{3}H]-VUF11211 to reassociate with the receptor. Using the k_{on} for [^{3}H]-VUF11211 obtained by the the association kinetic experiments, the k_{off} could be determined, yielding a k_{off} for [^{3}H]-VUF11211 of 0.020 ± 0.001 with an associated target residence time (RT) of approximately 50 min (figure 4b and table 1). The target residence time is a measure of how long a molecule resides on the receptor and is calculated by taking the reciprocal of the k_{off} value.

Next, a [^{3}H]-VUF11211 saturation binding (figure 4c) to CXCR3 was performed by incubating increasing concentrations of radioligand with HEK293/CXCR3 membranes in the presence or absence of 10 µM of NBI-74330 (to determine non-specific binding levels) for 2 hours. The assay revealed for [^{3}H]-VUF11211 an equilibrium affinity constant (K_d) of 0.65 ± 0.11 nM for [^{3}H]-VUF11211 (table 1), with a B_{max} value of 8.0 ± 1.0 pmol/mg protein.

In general, competition binding experiments are employed to determine the affinities of unlabeled compounds of different chemotypes (or analogs) without the need of radiolabeling each compound. As

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a test, homologous competition binding experiments were performed using 4 different concentrations (0.3, 1, 1.5, and 3 nM) of radioligand in the range of its K_d value (figure 4d). For each [^{3}H]-VUF11211 concentration a series of concentrations of unlabelled VUF11211 was incubated with HEK293/CXCR3 membranes to determine a pK_d value using global fitting of the data. The resulting pK_d for VUF11211 is 0.60 ± 0.16 nM with a B_{max} value of 7.9 ± 0.4 pmol/mg protein.

Displacement of [^{3}H]-VUF11211 binding to human CXCR3 with varying ligand chemotypes

A range of compounds with varying chemotypes and varying functional activities was tested to further characterize [^{3}H]-VUF11211 binding to HEK293/CXCR3 membranes (Figure 5A, Tables 2,3). This included CXCR3 antagonists (unlabeled VUF11211, NBI-74330 and VUF10085 (Verzijl et al., 2008), VUF10990 (Wijtmans et al., 2012b)) as well as CXCR3 agonists VUF10661 (Scholten et al., 2012b) and VUF11418 (Wijtmans et al., 2012b). In the case of VUF11211 and NBI-74330, pK_i values recorded with [^{125}I]-CXCL11 were significantly lower than those recorded with [^{3}H]-VUF11211, while for agonist VUF10661 the opposite was observed. The affinity of VUF10661 determined using [^{3}H]-VUF11211 ($pK_i \leq 4.5$) was at least 30 fold lower than in the case of [^{125}I]-CXCL11 (pK_i = 6.0 ± 0.1) (Figure 5B, Table 2).

To further substantiate the use of our radioligand in the context of structure-activity relationships (SAR), a few derivatives of the VUF11211 class were prepared as a proof of concept. All these derivatives originated from a common enantiopure precursor, for which the synthesis and stereochemical purity were previously disclosed by us as part of the synthesis of VUF11211 (Scholten et al., 2014). Table 3 shows exemplary compounds of this chemotype: amide VUF14479, α-methyl derivative VUF14263 and bromo-analogue VUF13948 (see Supplementary Information Fig. S2, S6-S14 for synthesis routes and chemical analyses). These three compounds display a diversity of affinities as measured using [^{3}H]-VUF11211 (from pK_i=7.7 to 9.0).

Different types of ligands, ranging from inverse agonists to agonists, might recognize different subsets of CXCR3 populations (Cox et al., 2001; Scholten et al., 2012b). For example, agonist binding to GPCRs is often dependent on G protein precoupling. The use of a non-hydrolyzable form of GTP, known as GTPγS, has been used previously to uncouple GPCRs (including CXCR3) from its G protein, leading to loss in agonist binding (Cox et al., 2001; Nijmeijer et al., 2010). As such, we set out to characterize the properties of [^{3}H]-VUF11211 binding to CXCR3 in this context. Different

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concentrations of GTPγS, ranging from 1 µM to 100 µM did not affect the total binding of [^{3}H]-VUF11211 to HEK293/CXCR3 membranes (Figure 5C), while applying 25 µM GTPγS resulted in a decrease of roughly 50% of total binding of [^{125}I]-CXCL11 binding to the same membranes (Figure 5D).

In addition, we performed a competition binding assay with HEK293/CXCR3 membranes and [^{125}I]-CXCL11 as the radioligand and unlabeled VUF11211 as the displacer and vice versa (Figure 5E). Interestingly, unlabelled VUF11211 was able to completely prevent [^{125}I]-CXCL11 binding to CXCR3, whereas in the opposite case saturating concentrations of CXCL11 only led to a maximum decrease of ±30% in [^{3}H]-VUF11211 binding (1 nM) to CXCR3 (Figure 5E).

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Discussion

The present study describes the pharmacological characterization of the (radiolabeled) small-molecule CXCR3 antagonist VUF11211 from the piperazinyl-piperidine class, which contains many compounds possessing (sub)nanomolar affinities for CXCR3 (McGuinness et al., 2009; Shao et al., 2011; Kim et al., 2011; Nair et al., 2014). VUF11211 showed antagonistic behavior in CRE reportergene- and β-arrestin recruitment assays where it completely blocked CXCL11-induced receptor activation (figure 2a and 2d) with good to high potency (pIC_{50} = 7.5 ± 0.1 and 8.3 ± 0.1, respectively). Interestingly, VUF11211 tends to inhibit basal signaling of CXCR3 WT to a small extent in the CRE-luciferase reportergene assay (fig.2). Therefore, the constitutively active mutant CXCR3-N3.35A was considered (Verzijl et al., 2008). Basal activity of CXCR3-N3.35A was significantly higher than for CXCR3 WT, confirming its constitutive activity (fig. 2) (Verzijl et al., 2008). Furthermore, VUF11211 dose-dependently decreases the constitutive receptor activity of CXCR3-N3.35A (fig.2b). These findings are in line with the earlier reported inverse agonistic properties of NBI-74330 and related compound VUF10085 (AMG487) from the 8-azaquinazolinone class. These compounds both inhibit constitutive activity of CXCR3-N3.35A in an inositol phosphate accumulation assay (Verzijl et al., 2008). VUF11211 inhibited basal activity of CXCR3 WT only to a minor extent, consistent with its very low constitutive activity compared to CXCR3-N3.35A. In most assay systems, constitutive activity of CXCR3 WT is not observed at all (Verzijl et al., 2008), yet a reportergene assay involves considerably more signal amplification than upstream assays like [^{35}S]-GTPγS or inositol phosphates accumulation, potentially unmasking the presence of low levels of constitutive activity of the CXCR3 WT.

Increasing evidence supports the allosteric nature of small-molecule ligands interacting with the CXCR3 receptor (Scholten et al., 2012b; Nedjai et al., 2012; Scholten et al., 2014; Nedjai et al., 2014). Recently, we reported evidence for an allosteric binding mechanism for VUF11211 and NBI-74330 (Scholten et al., 2014). Mutations in the TM domains of CXCR3 affected binding of these ligands, but not of the orthosteric chemokine ligand CXCL11. In addition, we assessed the mechanism of inhibition by VUF11211 by means of Schild Analysis using the CRE-luciferase reportergene assay. VUF11211 dose-dependently and insurmountably decreased the maximum efficacy of CXCL11, again suggesting non-competitive allosteric behavior.

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The allosteric properties of these ligands imposes limitations on the use of orthosteric radioligands for discovery and characterization of such molecules due to probe-dependence of allosteric effects (May et al., 2007). Therefore, we developed the radiolabeled enantiopure allosteric ligand [^{3}H]-VUF11211. Studies with [^{3}H]-VUF11211 are complementary to studies with the CXCR3 radioligand [^{3}H]-RAMX3 (Bernat et al., 2012), a derivative of NBI-74330, as both probe different parts of the TM domains of CXCR3. NBI-74330 predominantly binds to TMS1 while VUF11211 traverses both TMS1 and TMS2 (Scholten et al., 2014).

Kinetic radioligand binding experiments were employed to establish the kinetics of [^{3}H]-VUF11211 binding to CXCR3. The association rate of [^{3}H]-VUF11211 binding to CXCR3. was in the same range as reported for the 8-azaquinazolinone CXCR3 radioligand [^{3}H]-RAMX3 (k_{on} = 0.034 ± 0.002 nM^{-1} min^{-1} at 22°C vs. 0.045 ± 0.003 nM^{-1} min^{-1} at 37°C, respectively) (Bernat et al., 2012). However, it should be noted that kinetic parameters are dependent on temperature and as binding assays with [^{3}H]-RAMX3 were performed at 37°C, it might be expected that it would associate slower when incubated at 22°C. Another parameter that can be obtained from these data is the residence time of a ligand, which is inversely correlated with the dissociation rate (k_{off}). Dissociation kinetic binding experiments revealed a relatively short residence time for [^{3}H]-VUF11211 (RT ≈ 50 min) compared to other small-molecule chemokine receptor ligands, including SCH-527123 (CXCR2; RT ≈ 22 hr at 22°C) or maraviroc (CCR5; RT >136 hr at 4°C) (Guo et al., 2014).

Both types of kinetic experiments resulted in similar dissociation rate constants for [^{3}H]-VUF11211 (k_{off} = 0.022 ± 0.004 and 0.020 ± 0.001 min^{-1}, respectively) (figure 4A). Moreover, the affinity of [^{3}H]-VUF11211 for CXCR3 determined with both equilibrium saturation binding and association kinetic binding experiments are virtually identical (K_d = 0.65 ± 0.11 nM and 0.69 ± 0.10 nM, respectively), confirming that adequate assay conditions were applied (Hulme and Trevethick, 2010). In addition, the binding of [^{3}H]-VUF11211 to HEK293/CXCR3 membranes is saturable, and the amount of receptors recognized by the radioligand (B_{max}) is 8.0 ± 1.0 pmol/mg protein. However, the utility of saturation binding assays is limited to ligands that are radiolabeled. As such, the K_d and B_{max} were also established using a competition binding assay with unlabeled VUF11211 which resulted in comparable values (K_d = 0.65 ± 0.11 vs. 0.60 ± 0.16 nM and B_{max} = 8.0 ± 1.0 vs. 7.9 ± 0.4 pmol/mg protein, respectively), validating the use of [^{3}H]-VUF11211 competition binding for affinity determination.

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The affinities of different CXCR3 ligands, including agonists (VUF10661, VUF11418) and antagonists (VUF10085, NBI-74330, VUF10990), were determined by [^{3}H]-VUF11211 and [^{125}I]-CXCL11 competition binding assays (fig. 5a,b and table 2). The inverse agonists VUF11211 and NBI-74330 show lower affinities for CXCR3 in the case of [^{125}I]-CXCL11 compared to [^{3}H]-VUF11211. This is probably due to selection of a different subset of CXCR3 (inactive versus active) conformations by the two radioligands and associated efficiency of their displacement by the unlabeled ligands that have their own conformational preference. Inverse agonists generally prefer inactive conformations, whereas agonists also bind to active GPCR conformations (Bouvier, 2013). In accordance with this, VUF10661 was found to displace [^{3}H]-VUF11211 with >30-fold lower efficiency than [^{125}I]-CXCL11 from HEK293/CXCR3 membranes (fig. 2b and table 2). Although a similar trend is observed for other structurally-related antagonists (VUF10085 and VUF10990) and the agonist VUF11418, the differences were not statistically significant (table 2). However, we have shown previously that relatively minor changes in chemical structure can result in major pharmacological changes (Scholten *et al.*, 2012b). This might explain differences in conformational preference between structurally-related compounds.

A simple on-off model does not provide a full description of GPCR activation. The receptor rather exists as an ensemble of conformations, which are continuously sampled, including multiple active and inactive conformations. Ligands and signaling effectors as G proteins and other (cytosolic) proteins, limit the conformational dynamics of the GPCR, leading to a distinct functional outcome (Bouvier, 2013). The notion that ligand-chemokine receptor interaction is often dependent on other proteins, implicates that affinity and potency values are highly dependent on the cellular context and experimental conditions (Cox *et al.*, 2001; Scholten *et al*., 2012a). This is illustrated by our previous finding that CXCL11 exhibited a 10-fold lower affinity in binding experiments performed on whole cells compared to membranes prepared from those same cells (Scholten *et al*., 2012b).

To further characterize the binding of [^{3}H]-VUF11211 to CXCR3, its sensitivity to G protein uncoupling by GTPγS was investigated. Uncoupling CXCR3 receptors from their G proteins led to a ±50% decrease in [^{125}I]-CXCL11 binding, suggesting that CXCL11 binds to both G protein-coupled as well as –uncoupled conformations, confirming results reported before (Cox et al., 2001). In contrast,

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incubation of [^{3}H]-VUF11211 with GTPγS did not change specific binding of the radioligand to the same membranes, even at 100 µM of GTPγS. This indicates that [^{3}H]-VUF11211 binding to CXCR3 is unaffected by G protein coupling, which is expected for inverse agonists generally binding to (a subset of) inactive non-G protein-coupled GPCR conformations (Bouvier, 2013). Altogether, it can be concluded that CXCL11 and VUF11211 bind to distinct sets of CXCR3 conformations. Interestingly, unlabeled VUF11211 was able to fully displace [^{125}I]-CXCL11 binding to CXCR3, yet unlabeled CXCL11 only produced ±40% displacement of [^{3}H]-VUF11211. This either signifies that CXCL11 and VUF11211 bind to CXCR3 in an allosteric fashion or that VUF11211 binds to a distinct subset of CXCR3 conformations inaccessible by CXCL11. The latter notion is confirmed by the observation that the B_{max} value obtained for [^{3}H]-VUF11211 binding is ± 8-fold higher than was reported earlier for [^{125}I]-CXCL11 binding at HEK293/CXCR3 membrane preparations (8.0 ± 1.0 vs. 1.0 ± 0.3 pmol/mg protein, respectively) (Scholten et al., 2012b). A similar pattern is observed when the B_{max} values for preparations of COS-7 cells expressing the CXCR2 receptor were determined with [^{125}I]-CXCL8 or allosteric antagonist [^{3}H]-SB265610 (B_{max} = ± 0.1 vs. ± 50 pmol/mg protein, respectively) (de Kruijf et al., 2009).

To validate the use of [^{3}H]-VUF11211 in generation of SAR, a small set of VUF11211 derivatives was prepared and assayed as a proof of concept. We noted a significant drop in affinity when installing an amide functionality (compare VUF11211: pK_d = 9.0 ± 0.0 to VUF14479: pK_i = 7.7 ± 0.1) while the effect of α-methylation was less pronounced (compare VUF11211: pK_d = 9.0 ± 0.0 to VUF14263: pK_i = 8.8 ± 0.1), comparable to studies from Merck Sharp & Dohme with similar molecules (McGuinness et al., 2009; Shao et al., 2011). Replacing the chloro- by a bromo-moiety led to significant lower affinity (compare VUF11211: pK_d = 9.0 ± 0.0 to VUF13948: pK_i = 8.1 ± 0.1) (Table 3). Altogether, effects of changing or adding substituents to VUF11211 on affinity could be readily observed in the competition binding assay using [^{3}H]-VUF11211, suggesting that the radioligand is suitable for SAR studies.

Conclusion

In the current work, CRE-Luciferase and β-arrestin recruitment functional assays have been used to further characterize the pharmacology of VUF11211, revealing inverse agonist properties at a constitutively active mutant of CXCR3. We also describe the radiolabeling of VUF11211 to provide enantiopure [^{3}H]-VUF11211 with excellent specific activity. Different equilibrium binding assays,

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including saturation and displacement, as well as kinetic binding to CXCR3 show that [^{3}H]-VUF11211 is an allosteric small-molecule CXCR3 radioligand with relatively fast binding kinetics and high affinity for human CXCR3, recognizing a different receptor population compared to the endogenous chemokine CXCL11. Small-molecule CXCR3 ligands (antagonists and agonists) of diverse chemotypes displace [^{3}H]-VUF11211 binding to CXCR3 with different potencies as obtained in displacement studies with [^{125}I]-CXCL11. In all, the results of this study show that [^{3}H]-VUF11211 is a high-affinity allosteric radioligand for CXCR3 that can be used for screening, characterization and optimization of allosteric ligands that bind to the TM region of CXCR3.

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Authorship Contributions

Participated in research design: Scholten, Wijtmans, Custers, van Senten, Stunnenberg, de Esch, Smit, Leurs

Conducted experiments: Scholten, Wijtmans, van Senten, Custers, Stunnenberg

Contributed new reagents or analytic tools: Wijtmans, Custers, de Esch,

Performed data analysis: Scholten, Wijtmans, van Senten, Custers, Stunnenberg, Leurs

Wrote or contributed to the writing of the manuscript: Scholten, Wijtmans, de Esch, Smit, Leurs

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Footnotes

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Figure Legends

Figure 1

Chemical structure of the enantiopure CXCR3 antagonist VUF11211

Figure 2

(A-C) CRE-Luciferase reporter gene assays to investigate the properties of VUF11211 in a functional assay. (**A**) Cells expressing CXCR3 and CRE-luciferase reporter gene were either incubated with a concentration series of CXCL11 alone, or coincubated with 3 nM of CXCL11 (EC_{80}) and a concentration series of VUF11211. (**B**) HEK293T cells expressing the CRE-luciferase reporter gene and either CXCR3 WT or the constitutively active mutant N3.35A were incubated with a concentration series of VUF11211. (**C**) Schild analysis with a concentration series of CXCL11 in the absence or presence of constant concentrations of VUF11211. Data is grouped from three different experiments showing average ± SEM. Data is normalized towards forskolin-induced increase of CRE-luciferase reportergene activity. (**D**) A β-arrestin recruitment assay. Cells expressing CXCR3-Rluc and β-arrestin2-YFP were incubated with a concentration series of either CXCL11 or VUF11211 in the absence or presence of 10 nM of CXCL11.

Figure 3

Optimization of Radioligand binding assay conditions for [^{3}H]-VUF11211 binding to human CXCR3. (**A**) Binding of the radioligand to membranes prepared from HEK293 cells stably expressing CXCR3 (TB = total binding, NS = non-specific binding (determined using 10 µM unlabeled VUF11211)) or empty HEK293 cells (mock). Conditions that were used 50 mM Tris pH 7.4 + 100 mM NaCl with 1 nM of [^{3}H]-VUF11211. (**B**) Optimization of assay conditions to reduce non-specific binding to plastics and filter plates, using 1 nM of [^{3}H]-VUF11211 in 50 mM Tris pH 7.4 + 100 mM NaCl as buffer. The data is normalized towards non-specific binding, showing actual "specific binding". Under these conditions no more than 10% of the added radioligand was bound (no depletion). (**C**) Preincubation of the GF/C filter plates for 2 hours was done with either 0.5% PEI in H_2O or 0.5% BSA in H_2O for 4 different concentrations of [^{3}H]-VUF11211.

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Figure 4

Characterization of [^{3}H]-VUF11211 properties on membranes prepared from HEK293 cells stably expressing CXCR3 proteins. (2 µg/well of membranes were used for each experiment). (**A**) Association kinetics experiment performed with three different concentrations of [^{3}H]-VUF11211: 0.3 nM (open circles), 1 nM (filled squares), and 3 nM (filled circles). (**B**) Incubation of 1 nM of [^{3}H]-VUF11211 with HEK293/CXCR3 membranes for 1 hour to reach equilibrium and subsequent dissociation of [^{3}H]-VUF11211 initiated by addition of 10 µM of unlabeled VUF11211 at different time points. (**C**) Saturation binding experiment with increasing concentrations of [^{3}H]-VUF11211 in the presence or absence of 10^{-5}M NBI-74330 to determine non-specific binding. (**D**) Homologous competition binding experiments with four different radioligand concentrations: 0.3 nM (open circles), 1 nM (filled squares), 1.5 nM (open squares), and 3 nM (filled circles). Shown graphs are representative of at least 3 different experiments and each data point is performed in triplicate.

Figure 5

Characterization of the binding of [^{3}H]-VUF11211 and other ligand classes to CXCR3. (**A**) Competition binding using 1 nM of [^{3}H]-VUF11211 and concentration series of small-molecule CXCR3 antagonists. (**B**) Competition binding using displacement of 1 nM of [^{3}H]-VUF11211 or 70 pM of [^{125}I]-CXCL11 with CXCR3 agonists VUF11418 and VUF10661. (**C**) Investigation of the effect of receptor uncoupling from G proteins on 1 nM of [^{3}H]-VUF11211 binding using increasing concentrations of GTPγS. (**D**) Investigation of the effect of receptor uncoupling from G proteins on 70 pM of [^{125}I]-CXCL11 binding using increasing concentrations of GTPγS. (**E**) Competition binding experiment using co-incubation of 1 nM of [^{3}H]-VUF11211 with a concentration series of cold CXCL11, and using co-incubation of 70 pM [^{125}I]-CXCL11 with a concentration series of cold VUF11211. The dashed line indicates maximum homologous displacement (CXCL11 in the case of [^{125}I]-CXCL11 and VUF11211 in the case of [^{3}H]-VUF11211.

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Tables

Table 1: Properties of [^{3}H]-VUF11211

Binding assay type	k_{on} (min^{-1} nM^{-1})	k_{off} (min^{-1})	$t_{1/2}$ (min)	K_d	B_{max} (pmol/mg protein)	n
Association [a]	0.034 ± 0.002	0.022 ± 0.004	32 ± 6	0.69 ± 0.10	-	6
Dissociation [b]	- [e]	0.020 ± 0.001	35 ± 2	-	-	3
Saturation [c]	-	-	-	0.65 ± 0.11	8.0 ± 1.0	4
Competition [d]	-	-	-	0.60 ± 0.16	7.9 ± 0.4	3

Values resulting from experiments shown in figure 3. All values are average ± SEM of at least three independent experiments.

[a] Association kinetics experiments with [^{3}H]-VUF11211 (see figure 3A)

[b] Dissociation kinetics experiments with [^{3}H]-VUF11211 (see figure 3B)

[c] Saturation binding experiments (see figure 3C)

[d] Homologous competition of [^{3}H]-VUF11211 (see figure 3D)

[e] For calculation of the k_{off} the k_{on} data obtained from association kinetics were used.

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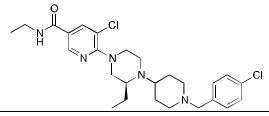
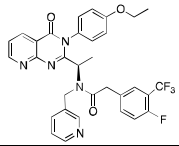
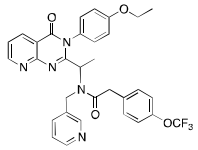
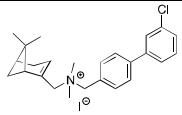
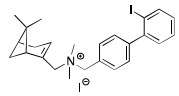
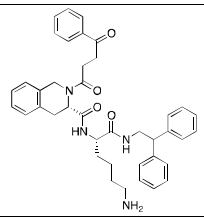
Table 2: SAR study on CXCR3 antagonists and agonists of various chemotypes determined using heterologous competition binding.

Compound structure	Compound name	[^{3}H]-VUF11211 (pK_i ± SEM)	[^{125}I]-CXCL11 (pK_i ± SEM)
	VUF11211	9.0 ± 0.0	7.8 ± 0.1***
	NBI-74330	8.4 ± 0.1	7.2 ± 0.1***
	VUF10085	7.1 ± 0.1	6.8 ± 0.1
	VUF10990	6.4 ± 0.1	6.0 ± 0.1
	VUF11418[a]	5.8 ± 0.1	6.0 ± 0.1
	VUF10661[a]	≤4.5	6.0 ± 0.1***

All data is presented as mean ± SEM of at least three independent experiments (*** p< 0.001 compared to affinity of the same compound determined using [^{3}H]-VUF11211).

[a] Ligand is classified as agonist (Scholten *et al.*, 2012b; Wijtmans *et al.*, 2012b)

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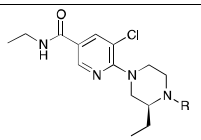
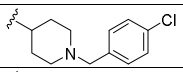
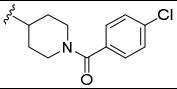
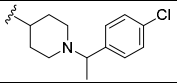
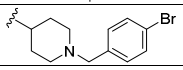
Table 3: Small SAR study on piperidinyl-piperazine CXCR3 ligands determined using heterologous competition binding.



R	VUF #	$pK_i \pm SEM$[a]
	VUF11211	9.0 ± 0.0[b]
	VUF14479	7.7 ± 0.1***
	VUF14263	8.8 ± 0.1
	VUF13948	8.1 ± 0.1***

All data are presented as mean ± SEM values from three independent experiments (*** $p < 0.001$, compared to VUF11211),

[a] Determined using [^{3}H]-VUF11211 competition binding experiments

[b] This value is a pK_d as it is the result of homologous competition binding

Figure 1

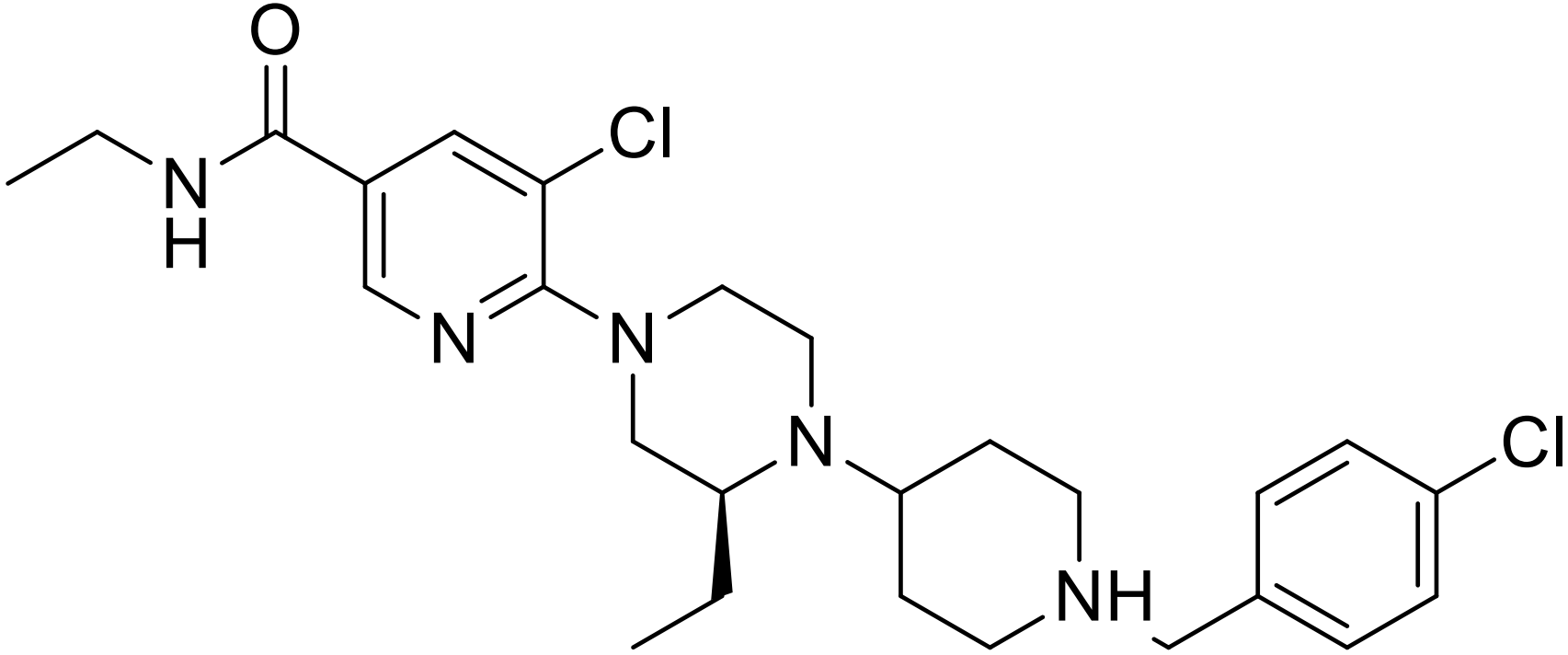


Figure 2

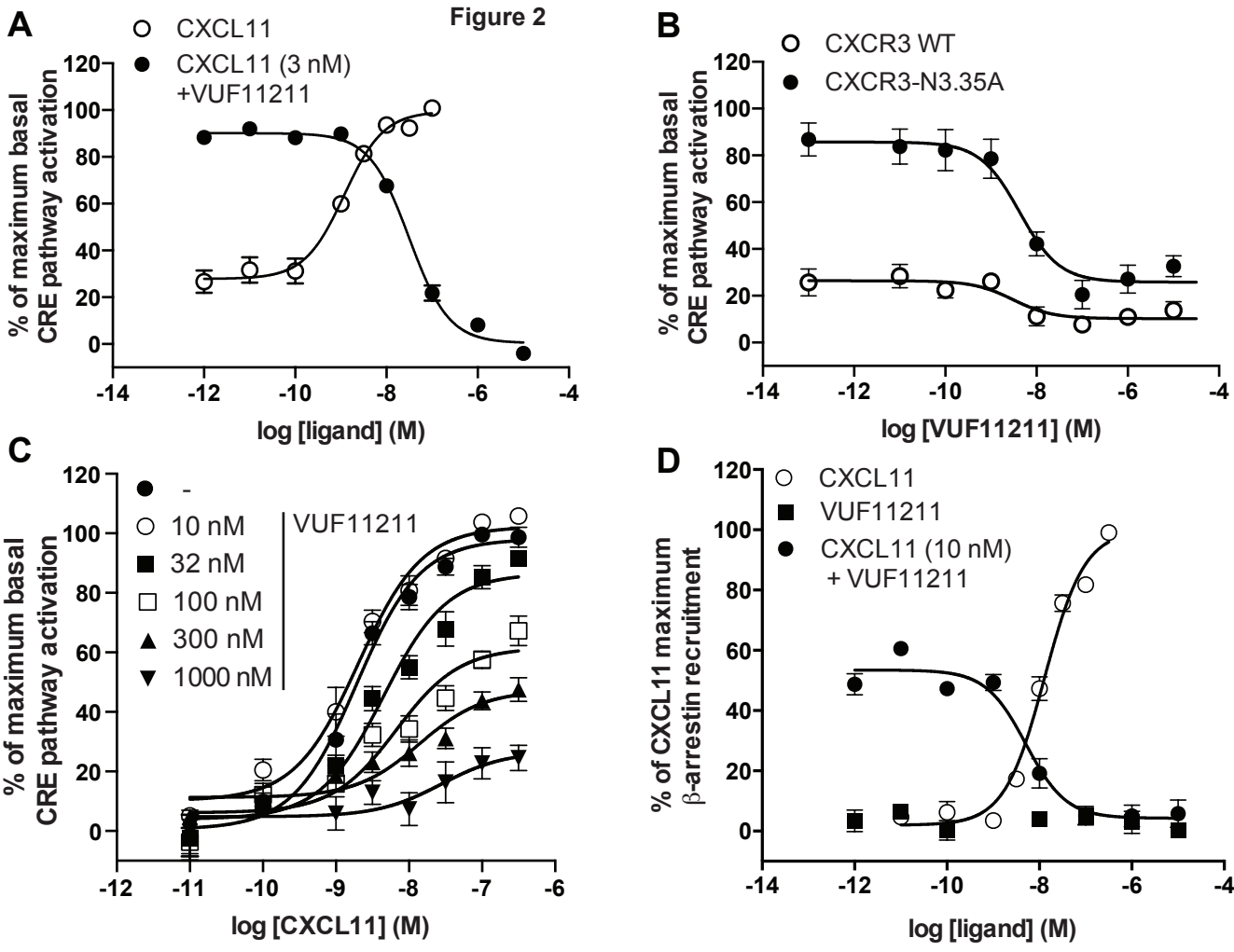

A
CXCL11
CXCL11 (3 nM)
+VUF11211
% of maximum basal
CRE pathway activation
log [ligand] (M)
B
CXCR3 WT
CXCR3-N3.35A
% of maximum basal
CRE pathway activation
log [VUF11211] (M)
C
-
10 nM
32 nM
100 nM
300 nM
1000 nM
VUF11211
% of maximum basal
CRE pathway activation
log [CXCL11] (M)
D
CXCL11
VUF11211
CXCL11 (10 nM)
+ VUF11211
% of CXCL11 maximum
β-arrestin recruitment
log [ligand] (M)

Figure 3

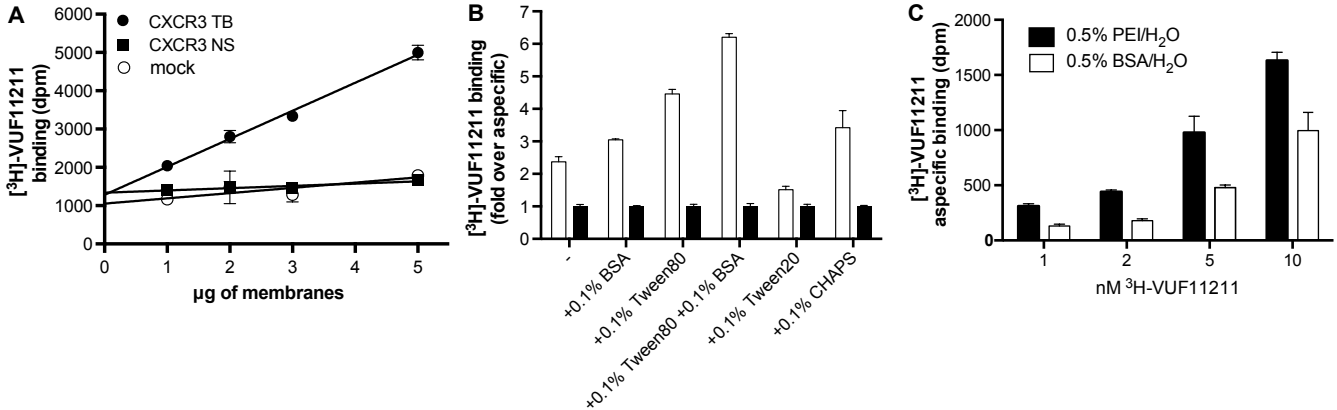

A
CXCR3 TB
CXCR3 NS
mock
[3H]-VUF11211 binding (dpm)
µg of membranes
B
[3H]-VUF11211 binding (fold over aspecific)
+0.1% BSA
+0.1% Tween80
+0.1% Tween80 +0.1% BSA
+0.1% Tween20
+0.1% CHAPS
C
0.5% PEI/H2O
0.5% BSA/H2O
[3H]-VUF11211 aspecific binding (dpm)
nM 3H-VUF11211

Figure 4

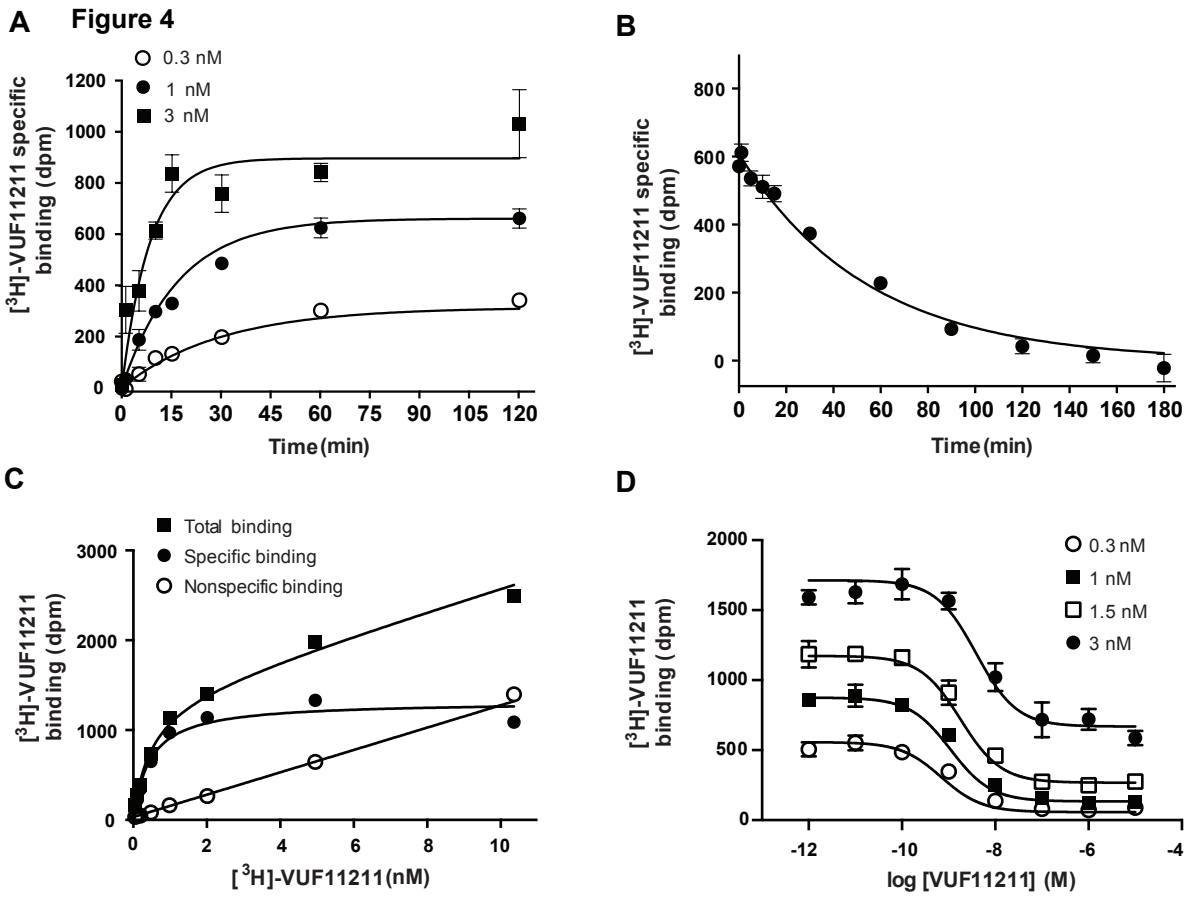
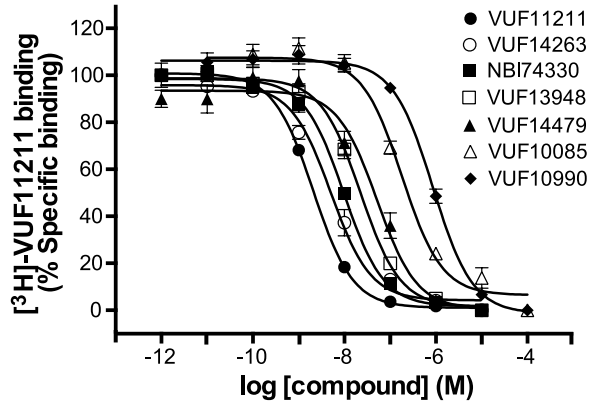
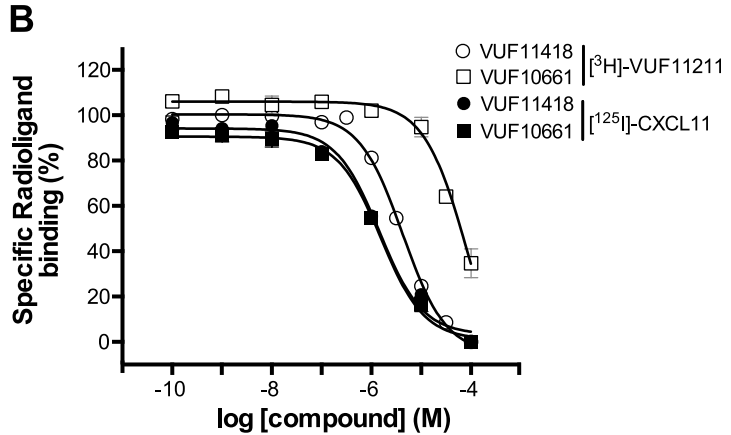
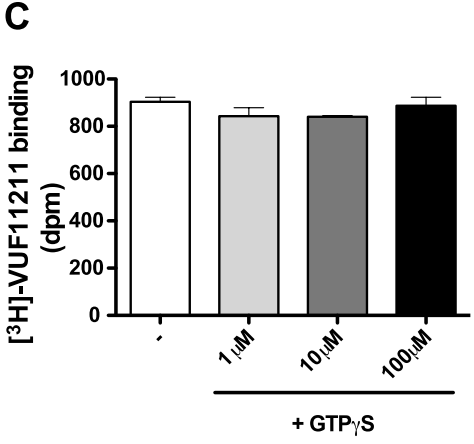
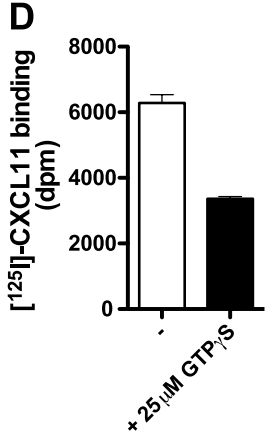

A
0.3 nM
1 nM
3 nM
[3H]-VUF11211 specific binding (dpm)
Time (min)
B
[3H]-VUF11211 specific binding (dpm)
Time (min)
C
Total binding
Specific binding
Nonspecific binding
[3H]-VUF11211 binding (dpm)
[3H]-VUF11211(nM)
D
0.3 nM
1 nM
1.5 nM
3 nM
[3H]-VUF11211 binding (dpm)
log [VUF11211] (M)

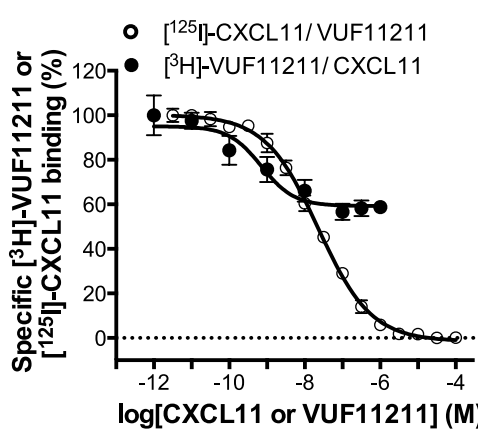
Figure 5


A
[3H]-VUF11211 binding (% Specific binding)
log [compound] (M)
VUF11211
VUF14263
NBI74330
VUF13948
VUF14479
VUF10085
VUF10990


B
Specific Radioligand binding (%)
log [compound] (M)
VUF11418
VUF10661
[3H]-VUF11211
VUF11418
VUF10661
[125I]-CXCL11


C
[3H]-VUF11211 binding (dpm)
-
1 μM
10 μM
100 μM
+ GTPγS


D
[125I]-CXCL11 binding (dpm)
-
+ 25 μM GTPγS


E
[125I]-CXCL11/ VUF11211
[3H]-VUF11211/ CXCL11
Specific [3H]-VUF11211 or [125I]-CXCL11 binding (%)
log[CXCL11 or VUF11211] (M)