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


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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 for investigating 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 orthosteric probe that is used. 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 [(S)-5-chloro-6-[(4-[(4-chlorobenzyl)piperidin-4-yl]-3-ethylpiperazin-1-yl)-N-ethylnicotinamide], 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 ($K_d = 0.65 \text{ nM}$) and reasonably fast association ($k_{on} = 0.03 \text{ minute}^{-1} \text{nM}^{-1}$) and dissociation kinetics ($k_{off} = 0.02 \text{ minute}^{-1}$). 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 with the CXCR3 agonist XCC chemokine ligand 11 (CXCL11), VUF11418[1-[(1R,5S)-6,6-dimethylbicyclo[3.1.1]hept-2-en-2-yl]-(2‘-iodobiphenyl-4-yl)methyl]-N,N-dimethylmethanaminium iodide], and VUF10661 [N-(6-amino-1-(2,2-diphenylethylamino)-1-oxohexan-2-yl)-(2-(4-oxo-4-phenylbutanoyl))-1,2,3,4-tetrahydroisoquinoline-3-carboxamide]. These findings, taken together, indicate that this allosteric inverse agonist radioligand for CXCR3 may facilitate the discovery, characterization, and optimization of allosteric modulators for the chemokine receptor CXCR3.

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 CC chemokine receptor 5 (CCR5) antagonist maraviroc, which is currently being marketed as Selzentry, and is used in combination treatment of human immunodeficiency virus-1 (HIV-1) infection. This molecule shows noncompetitive allosteric behavior toward 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 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

ABBREVIATIONS: AMG487, (R)-N-(1-(4-(4-ethoxyphenyl)-4-oxo-3,4-dihydropyrido[2,3-d]pyrimidin-2-yl)ethyl)-N-(pyridin-3-3-ylmethyl)-2-(4-trifluoromethoxy)phenyl)acetamide; BRET, bioluminescence resonance energy transfer; BSA, bovine serum albumin; GPCR, G-protein–coupled receptor; GTP $\gamma$S, GTP $\gamma$-O-(3-thiotriphosphate; NBI-74330, (R)-N-(1-(3-ethylpiperazin-4-yl)-4-oxo-3,4-dihydropyrido[2,3-d]pyrimidin-2-yl)ethyl)-2-(4-fluoro-3-(trifluoromethyl)phenyl)acetamide; PT, polyethyleneimine; RT, residence time; SAR, structure–activity relationship; SCH-527123, (R)-2-hydroxy-N,N-dimethyl-3-((6-(1-(5-methylfuran-2-yl)propargylamino)-3-dioxocyclobut-1-en-1-yl)amino)benzamide; TM, transmembrane; TMS, transmembrane site; VUF10661, N-(6-amino-1-(2,2-diphenylethylamino)-1-oxohexan-2-yl)-(2-(4-oxo-4-phenylbutanoyl))-1,2,3,4-tetrahydroisoquinoline-3-carboxamide; VUF11211, (S)-5-chloro-6-[(4-[[4-(4-chlorobenzyl)piperidin-4-yl]-3-ethylpiperazin-1-yl]-N-ethylnicotinamide; VUF11418, 1-1[(1R,5S)-6,6-dimethylbicyclo[3.1.1]hept-2-en-2-yl]-(2'-iodobiphenyl-4-yl)methyl]-N,N-dimethylmethanaminium iodide; WT, wild-type.
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), such as the 8-azaquinazolinonone compounds from Amgen Inc. (AMG487, (R)-N-(1-(3-(4-ethoxyphenyl)-4-oxo-3,4-dihydropyrido[2,3-d]pyrimidin-2-ylmethyl)-(pyridin-3-ylmethyl)-2-(4-trifluoromethoxy)phenyl)acetamide; Washington, D.C.) and Neurocrine Biosciences (NBI-74330, (R)-N-(1-(3-(4-ethoxyphenyl)-4-oxo-3,4-dihydropyrido[2,3-d]pyrimidin-2-yl)methyl)-2-(4-fluoro-3-(trifluoromethyl)phenyl)N-(pyridin-3-ylmethyl)acetamide; San Diego, CA), 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-piperidin compound class containing ligands with nanomolar CXCR3 affinities was reported by Schering Plough (now Merck Sharp & Dohme, Kenilworth, NJ) (Meguinness et al., 2009; Kim et al., 2011; Shao 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 (Bernat et al., 2012; Nedjai et al., 2012; Scholten et al., 2012b, 2014). In a recent study (Scholten et al., 2014), we reported on the binding mode of two CXCR3 ligands originating from the 8-azaquinazolinonone class (NBI-74330) and the piperazinyl-piperidin class (VUF11211, (S)-5-chloro-6-(4-(1-(4-chlorobenzyl)piperizin-4-yl)-3-ethylpiperazin-1-yl)-N-ethylnicotinamide) using site-directed mutagenesis in conjunction with in silico modeling of CXCR3. We showed that NBI-74330 binds mainly to transmembrane site 1 (TMS1), between TMs 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 used predominantly for receptor-ligand interaction studies. Since these radioligands are orthosteric ligands, they potentially limit the understanding of these interactions has started to emerge in the past years (Bernat et al., 2012; Nedjai et al., 2012; Scholten et al., 2012b, 2014). In a recent study (Scholten et al., 2014), we reported on the binding mode of two CXCR3 ligands originating from the 8-azaquinazolinonone class (NBI-74330) and the piperazinyl-piperidin class (VUF11211, (S)-5-chloro-6-(4-(1-(4-chlorobenzyl)piperizin-4-yl)-3-ethylpiperazin-1-yl)-N-ethylnicotinamide) using site-directed mutagenesis in conjunction with in silico modeling of CXCR3. We showed that NBI-74330 binds mainly to transmembrane site 1 (TMS1), between TMs 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 used predominantly 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 [3H]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 ([3H]VUF11211, Fig. 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.

**Materials and Methods**

Dulbecco’s modified Eagle’s medium and trypsin were purchased from Sigma-Aldrich (St. Louis, MO); penicillin and streptomycin were obtained from Lonza (Verviers, Belgium); fetal bovine serum was purchased from PAA Laboratories GmbH (Pasching, Austria); and [35S]cycloheximide chemokine ligand 11 (CXCL11) (± 100 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Unlabeled chemokines were purchased from PeproTech (Rocky Hill, NJ). Polyethyleneimine (PEI, linear 25 kDa) for transfection was obtained from Polysciences (Warrington, PA). Unless stated otherwise, all other chemicals were obtained from Sigma-Aldrich.

**CXCR3 Ligands.** VUF11211 (compound 181 in (Shao et al., 2011)) was prepared in enantiopure form in our laboratory as described previously (Scholten et al., 2014). [3H]VUF11211 was prepared from VUF11211 (Supplemental Fig. S1) and analyzed (Supplemental Figs. S5–S5) by PerkinElmer Health Sciences (38.4 Ci/mmol). VUF14479, VUF14263, and VUF13948 were synthesized (Supplemental Fig. S2) and analyzed (Supplemental Figs. S6–S14) in our laboratory.

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

**Cell Culture and Transfection.** Human embryonic kidney 293T cells (HEK293T) stably expressing CXCR3 were grown at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 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 PEI as DNA carrier agent. 8 µg of DNA per 4 · 106 cells was combined with 48 µg PEI in a total volume of 500 µl 150 mM NaCl and incubated for 10 minutes at room temperature. Subsequently, the DNA/PEI mix was added to a suspension of HEK293T cells in full growth medium (0.5 · 106 cells/ml) and subsequently plated out in a 96-well plate and cultured overnight at standard culture conditions.

**CRE-Luciferase Reporter Gene Assay.** pcDEF3-CXCR3, CRE-Luc reporter gene DNA, and empty plasmid DNA were transfected with a weight ratio of 1:3.4. Twenty-four hours after transfection, the ligands were added to the cells (homogeneous format) and incubated for 6 hours at standard growth conditions. Subsequently, the medium was replaced by 25 µl of substrate buffer (39 mM Tris–HCl, pH 7.8, 39% glycerol, 2.6% Triton X-100, 0.9 mM dithiothreitol, 18 mM MgCl2, 0.8 mM ATP, 77 µM dioxolane pyrophosphate, 230 µg/ml beetle luciferin), and luminescence was measured after 30 minutes with a Victor3 plate reader (PerkinElmer).

**Bioluminescence Energy Transfer β-Arrestin Recruitment Assay.** pcDEF3-CXCR3-Rltc and pcDEF3-β-arrestin-yFP were transfected in a ratio of 1:4. The next day, medium was aspirated and cells were washed with Hanks’ balanced salt solution. Next, cells were incubated in Hanks’ balanced salt solution with or without antagonist 30 minutes before agonist addition. To assess bioluminescence energy transfer (BRET), agonists were added 10 minutes after incubation with coelenterazine–h and incubated for an additional 10 minutes 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 phosphate-buffered saline, collected in tubes, and centrifuged at 1500g for 10 minutes. The cells were resuspended in ice-cold membrane buffer.

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**Fig. 1.** Chemical structure of the enantiopure CXCR3 antagonist VUF11211.
(15 mM Tris, pH 7.5, 1 mM EGTA, 0.3 mM EDTA, and 2 mM MgCl₂) 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 40,000g for 25 minutes. 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. HEK293/CXCR3 membranes (2 μg) 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 CaCl₂, 5 mM MgCl₂, 100 mM NaCl, and 0.5% (w/v) Tween80, 0.1% (w/v) BSA fraction V) with approximately 70 pM [125I]CXCL11 and a concentration range of cold CXCL11, VUF11211, or NBI-74330 for 2 hours at room temperature. Next, the membranes were harvested by filtration through Unifilter 96-well GF/C plates (PerkinElmer) presoaked with 0.5% PEG using ice-cold wash buffer (chemokine binding buffer supplemented with 0.5% NaCl). Bound radioactivity was determined with a MicroBeta scintillation counter using a [125I]protocol (PerkinElmer).

**[3H]VUF11211 Binding Assay.** HEK293/CXCR3 membranes (2 μg) were used per well in 96-well clear plates and incubated with varying amounts of [3H]VUF11211, depending on the assay type (see respective figures and legends). All ligands and membranes were prepared in binding buffer (50 mM 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 [3H]VUF11211 was coincubated with a concentration series of different cold ligands. In saturation binding experiments, increasing concentrations of [3H]VUF11211 were used, and NBI-74330 was used to determine nonspecific binding levels. All equilibrium binding assays were performed using a MicroBeta scintillation counter using a tritium protocol.

**Data Analysis.** Prism v6.0b from GraphPad Software (San Diego, CA) was used to plot and analyze the data. Curve fitting was performed using nonlinear 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]/(Kd + [L])) × (1 - e^(-Kd × X)), where [L] is the concentration of radioligand in nM; dissociation kinetics with one-phase exponential decay: Y = (Yo - NS) × e^(-K × X) + NS, Yo is the Y value at t = 0, NS is the minimum binding plateau reached after longer incubations in units of X, and K is the rate constant of dissociation (inverse units of X); saturation binding: Y = Bmax × X/(Kd + X), where X depicts increasing concentrations of [3H]VUF11211, Kd is the equilibrium dissociation constant in units of X, and Bmax is maximum binding in units of Y; one-site homologous competition binding: Y = (Bmax × [L] × (1 + 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^logKd + 9) × [L]), where [L] is the radioligand concentration in nM, and Kd 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 with the used radioligands, the observation that these ligands completely displace the radioligand indicates strong negative cooperativity. Therefore, use of competitive analysis for affinity determination is justified.

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

**Results**

**VUF11211 Pharmacology.** First, we used functional readouts to characterize more fully 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 CRE-Luc reporter gene assay was used to measure CXCR3 receptor activity (Wijtmans et al., 2012b). As expected, CXCL11 acted as an agonist in this assay with a pEC50 of 8.9 ± 0.1 (Fig. 2A). Next, an EC50 concentration of CXCL11 (3 nM) and a concentration series of VUF11211 were coincubated, resulting in dose-dependent antagonism of CXCL11-induced receptor activity by VUF11211 (pIC50 = 7.5 ± 0.1) (Fig. 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 wild-type (WT) in this assay. Hence, a concentration range 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 (pIC50 = 8.4 ± 0.2) and, to a much lesser extent, at CXCR3 WT (Fig. 2B). To probe further the mechanism of action of VUF11211, the compound was investigated in a Schild analysis approach. A concentration range of CXCL11 was coincubated 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 (Fig. 2C).

VUF11211 was also studied in the context of β-arrestin recruitment toward CXCR3 (Fig. 2D). Whereas CXCL11 was an agonist in this assay (pEC50 = 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 (pIC50 = 8.3 ± 0.1). Moreover, VUF11211 did not induce β-arrestin recruitment on its own (Fig. 2D).

**Radiolabeling of VUF11211.** As VUF11211 had proven to be 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 and BRET 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., 2009; Scholten et al., 2014) were considered and some subjected to initial unlabeled trials, but eventually all were considered not to be attractive because of the lack of required 3H 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 [3H]VUF11211 with a specific activity of 38.4 Ci/mmol and 99.6% radiochemical purity (Supplemental Figs. S1 and S3–S5). Mass spectrometry analysis revealed the incorporation of, on average, two 3H atoms per molecule VUF11211.

**Assay Development for [3H]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 (Fig. 3a). Moreover, coincubation of 1 nM of [3H]VUF11211 with a saturating concentration of NBI-74330 (10 μM) led to radioligand binding levels
comparable to nonspecific binding as detected in membranes without CXCR3 receptors (Fig. 3A). As expected, specific \[^{3}H\]VUF11211 binding was proportional to the amount of membranes, whereas nonspecific \[^{3}H\]VUF11211 binding remained largely unchanged when membrane amounts were increased, suggesting that nonspecific binding is mostly dependent on constituents other than membranes. HEK293/ CXCR3 membranes (3 μg/well) was selected for further \[^{3}H\]VUF11211 binding experiments.

Preliminary experiments (not shown) revealed lower \[^{3}H\]VUF11211 concentrations in the assay than expected from calculations based on the properties of the radioligand. This result suggests loss of radioligand by binding to, for example, plastics. Therefore, different agents were considered to decrease these potential nonspecific binding issues (Fig. 3B). As can be seen in Fig. 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 nonspecific 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 filter plate used to harvest the

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Fig. 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 incubated with either a concentration series of CXCL11 alone or co-incubated with 3 nM CXCL11 (EC_{50}) 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 are grouped from three different experiments showing average ± S.E.M. Data are normalized toward forskolin-induced increase of CRE-luciferase reporter gene activity.

Fig. 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, nonspecific binding (determined using 10 μM unlabeled VUF11211) or empty HEK293 cells (mock). Conditions were 50 mM Tris pH 7.4 + 100 mM NaCl with 1 nM of \[^{3}H\]VUF11211. (B) Optimization of assay conditions to reduce nonspecific binding to plastics and filter plates, using 1 nM \[^{3}H\]VUF11211 in 50 mM Tris pH 7.4 + 100 mM NaCl as buffer. The data are normalized toward nonspecific 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_{2}O or 0.5% BSA in H_{2}O for four different concentrations of \[^{3}H\]VUF11211.
membranes is preincubated with PEI, to counteract nonspecific binding to glass fibers. In our study, either 0.5% w/v PEI or BSA was compared; the latter resulted in significantly lower nonspecific binding counts (roughly 50% lower) for a range of radioligand concentrations (Fig. 3c). Consequently, BSA was selected as an agent to counteract nonspecific binding in future experiments.

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

Next, a \([H]VUF11211\) saturation binding (Fig. 4C) to CXCR3 was performed by incubating increasing concentrations of radioligand with HEK293/CXCR3 membranes in the presence or absence of 10 \( \mu \)M of NBI-74330 (to determine nonspecific binding). In our study, either 0.5% w/v PEI or BSA was compared; the latter resulted in significantly lower nonspecific binding counts (roughly 50% lower) for a range of radioligand concentrations (Fig. 3c). Consequently, BSA was selected as an agent to counteract nonspecific binding in future experiments.

![Figure 4](https://example.com/fig4.png)

**Figure 4.** Characterization of \([H]VUF11211\) properties on membranes prepared from HEK293 cells stably expressing CXCR3 proteins (2 \( \mu \)g/well of membranes were used for each experiment). (A) Association kinetic experiment performed with three different concentrations of \([H]VUF11211\): 0.3 nM (open circles), 1 nM (filled squares), and 3 nM (filled circles). (B) Incubation of 1 nM \([H]VUF11211\) with HEK293/CXCR3 membranes for 1 hour to reach equilibrium and subsequent dissociation of \([H]VUF11211\) initiated by the addition of 10 \( \mu \)M unlabeled VUF11211 at different time points. (C) Saturation binding experiment with increasing concentrations of \([H]VUF11211\) in the presence or absence of 10 \( \mu \)M NBI-74330 to determine nonspecific 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). Graphs are representative of at least three different experiments, and each data point is performed in triplicate.
binding levels) for 2 hours. The assay revealed for [3H]VUF11211 an equilibrium affinity constant ($K_d$) of 0.65 ± 0.11 nM for [3H]VUF11211 (Table 1), with a $B_{\text{max}}$ value of 8.0 ± 1.0 pmol/mg protein.

In general, competition binding experiments are used to determine the affinities of unlabeled compounds of different chemotypes (or analogs) without the need for radiolabeling each compound. As a test, homologous competition binding experiments were performed using four different concentrations (0.3, 1.0, 1.5, and 3 nM) of radioligand in the range of its $K_d$ value (Fig. 4d). For each [3H]VUF11211 concentration, a series of concentrations of unlabeled VUF11211 was incubated with HEK293/CXCR3 membranes to determine a $K_d$ value using global fitting of the data. The resulting $K_d$ for VUF11211 is 0.60 ± 0.16 nM with a $B_{\text{max}}$ value of 7.9 ± 0.4 pmol/mg protein.

**Displacement of [3H]VUF11211 Binding to Human CXCR3 with Varying Ligand Chemotypes.** A range of compounds with varying chemotypes and varying functional activities was tested to characterize more completely [3H]VUF11211 binding to HEK293/CXCR3 membranes (Fig. 5A; Tables 2 and 3), including CXCR3 antagonists (unlabeled VUF11211, NBI-74330, and VUF11085) (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, $K_d$ values recorded with [125I]CXCL11 were significantly lower than those recorded with [3H]VUF11211, whereas for agonist VUF10661, the opposite was observed. The affinity of VUF10661 determined using [125I]CXCL11 ($pK_i$ = 4.5) was at least 30-fold lower than in the case of [125I]CXCL11 ($pK_i$ = 6.0 ± 0.1) (Fig. 5B; Table 2).

To substantiate more fully 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 we had previously disclosed the synthesis of VUF11211 (Scholten et al., 2014). Table 3 shows exemplary compounds of this chemotype: amide VUF14479, $\alpha$-methyl derivative VUF14263 and bromo-analog VUF13948 (see Supplemental Figs. S2, S6–S14 for synthesis routes and chemical analyses). These three compounds display a diversity of affinities as measured using [3H]VUF11211 (from $pK_i$ = 7.7–9.0).

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 nonhydrolyzable form of GTP, known as GTP 5-y-O-(3-thio)triphosphate (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 [3H]VUF11211 binding to CXCR3 in this context. Different concentrations of GTPγS, ranging from 1 to 100 μM, did not affect the total binding of [3H]VUF11211 to HEK293/CXCR3 membranes (Fig. 5C), whereas applying 25 μM GTPγS resulted in a decrease of roughly 50% of total binding of [125I]CXCL11 binding to the same membranes (Fig. 5D).

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

**Discussion**

The purpose of the present study was to characterize the pharmacological properties 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; Kim et al., 2011; Shao et al., 2011; Nair et al., 2014). VUF11211 showed antagonistic activity of CXCR3 from the piperazinyl-piperidine class, which contains many compounds possessing (sub)nanomolar affinities for CXCR3 (McGuinness et al., 2009; Kim et al., 2011; Shao et al., 2011; Nair et al., 2014). VUF11211 showed antagonistic activity of CXCR3 WT to a small extent in the CRE-luciferase reporter assay (Fig. 2). Therefore, the constitutively active mutant CXCR3-N3.35A was significantly higher than for CXCR3 WT, confirming its constitutive activity (Fig. 2) (Verzijl et al., 2008). Furthermore, VUF11211 dose dependently decreased 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-azaquinazololine class. These compounds both inhibit constitutive activity of CXCR3 WT only to a minor extent, consistent with its very low
constitutive activity compared with CXCR3-N3.35A. In most assay systems, constitutive activity of CXCR3 WT has not been observed at all (Verzijl et al., 2008), yet a reporter gene assay involves considerably more signal amplification than upstream assays like [35S]GTP\(_\text{S}\) 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 (Nedjai et al., 2012; Scholten et al., 2012b, 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 reporter gene assay. VUF11211 dose dependently and insurmountably decreased the maximum efficacy of CXCL11, again suggesting noncompetitive allosteric behavior.

The allosteric properties of these ligands impose limitations on the use of orthosteric radioligands for the discovery and characterization of such molecules as a result of the 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, whereas VUF11211 traverses both TMS1 and TMS2 (Scholten et al., 2014).

Kinetic radioligand binding experiments were performed 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 (\(k_{\text{on}} = 0.034 \pm 0.002 \text{ nM}^{-1} \text{min}^{-1}\) at 22°C versus 0.045 \(\pm 0.003 \text{ nM}^{-1} \text{min}^{-1}\) at 37°C, respectively) (Bernat et al., 2012). However, it should be noted that kinetic parameters are dependent on temperature; binding assays with \(^{3}H\)RAMX3 were performed at 37°C, so 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 (\(k_{\text{off}}\)). Dissociation kinetic binding experiments revealed a relatively short residence time for \(^{3}H\)VUF11211 (\(RT_{25} / C25 = 50\) minutes) compared with other small-molecule chemokine receptor ligands, including SCH-527123 [(R)-2-hydroxy-N,N-dimethyl-3-((2-(1-(5-methylfuran-2-ylpropyl)amino)-3,4-dioxocobut-1-en-1-yl)amino)benzamide] (CXCR2; \(RT \approx 22\) hours at 22°C) or maraviroc (CCR5; \(RT > 136\) hours at 4°C) (Guo et al., 2014).

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

The affinities of different CXCR3 ligands, including agonists (VUF10661, VUF11418) and antagonists (VUF10085, NBI-74330, VUF10990), were determined by [3H]VUF11211 and [125I]CXCL11 competition binding assays (Fig. 5, A and B; Table 2). The inverse agonists VUF11211 and NBI-74330 showed lower affinities for CXCR3 in the case of [125I]CXCL11 compared with [3H]VUF11211. This result 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 [3H]VUF11211 with >30-fold lower efficiency than [125I]CXCL11 from HEK293/CXCR3 membranes (Fig. 2B; 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

<table>
<thead>
<tr>
<th>Compound Structure</th>
<th>Compound Name</th>
<th>$[3H]$VUF11211 $pK_i \pm$ S.E.M.</th>
<th>$[125I]$CXCL11 $pK_i \pm$ S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Compound Structure" /></td>
<td>VUF11211</td>
<td>9.0 $\pm$ 0.0</td>
<td>7.8 $\pm$ 0.1***</td>
</tr>
<tr>
<td><img src="image2.png" alt="Compound Structure" /></td>
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<td>7.2 $\pm$ 0.1***</td>
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<td><img src="image3.png" alt="Compound Structure" /></td>
<td>VUF10085</td>
<td>7.1 $\pm$ 0.1</td>
<td>6.8 $\pm$ 0.1</td>
</tr>
<tr>
<td><img src="image4.png" alt="Compound Structure" /></td>
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<td>6.4 $\pm$ 0.1</td>
<td>6.0 $\pm$ 0.1</td>
</tr>
<tr>
<td><img src="image5.png" alt="Compound Structure" /></td>
<td>VUF11418*</td>
<td>5.8 $\pm$ 0.1</td>
<td>6.0 $\pm$ 0.1</td>
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<tr>
<td><img src="image6.png" alt="Compound Structure" /></td>
<td>VUF10661*</td>
<td>$\leq$4.5</td>
<td>6.0 $\pm$ 0.1***</td>
</tr>
</tbody>
</table>

*Ligand is classified as agonist (Scholten et al., 2012b; Wijtmans et al., 2012b).
led to a investigated. Uncoupling CXCR3 receptors from their G proteins to CXCR3, its sensitivity to G-protein uncoupling by GTP membranes, even at 100 m not change specific binding of the radioligand to the same indicates that [3H]VUF11211 binding to CXCR3 is unaffected (2001). In contrast, incubation of [3H]VUF11211 with GTP CXCL11 binds to both G-protein coupled GPCR conformations (Bouvier, 2013). Taken together, this notion is confirmed by the observation that the Bmax value obtained for [3H]VUF11211 binding is ±8-fold higher than was reported earlier for [125I]CXCL11 binding at HEK293/CXCR3 membrane preparations (8.0 ± 1.0 versus 1.0 ± 0.3 pmol/mg protein, respectively) (Scholten et al., 2012b). A similar pattern is observed when the Bmax values for preparations of COS-7 cells expressing the CXCR2 receptor were determined with [125I]CXCL8 or allosteric antagonist [3H]SB265610 (Bmax ± 0.1 versus ± 50 pmol/mg protein, respectively) (de Kruijf et al., 2009).

To validate the use of [3H]VUF11211 in the 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: pKd = 9.0 ± 0.0 with VUF14479: pKd = 7.7 ± 0.1), whereas the effect of α-methylation was less pronounced (compare VUF11211: pKd = 9.0 ± 0.0 with VUF14263: pKd = 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: pKd = 9.0 ± 0.0 to VUF13948: pKd = 8.1 ± 0.1) (Table 3). Taken together, the effects of changing or adding substituents to VUF11211 on affinity could be readily observed in the competition binding assay using [3H]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 characterize more completely 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 [3H]VUF11211 with excellent specific activity. Different equilibrium binding assays, including saturation and displacement, as well as kinetic binding to CXCR3, have shown that [3H]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 with the endogenous chemokine CXCL11. Small-molecule CXCR3 ligands (agonists and antagonists) of diverse chemotypes displace [3H]VUF11211 binding to CXCR3 with different potencies as obtained in displacement studies with [125I]CXCL11. In all, the results of this study show that [3H]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.

**Acknowledgments**

The authors thank Johan Grootjans (PerkinElmer) for technical assistance on radiolabeling.

**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.

**TABLE 3**

Small structure-activity relationship study on piperidinyl-piperazine CXCR3 ligands determined using heterologous competition binding

All data are presented as mean ± S.E.M. values from three independent experiments (***P < 0.001 compared with VUF11211).

<table>
<thead>
<tr>
<th>R</th>
<th>Compound Name</th>
<th>Affinitya</th>
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<td></td>
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<td>VUF13948</td>
<td>8.1 ± 0.1***</td>
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</table>

aDetermined using [3H]VUF11211 competition binding experiments.

bThis value is a pKd as it is the result of homologous competition binding.

to CXCR3, yet unlabeled CXCL11 produced only ±40% displacement of [3H]VUF11211. This finding signifies either 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 Bmax value for [3H]VUF11211 binding is ±8-fold higher than was reported earlier for [125I]CXCL11 binding at HEK293/CXCR3 membrane preparations (8.0 ± 1.0 versus 1.0 ± 0.3 pmol/mg protein, respectively) (Scholten et al., 2012b). A similar pattern is observed when the Bmax values for preparations of COS-7 cells expressing the CXCR2 receptor were determined with [125I]CXCL8 or allosteric antagonist [3H]SB265610 (Bmax ± 0.1 versus ± 50 pmol/mg protein, respectively) (de Kruijf et al., 2009).

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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.

References


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