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Nonpeptidergic Allosteric Antagonists Differentially Bind to the CXCR2 Chemokine Receptor

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ABSTRACT

The chemokine receptor CXCR2 is involved in different inflammatory diseases, like chronic obstructive pulmonary disease, psoriasis, rheumatoid arthritis, and ulcerative colitis; therefore, it is considered an attractive drug target. Different classes of small CXCR2 antagonists have been developed. In this study, we selected seven CXCR2 antagonists from the diarylurea, imidazolylpyrimide, and thiazolopyrimidine class and studied their mechanisms of action at human CXCR2. All compounds are able to displace ^{125}I-CXCL8 and inhibit CXCL8-induced β-arrestin2 recruitment. Detailed studies with representatives of each class showed that these compounds displace and antagonize CXCL8, most probably via a noncompetitive, allosteric mechanism. In addition, we radiolabeled the high-affinity CXCR2 antagonist SB265610 [1-(2-bromophenyl)-3-(4-cyano-1*H*-benzo[*d*] [1,2,3]-triazol-7-yl)urea] and subjected [^{3}H]SB265610 to a detailed analysis. The binding of this radioligand was saturable and reversible. Using [^{3}H]SB265610, we found that compounds of the different chemical classes bind to distinct binding sites. Hence, the use of a radiolabeled low-molecular weight CXCR2 antagonist serves as a tool to investigate the different binding sites of CXCR2 antagonists in more detail.

Chemokine receptors, belonging to the rhodopsin-like family of G protein-coupled receptors (GPCRs), play a major role in the control and regulation of the immune system (Murphy et al., 2000). These GPCRs are expressed on the cell membrane of leukocytes, driving the trafficking of leukocytes to sites of inflammation, upon sensing chemoattractant cytokines. To date, approximately 50 chemokines and 20 chemokine receptors have been identified (Viola and Luster, 2008). Dysregulation of chemokine expression and/or their GPCR targets is implicated in various human diseases, including chronic inflammatory diseases, autoimmune diseases, and cancer (Rotondi et al., 2007; Singh et al., 2007). As a consequence, chemokine receptor antagonists are seen currently as a promising approach for new therapeutic options in a wide variety of disorders (Donnelly and Barnes, 2006).

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ABBREVIATIONS: GPCR, G protein-coupled receptor; SB332235, 6-chloro-3-(3-(2,3-dichlorophenyl)ureido)-2-hydroxybenzenesulfonamide; SB225002, 1-(2-bromophenyl)-3-(4-cyano-1*H*-benzo[*d*][1,2,3]triazol-7-yl)urea; SB265610, 1-(2-bromophenyl)-3-(4-cyano-1*H*-benzo[*d*] [1,2,3]triazol-7-yl)urea; VUF10948, (*R*)-5-(benzylthio)-7-(1-hydroxybutan-2-ylamino)thiazolo[4,5-*d*]pyrimidin-2-ol; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney; compound 1, (*S*)-2-(2-(1*H*-imidazol-1-yl)-6-(octylthio)pyrimidin-4-ylamino)-*N*-(3-ethoxypropyl)-4-methylpentanamide; compound 2, (*S*)-*N*-(2-(2,5-dihydro-1*H*-pyrrol-1-yl)ethyl)-4-methyl-2-(6-methyl-2-(4-(4-(trifluoromethoxy)phenyl)-1*H*-imidazol-1-yl)pyrimidin-4-ylamino)pentanamide); compound 3, (*S*)-2-(6-butyl-2-(4-(4-chloro-3-(trifluoromethyl)phenyl)-1*H*-imidazol-1-yl)pyrimidin-4-ylamino)-4-methyl-*N*-(4,4,4-trifluorobutyl)pentanamide); GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; Sch527123, 2-hydroxy-*N*,*N*-dimethyl-3-{2-[[(R)-1-(5-methyl-furan-2-yl)-propyl]amino]-3,4-dioxo-cyclobut-1-enylamino}-benzamide; AMD3100, 1,1′-[1,4-phenylenebis(methylene)]bis [1,4,8,11-tetraazacyclotetradecane] octohydrobromide dehydrate; TAK-799 (*N*,*N*-dimethyl-*N*-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5*H*-benzocyclohepten-8-yl]carbon-yl]amino]benzyl]-tetrahydro-2*H*-pyran4-aminium chloride; BX471 *N*-(5-chloro-2-(2-(4-((4-fluorophenyl)methyl)-2-methyl-1-piperazinyl)-2-oxoethoxy)phenyl)urea hydrochloric acid.

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The CXCR2 receptor is one of the chemokine receptors that currently attracts a lot of attention in drug discovery. It is a promiscuous receptor that binds with high affinity to CXCL1, CXCL2, CXCL3 (growth-related protein α, β, or γ, respectively), CXCL5 (epithelial cell-derived neutrophil attractant-78), CXCL6 (granulocyte chemotactic peptide-2), CXCL7 (neutrophil activating peptide-2), and CXCL8 (interleukin-8). CXCR2 is expressed on, e.g., endothelial cells, eosinophils, neutrophils, macrophages, and monocytes (Murphy et al., 2000; Bizzarri et al., 2006) but also on various tumor cells. An important role for CXCR2 and its ligands has been shown in cancer and different inflammatory diseases, like chronic obstructive pulmonary disease (Donnelly and Barnes, 2006), psoriasis (Kulke et al., 1998), rheumatoid arthritis (Podolin et al., 2002), and ulcerative colitis (Buanne et al., 2007). It has been reported that neutralizing CXCR2 antibodies inhibit the early influx of neutrophils in the colon in a rat colitis model (Ajuebor et al., 2004) and CXCL8-mediated angiogenesis in rat (Addison et al., 2000). In addition, in CXCR2 knockout mice, both angiogenesis and primary tumor growth were reduced compared with wild-type mice (Addison et al., 2000; Keane et al., 2004). Moreover, CXCR2 knockout mice also showed a decrease in PMN infiltration into the mucosa and limited signs of mucosal damage compared with wild-type mice in a colitis model (Buanne et al., 2007). Furthermore, in vivo studies with mice, rat, and primates, exposed to cigarette smoke or lipopolysaccharide, demonstrated that the small CXCR2 antagonist Sch527123 (Chapman et al., 2007) reduces neutrophil infiltration into the bronchoalveolar lavage (BAL) fluid, thereby reducing the associated lung tissue damage (Thatcher et al., 2005; Chapman et al., 2007). Thus, in CXCR2 knockout mice or wild-type mice treated with a CXCR2 antagonist or neutralizing antibody, lung tissue damage and ulcerative colitis are reduced, suggesting that CXCR2 is an important drug target (Buanne et al., 2007). In view of this therapeutic potential, different classes of small CXCR2 antagonist have been developed, including diarylureas (Widdowson et al., 2004), thiazolo- and imidazolylpyrimidines (Baxter et al., 2006; Ho et al., 2006), quinoxalines (Li et al., 2003), nicotinamide *N*-oxides (Cutshall et al., 2001), indole carboxylic acids (Barth et al., 2002), and arylpropionic acids (Allegretti et al., 2005). So far, most literature describes in vitro data of the different CXCR2 antagonist classes. However, both diarylurea and arylpropionic compounds have shown promising in vivo data, and clinical trials are ongoing with some of these compounds (Bizzarri et al., 2006).

Despite the clinical interest in CXCR2 antagonists, little is known about their molecular mechanism of action. The large peptidergic chemokines bind to the N terminus and extracellular loops of their receptors, but small-molecule antagonists are considered generally to bind to the 7TM domains (Rajagopalan and Rajarathnam, 2006; Allen et al., 2007; Viola and Luster, 2008), suggesting allosteric interactions between chemokines and small-molecule antagonists. It is interesting that recently, the CXCR2 antagonist SB332235 was suggested to bind to the intracellular domain of CXCR2 (Nicholls et al., 2008).

In this study, seven different CXCR2 antagonists of three classes have been selected and subjected to a detailed pharmacological characterization. Three compounds of the diarylurea class have been chosen (SB225002, SB332235, and SB265610) (Bizzarri et al., 2006), as well as three imidazolylpyrimidine compounds (Conti et al., 2004; Erickson et al., 2004; Ho et al., 2006) and one thiazolopyrimidine compound from patent literature, named herein VUF10948 (Willis et al., 2001).

The studies presented in this article show that all compounds are both able to displace ^{125}I-CXCL8 from human CXCR2 and to inhibit CXCR2-induced β-arrestin2 recruitment. By investigating one representative of each class in more detail, we suggest that the compounds are allosteric modulators at CXCR2. By radiolabeling the potent CXCR2 antagonist SB265610, we found that compounds of the different chemical classes bind to distinct binding sites.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, penicillin, and streptomycin were all obtained from PAA Laboratories GmbH (Linz, Austria). Fetal bovine serum was purchased from Integro B.V. (Dieren, The Netherlands). DMEM containing 25 mM HEPES and L-glutamine, Opti MEM I, hygromycin B, and G-418 (Geneticin) were obtained from Invitrogen (Paisley, UK), and fetal calf serum was purchased from Cambrex Bio Sciences (Verviers, Belgium). Chloroquine diphosphate and DEAE-dextran were obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin fraction V was purchased from Roche Diagnostics (Mannheim, Germany). ^{125}I-CXCL8 (2200 Ci/mmol) or ^{125}I was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA), whereas the unlabeled chemokines were purchased from PeproTech (Rocky Hill, NJ) or from R&D Systems (Minneapolis, MN). All CXCR2 antagonists and [^{3}H]SB265610 (26.07 Ci/mmol) were synthesized at the Schering-Plough Research Institute (Oss, The Netherlands).

Cell Culture and Transfection of hCXCR2. COS-7 cells were grown at 5% CO_2 and 37°C in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal bovine serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin. COS-7 cells were transiently transfected using the DEAE-dextran method (Brakenhoff et al., 1994). In brief, cells were trypsinized, washed once in RPMI 1640, supplemented with 2% fetal bovine serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin, and resuspended in the same solution containing 100 μM chloroquine, 0.8 mg/ml DEAE-dextran, and 2 μg of pcDEF$_3$-hCXCR2 (Goldman et al., 1996) or pcDNA3-hCXCR1 cDNA per 10^6 cells. Cells were incubated at 5% CO_2 and 37°C for 1 h and then plated out in growth medium. After 48 h, the cells were washed once in phosphate-buffered saline, scraped, and pelleted for preparation of membranes.

PathHunter HEK293-CXCR2 cells (DiscoveRx Corporation, Fremont, CA) were grown at 5% CO_2 and 37°C in DMEM with 25 mM HEPES and L-glutamine supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, 800 μg/ml G-418, and 200 μg/ml hygromycin B.

Radioligand Binding Assays. Pellets of COS-7 membranes expressing hCXCR1 or hCXCR2 were resuspended in ice-cold binding buffer (50 mM Na_2HPO_4 and 50 mM KH_2PO_4, pH 7.4) and homogenized 15 times with a Dounce homogenizer. Protein concentration in membrane preparations was determined using the BioRad Protein Determination assay 18 from Bio-Rad (Hercules, CA).

Competition binding, saturation binding, and binding kinetics analyses of ^{125}I-CXCL8 and [^{3}H]SB265610 were all performed at COS-7 membranes expressing human CXCR1 or CXCR2 in binding buffer (50 mM Na_2HPO_4 and 50 mM KH_2PO_4, pH 7.4) at room temperature in a final volume of 100 to 200 μl. After the indicated incubation times, membranes were harvested with a Brandel harvester or with rapid filtration through Unifilter GF/C 96-well filter plates (PerkinElmer Life and Analytical Sciences) pretreated with 0.3% polyethylenimine and washed three times with ice-cold wash buffer (50 mM Na_2HPO_4 and 50 mM KH_2PO_4, pH 7.4). Bound ra-

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dioactivity was determined using a Tri-Carb 1900 Hewlett Packard counter (PerkinElmer Life and Analytical Sciences) or a MicroBeta counter (PerkinElmer Life and Analytical Sciences).

For ^{125}I-CXCL8 competition binding assays, membranes (approximately 10 μg/data point) were incubated with indicated concentrations of antagonists and approximately 300 pM ^{125}I-CXCL8 for 1 h. To determine saturation binding of ^{125}I-CXCL8, membranes (approximately 20 μg/data point) were incubated with indicated concentrations of ^{125}I-CXCL8 in the absence or presence of 20 nM SB265610, 50 nM compound 1, or 200 nM VUF10948. Nonspecific binding was determined with 30 nM CXCL1.

Single-point [^{3}H]SB265610 competition binding was performed with 8-μg membranes, in absence or presence of 10 μM VUF10948 and 3.8 nM [^{3}H]SB265610 for 1 h at room. For experiments to determine the association rate of [^{3}H]SB265610, membranes (approximately 2 μg/data point) were incubated for the indicated times with 7 nM [^{3}H]SB265610 in the absence or presence of 10 μM VUF10948. To measure the dissociation rate of [^{3}H]SB265610, membranes (approximately 2 μg/data point) were incubated for 1 h with 7 nM [^{3}H]SB265610 before the addition of 10 μM VUF10948. Samples were taken at the indicated times, bound and free radioactivities were separated and determined by liquid scintillation. For saturation binding with [^{3}H]SB265610, membranes (approximately 6 μg/data point) were incubated with indicated concentrations of [^{3}H]SB265610 for 1 h in the absence or presence of 10 μM VUF10948 to determine total and nonspecific binding. In competition binding experiments with various concentrations of cold ligands, membranes (approximately 4 μg/data point) were incubated with indicated concentrations of antagonist or chemokines and approximately 10 nM [^{3}H]SB265610 for 1 h at room temperature.

Binding data were evaluated by a nonlinear curve fitting procedure using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA). Ligand affinities (pK_i) from competition binding experiments were calculated from binding IC_{50} using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

β-Arrestin Recruitment Assay. PathHunter HEK293-CXCR2 cells were plated out overnight at 10,000 cells/well (384-well format) in 20 μl of Opti MEM I. A preincubation with CXCR2 antagonists or vehicle (phosphate-buffered saline + 0.1% bovine serum albumin) of 30 min at 37°C and 5% CO_2 was followed by 60-min CXCL8 stimulation at 37°C and 5% CO_2. Next, the plate was placed at room temperature for 30 min; thereafter, 12 μl of PathHunter Detection Reagents (DiscoveRx Corporation) was added. After an incubation of 60 min at room temperature, β-galactosidase-induced luminescence upon β-arrestin-CXCR2 interaction was measured for 0.3 s in an Envision 2102 Multilabel Reader (PerkinElmer Life and Analytical Sciences). Functional data were evaluated by a nonlinear curve fitting procedure using GraphPad Prism 4.0 (GraphPad Software Inc.).

Results

Competition Binding Analysis with ^{125}I-CXCL8 at hCXCR2. Various nonpeptidergic ligands with distinct structural features (Fig. 1) recently have been reported to effectively inhibit CXCR2 function (White et al., 1998; Podolin et al., 2002; Ho et al., 2006). In this study, we examined a selection of recently developed diarylurea- and pyrimidine-based CXCR2 antagonists in more detail. Membranes of COS-7 cells transiently transfected with human CXCR2 were incubated with ^{125}I-CXCL8 and indicated concentrations of CXCL8, SB265610, compound 1, or VUF10948 (Fig. 2A). Analysis of homologous displacement with CXCL8 revealed binding of ^{125}I-CXCL8, with a K_d of 0.49 ± 0.07 nM and a B_{max} of 27.5 ± 5.8 fmol/mg protein (n = 3). All tested CXCR2 antagonists dose-dependently displaced ^{125}I-CXCL8 binding to human CXCR2. The pK_i values of the diarylurea compounds (SB225002, SB332235, and SB265610) are approximately 7.7, whereas the pK_i values of the tested pyrimidine derivatives (VUF10948, compound 1, compound 2, and compound 3) are in the range of 6.4 to 7.3 (Table 1).

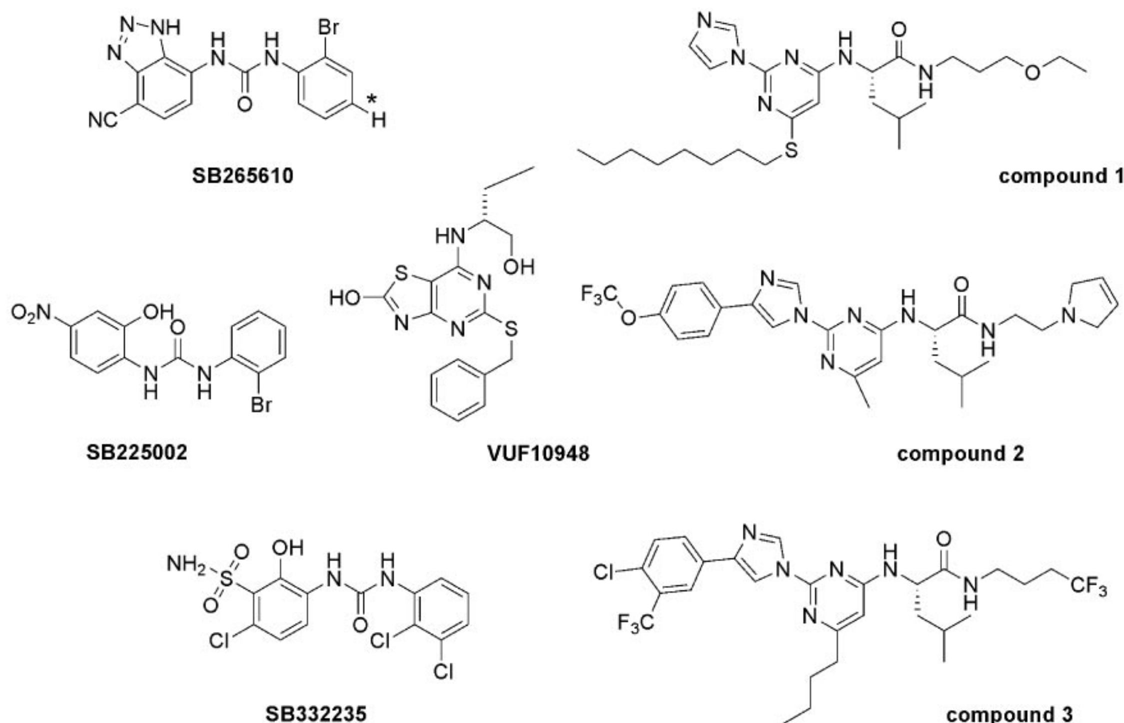

Fig. 1. The structures of nonpeptidergic CXCR2 antagonists are shown. SB265610, SB225002, and SB332235 belong to the diarylurea class. VUF10948 belongs to the thiazolopyrimidine class, and compound 1, compound 2, and compound 3 belong to the imidazolylpyrimidine class. SB265610 has been labeled with a tritium atom, indicated in the figure on an H-atom (*).

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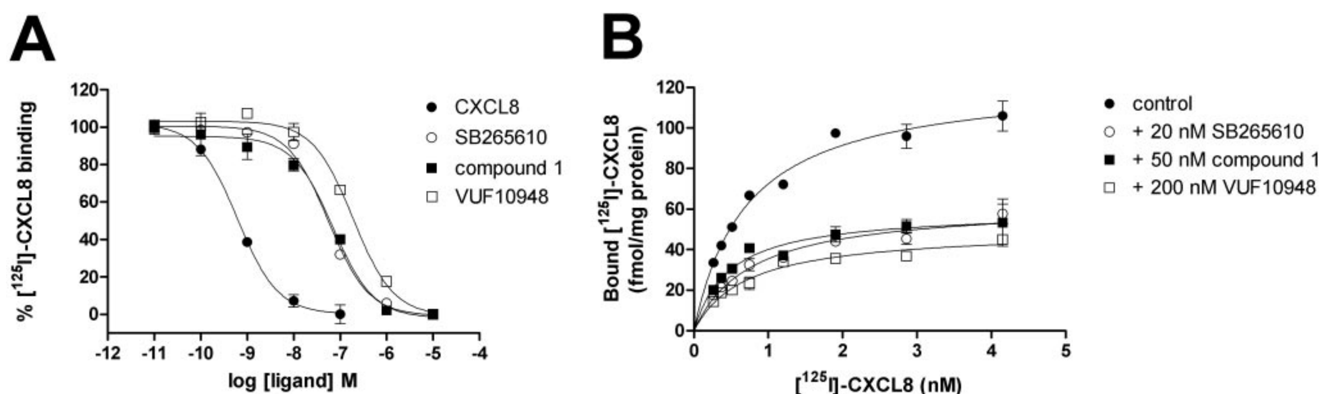

Fig. 2. Displacement of ^{125}I-CXCl8 binding to COS-7 cell membranes with CXCL8, SB265610, compound 1, and VUF10948 (A). Membranes were incubated with the indicated concentrations of CXCL8 (●), SB265610 (○), compound 1 (■), or VUF10948 (□) and approximately 300 pM ^{125}I-CXCL8. Data of triplicate determinations from a representative experiment (n = 3–6) are expressed as the percentage of ^{125}I-CXCL8 binding ± S.E.M. Saturation binding analysis with ^{125}I-CXCL8 at COS-7 membranes expressing hCXCR2 (B). Membranes were incubated with the indicated concentrations of ^{125}I-CXCL8 in the absence (●) or presence of SB265610 (○), compound 1 (■), or VUF10948 (□). Data show the mean specific binding ± S.E.M. of triplicate determinations from a representative experiment (n = 2–4).

TABLE 1

Properties of small nonpeptidergic antagonists at human CXCR2

Competition binding with nonpeptidergic CXCR2 antagonists at COS-7 membranes expressing hCXCR2 and inhibition of CXCL8-stimulated β-arrestin2 recruitment of these antagonists in PathHunter HEK293-hCXCR2 cells. COS-7-hCXCR2 membranes were incubated with nonpeptidergic CXCR2 antagonists (100 nM–10 μM) and approximately 300 pM [^{125}I]CXCL8 or approximately 10 nM [^{3}H]SB265610, respectively. Data shown are mean values of triplicate determinations (n = 2–3) ± S.E.M. PathHunter HEK293-hCXCR2 cells were preincubated for 30 min with nonpeptidergic CXCR2 antagonists (1 pM–100 μM), followed by stimulation with 7.6 nM CXCL8. Data shown are mean values of triplicate determinations (n = 3–6) ± S.E.M.

Antagonist	[^{125}I]CXCL8 Displacement (pK_i ± S.E.M.)	Inhibition of CXCL8-Induced β-Arrestin2 Recruitment (pK_b ± S.E.M.)	[^{3}H]SB265610 Displacement (pK_i ± S.E.M.)
SB265610	7.72 ± 0.08	8.63 ± 0.14	8.46 ± 0.06
SB332235	7.70 ± 0.15	8.92 ± 0.13	9.12 ± 0.18
SB225002	7.69 ± 0.21	7.88 ± 0.18	8.26 ± 0.25
VUF10948	6.78 ± 0.10	7.68 ± 0.09	7.71 ± 0.24
Compound 1	7.33 ± 0.09	7.16 ± 0.09	N.D.
Compound 2	6.37 ± 0.17	6.31 ± 0.20	N.D.
Compound 3	6.63 ± 0.11	6.88 ± 0.00	N.D.

N.D., no displacement of the radiolabeled SB265610 compound by cold ligand.

Saturation Binding of ^{125}I-CXCL8 at hCXCR2. Saturation binding analysis of ^{125}I-CXCL8 binding to COS-7 cell membranes expressing human CXCR2 (Fig. 2B) resulted in a K_d value of 0.66 ± 0.1 nM and a B_{max} value of 131.11 ± 4.1 fmol/mg protein (n = 4). In the presence of 20 nM SB265610, the K_d value of CXCL8 is not affected (0.56 ± 0.2 nM), whereas the B_{max} decreased to 56.3 ± 6.5 fmol/mg. This indicates that SB265610 is a noncompetitive antagonist. Likewise, compound 1, a representative of the imidazolylpyrimidine-based CXCR2 antagonists, did not affect the K_d value of CXCL8 (0.36 ± 0.1 nM) but decreased the B_{max} value (52.0 ± 7.1 fmol/mg protein). Furthermore, the representative of the thiazolopyrimidine class (VUF10948) also did not affect the K_d value of CXCL8 (0.76 ± 0.1 nM), whereas the B_{max} value decreased to 65.8 ± 16.1 fmol/mg.

Antagonism of CXCL8-Stimulated β-Arrestin2 Recruitment. Activation of CXCR2 by CXCL8 has been shown to lead to recruitment of β-arrestin (Richardson et al., 2003). To monitor direct interaction of CXCR2 with β-arrestin2, we used a β-arrestin2 recruitment assay for CXCR2 based on enzyme complementation of β-galactosidase (Olson and Eglen, 2007), as established by DiscoveRx Corporation (PathHunter HEK293-hCXCR2). Stimulation of the PathHunter HEK293-hCXCR2 cells with CXCL8 induces β-arrestin2 recruitment, as indicated by a 18.8- ± 1.3-fold increase in β-galactosidase activity (pEC_{50} = 8.49 ± 0.04, n = 16) (Fig. 3, C–E). All CXCR2-antagonists were able to dose-dependently inhibit the CXCL8-induced (at EC_{80} concentration of 7.6 nM) β-arrestin2 recruitment (Fig. 3A). Data obtained with this functional assay correlate with the pK_i values of the CXCR2 ligands obtained in the ^{125}I-CXCL8 binding studies (r = 0.73; Fig. 3B). The pK_b values of the diarylurea compounds are in the range of 7.9 to 8.9, whereas the pK_b values of the pyrimidine derivatives are in the range of 6.3 to 7.7 (Table 1). It is interesting that VUF10948 was the only tested CXCR2 antagonist that was not able to fully inhibit the CXCL8-induced signal, suggesting that this compound behaves as a noncompetitive antagonist or as a partial agonist.

From the seven compounds tested, a diarylurea (SB265610), an imidazolylpyrimidine (compound 1), and a thiazolopyrimidine (VUF10948) were chosen as representatives of their class to evaluate their mode of action in more detail. Cells were stimulated with increasing concentrations of CXCL8 in the absence or presence of SB265610 (Fig. 3C), compound 1 (Fig. 3D), or VUF10948 (Fig. 3E). Preincubation with SB265610, compound 1, or VUF10948 results in a rightward shift of the CXCL8 dose-response curve but also reduces the maximal response of CXCL8-induced β-arrestin2 recruitment, indicating again that these compounds behave as noncompetitive antagonists. It is interesting that VUF10948 showed to be a weak partial CXCR2 agonist (Fig. 3F), whereas the other tested compounds showed no partial agonistic effects (data not shown).

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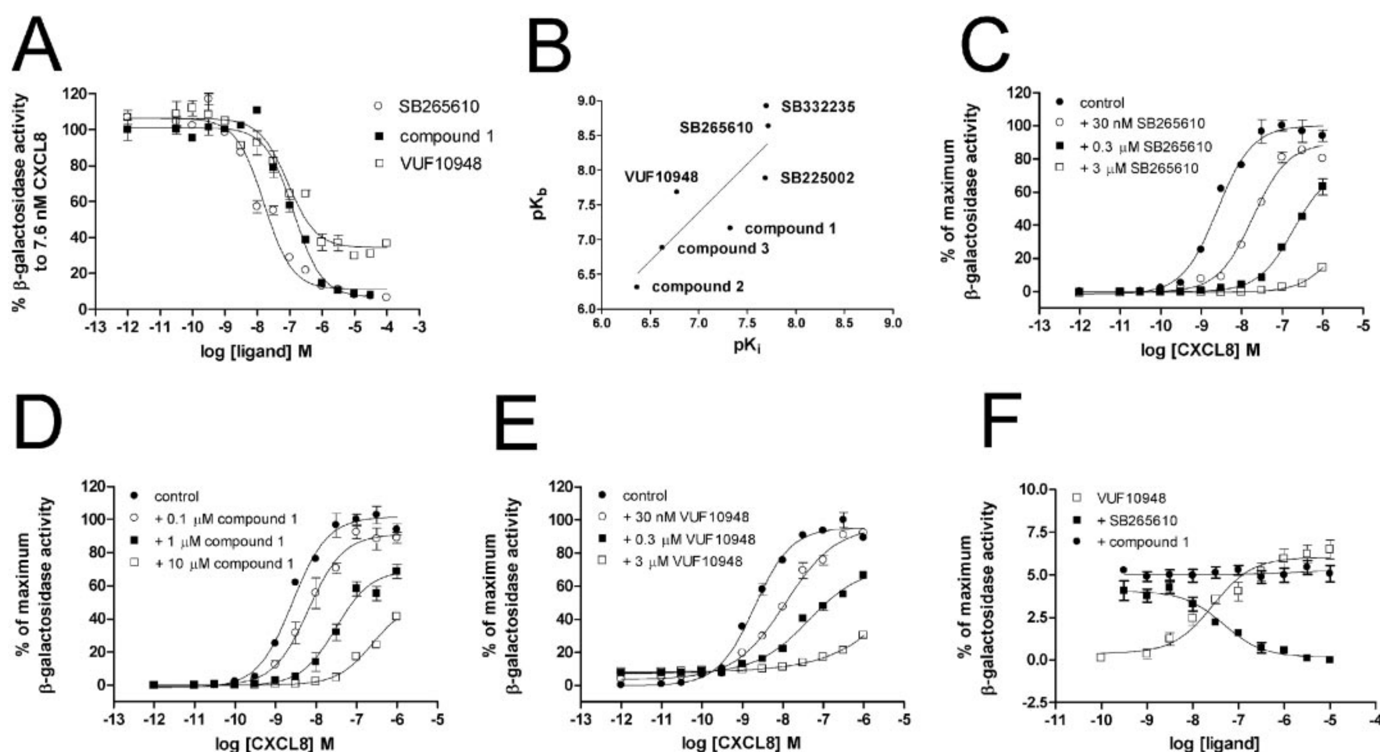

Fig. 3. Effect of nonpeptidergic CXCR2 antagonists on CXCL8-stimulated β-arrestin2 recruitment in PathHunter HEK293-hCXCR2 cells. CXCL8-induced β-arrestin2 recruitment is dose-dependent (pEC_{50} = 8.49 ± 0.04, n = 16). Data are expressed as percentage of maximal β-galactosidase activity ± S.E.M. of a representative experiment performed in triplicate (n = 16) (C–E). PathHunter HEK293-hCXCR2 cells were pretreated for 30 min with indicated concentrations of SB265610 (○), compound 1 (■), or VUF10948 (□) followed by stimulation with 7.6 nM CXCL8 (EC_{80}). Data of triplicate determinations from a representative experiment (n = 3–6) are expressed as the percentage of β-galactosidase activity in response to 7.6 nM CXCL8 ± S.E.M. (A). Relationship between the pK_i values as determined in ^{125}I-CXCL8 binding assay and the antagonistic potency as determined in the β-arrestin 2 recruitment assay is shown (r^2 = 0.73) (B). Furthermore, PathHunter HEK293-hCXCR2 cells were pretreated with the indicated concentrations of SB265610 (C), compound 1 (D), or VUF10948 (E), followed by dose-dependent stimulation of CXCL8 stimulation. Data of triplicate determinations from a representative experiment (n = 2–4) are expressed as percentage of maximum β-galactosidase activity ± S.E.M. VUF10948 dose-dependently (pEC_{50} = 7.39 ± 0.14) partially activates β-arrestin recruitment (□, n = 5) (F). Pretreatment for 30 min with indicated concentrations of SB265610 (■) or compound 1 (●) was followed by stimulation with 0.3 μM VUF10948 (EC_{90}). Data of triplicate determinations from a representative experiment (n = 3) are expressed as the percentage of β-galactosidase activity in response to 100 nM CXCL8 ± S.E.M.

Stimulation of cells with 10 μM VUF10948 resulted in 6.18 ± 1.0% (n = 5) β-galactosidase activity upon β-arrestin2 recruitment (pEC_{50} = 7.39 ± 0.14). At 0.3 μM (EC_{90} concentration), this signal was dose-dependently inhibited by SB265610 (pK_b = 8.11 ± 0.02). It is interesting that compound 1 was not able to inhibit the partial agonistic effect of VUF10948 (Fig. 3F).

[^{3}H]SB265610 as Radioligand for CXCR2. Because the diarylurea class of CXCR2 antagonists has distinct structural features compared with the pyrimidine derivatives (Fig. 1), we set out to determine whether they bind to the same site at the human CXCR2. To this end, we radiolabeled the high-affinity CXCR2 antagonist SB265610 with tritium and subjected [^{3}H]SB265610 to a detailed analysis. Binding of [^{3}H]SB265610 is proportional to the amount of membrane protein present (data not shown). Moreover, [^{3}H]SB265610 binds specifically to human CXCR2 and does not bind to mock or CXCR1-expressing COS-7 cell membranes (Fig. 4A).

In association studies, [^{3}H]SB265610 rapidly binds to membranes of COS-7 cells expressing human CXCR2. Half-maximal specific binding was reached within 5.2 ± 0.6 min and equilibrium at 30 min, remaining stable thereafter (Fig. 4B). [^{3}H]SB265610 binding was rapidly reversed ($t_{1/2}$ = 7.5 ± 2.7 min) by the addition of 10 μM VUF10948 (Fig. 4C). The association and dissociation constants of [^{3}H]SB265610 calculated from the kinetic data given in Fig. 4 are 8.3 × 10^6 min/M (derived from observed K_{on} 0.19 ± 0.02 min^{-1}) and 0.13 ± 0.06 min^{-1}, respectively, yielding a K_d of 16 nM.

Incubation of membranes of cells expressing human CXCR2 with increasing concentrations of [^{3}H]SB265610 in the absence or presence of 10 μM VUF10948 showed that the specific binding of [^{3}H]SB265610 was saturable (Fig. 4D). The K_d and B_{max} values obtained from saturation binding experiments were 2.51 ± 1.5 nM and around 50 pmol/mg, respectively.

Effect of GTPγS on ^{125}I-CXCL8 and [^{3}H]SB26510 Binding. To determine whether G protein coupling affects ^{125}I-CXCL8 and [^{3}H]SB265610 binding to CXCR2, binding experiments were performed in the absence and presence of GTPγS. ^{125}I-CXCL8 binding analysis in the presence of indicated concentrations of GTPγS to COS-7 cell membranes expressing human CXCR2 shows a dose-dependent inhibition of ^{125}I-CXCL8 binding (Fig. 5). It is noteworthy that GTPγS cannot fully inhibit the ^{125}I-CXCL8 binding. In contrast, the binding of [^{3}H]SB265610 is not affected by GTPγS at 10 μM (insert, Fig. 5). These results indicate that ^{125}I-CXCL8 mainly binds to human CXCR2 coupled to G proteins, whereas [^{3}H]SB265610 can bind to both G protein-coupled and uncoupled human CXCR2 conformations.

Competition Binding Analysis with [^{3}H]SB256510 at hCXCR2. Membranes of COS-7 cells transiently transfected

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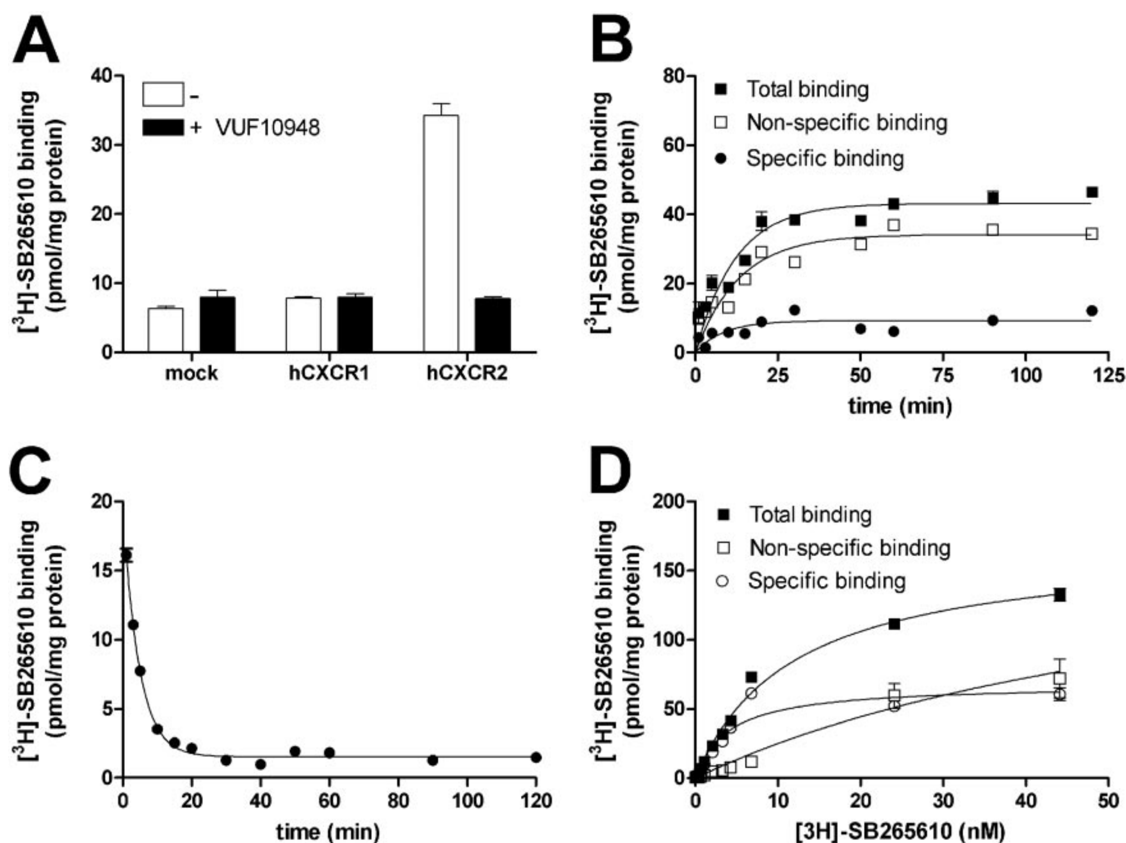

Fig. 4. Binding of [^{3}H]SB265610 to COS-7 membranes expressing hCXCR1 or hCXCR2 (A) in the absence (open bars) or presence of 10 μM VUF10948 (filled bars). Membranes were incubated at room temperature for 1 h with 3.8 nM [^{3}H]SB265610 (n = 2). To measure the association rate of [^{3}H]SB265610 (B), membranes were incubated at room temperature for the indicated times with 7 nM [^{3}H]SB265610 in the absence (■, total binding) or presence (□, nonspecific binding) of 10 μM VUF10948, resulting in specific binding (●). Data show the mean ± S.E.M. of triplicate determinations measured at the indicated times from a representative experiment (n = 6). To measure the dissociation rate of [^{3}H]SB265610 (D), membranes from COS-7-hCXCR2 were incubated with 7 nM [^{3}H]SB265610 for 1 h at room temperature before the addition of 10 μM VUF10948. Data show the mean ± S.E.M. of triplicate determinations measured at the indicated times from a representative experiment (n = 4). Saturation binding analysis of [^{3}H]SB265610 binding to COS-7 membranes expressing hCXCR2 (D). Membranes where incubated with the indicated concentrations of [^{3}H]SB265610 for 1 h at room temperature. Nonspecific binding was determined in the presence of 10 μM VUF10948. Data show the mean binding ± S.E.M. of triplicate, total binding (■), nonspecific binding (□), or specific binding (○) from a representative experiment (n = 2).

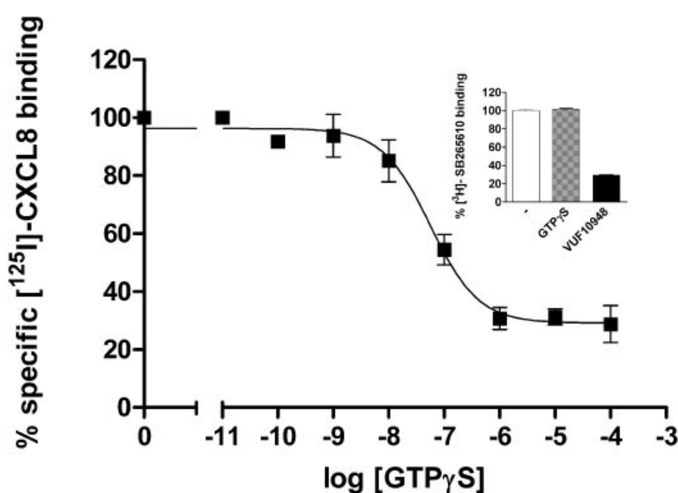

Fig. 5. Competition binding of ^{125}I-CXCL8 or [^{3}H]SB265610 and GTPγS at COS-7 membranes expressing hCXCR2. Membranes were incubated with the indicated concentrations of GTPγS and approximately 300 pM ^{125}I-CXCL8 or 10 μM GTPγS and 5 nM [^{3}H]SB265610 (insert). Nonspecific binding was determined in the presence of 10 μM VUF10948. Data of triplicate determinations from a representative experiment (n = 2) are expressed as the percentage of ^{125}I-CXCL8 binding or [^{3}H]SB265610 binding ± S.E.M., respectively.

with human CXCR2 were incubated for 1 h at room temperature with [^{3}H]SB265610 and the indicated concentrations of CXCL1, CXCL8, SB265610 (Fig. 6A), VUF10948, or compound 1 (Fig. 6B). The K_d and B_{max} values for SB265610 obtained from homologous displacement were 3.48 ± 0.63 nM and 25.7 ± 8.1 pmol/mg protein, respectively. These values are in good agreement with the data obtained in the saturation binding analysis. As expected, the diarylurea compounds (SB225002, SB332235, and SB265610) all displaced [^{3}H]SB265610 with pK_i values in the range of 7.7 to 9.1 (Table 1). However, the chemokines CXCL8, CXCL1, and the imidizolylpyrimidine compounds (compound 1, compound 2, and compound 3) do not displace [^{3}H]SB265610 up to 0.1 and 10 μM, respectively (Fig. 6; Table 1).

Discussion

CXCR2 has attracted considerable attention as a potential drug target because of its involvement in different inflammatory diseases, like chronic obstructive pulmonary disease, psoriasis, rheumatoid arthritis, and ulcerative colitis (Kulke et al., 1998; Podolin et al., 2002; Donnelly and Barnes, 2006; Buanne et al., 2007). As a consequence, different classes of small CXCR2 antagonists have been developed, including

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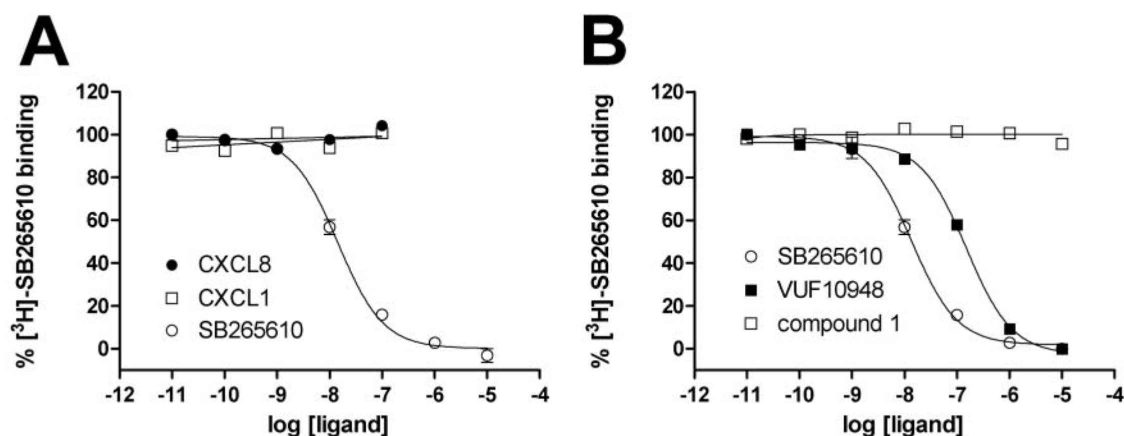

Fig. 6. Competition binding of [^{3}H]SB265610 and CXCL8, CXCL1, SB265610, VUF10948, and compound 1 at COS-7 membranes expressing hCXCR2. Membranes were incubated with the indicated concentrations of CXCL8 (●), CXCL1 (□), SB265610 (○) (A), VUF10948 (■), or compound 1 (□) (B) and approximately 10 nM [^{3}H]SB265610 for 1 h at room temperature. Data of triplicate determinations from a representative experiment (n = 2) are expressed as the percentage of [^{3}H]SB265610 binding ± S.E.M.

diarylureas, thiazolo- and imidazolylpyrimidines, quinoxalines, nicotinamide *N*-oxides, indole carboxylic acids, and arylpropionic acids (Cutshall et al., 2001; Barth et al., 2002; Li et al., 2003; Widdowson et al., 2004; Allegretti et al., 2005; Baxter et al., 2006; Ho et al., 2006). In this study, we selected seven different CXCR2 antagonists from the diarylurea, imidazolylpyrimidine, and thiazolopyrimidine class and studied their mechanisms of action at human CXCR2. In addition, the potent CXCR2 antagonist SB265610 was radiolabeled and used to identify distinct binding sites at the human CXCR2 for the studied CXCR2 antagonists.

The data presented in this study show ^{125}I-CXCL8 displacement by all nonpeptidergic CXCR2 antagonists (Fig. 2). The obtained pK_i values of both the diarylurea and pyrimidine compounds are in good agreement with earlier published data (White et al., 1998; Podolin et al., 2002; Catusse et al., 2003; Ho et al., 2006; Gonsiorek et al., 2007). All tested compounds were able to inhibit CXCL8-induced β-arrestin2 recruitment in human CXCR2-transfected cells, with a rank order of SB332235 ~ SB265610 > SB225002 ~ VUF10948 > compound 1 > compound 3 > compound 2 (Fig. 3). This rank order correlates well with the binding affinity of these compounds to human CXCR2. Furthermore, these data are consistent with previous reported values for SB225002 inhibiting CXCL8-induced β-arrestin2 recruitment (Yan et al., 2002).

The compounds SB265610, compound 1, and VUF10948 were chosen as representatives of the different chemical CXCR2 antagonist classes and subjected to a detailed study to determine their antagonistic behavior. Schild plot analysis using the β-arrestin2 recruitment assay showed that the dose-response curves of CXCL8 in the presence of SB265610, compound 1, or VUF10948 did not reach the maximal response (Fig. 3). This indicates that all studied nonpeptidergic antagonists behave as noncompetitive antagonists at human CXCR2. Of the different CXCR2 antagonists tested, only VUF10948 was not able to fully inhibit CXCL8-induced β-arrestin2 recruitment. This can be ascribed to the partial agonistic properties of this compound at high concentrations. ^{125}I-CXCL8 saturation binding studies in the presence of SB265610, compound 1, or VUF10948 showed a decrease of the maximal number of ^{125}I-CXCL8 binding sites but no alteration in the binding affinity of ^{125}I-CXCL8 (Fig. 2). Hence, all the CXCR2 antagonists of the different chemical classes tested in this study displace and antagonize CXCL8, most probably via a noncompetitive, allosteric mechanism. This mechanism of action is common for other small antagonists targeting chemokine receptors (Gonsiorek et al., 2007; Verzijl et al., 2008). The allosteric inhibition by the tested CXCR2 antagonists is expected, as in general chemokines, like CXCL8, are thought to bind to the extracellular part of the GPCR protein (Rajagopalan and Rajarathnam, 2006; Allen et al., 2007; Viola and Luster, 2008), notably the N terminus and the extracellular loops. In contrast, small antagonists are considered to bind to the 7TM domains of GPCRs, as shown for AMD3100 at CXCR4, TAK-779 at CCR5, and BX 471 at CCR1 (Allen et al., 2007) or possibly to the intracellular site of the receptor, as recently suggested for a thiazolopyrimidine compound and SB332235 at CXCR2 (Nicholls et al., 2008). However, it should be noted that an earlier study on a derivative on SB332235, SB225002, implicated the involvement of the N terminus and amino acids in the extracellular loops and transmembrane domains in the binding of the CXCR2 antagonist (Catusse et al., 2003).

Subsequently, we tritium-labeled SB265610 and used [^{3}H]-SB265610 as a new tool to investigate the nature of the binding sites of CXCR2 antagonists at the human CXCR2. The binding of this radioligand is reversible and selective for human CXCR2 (Fig. 4). Compared with the recently radiolabeled CXCR2 antagonist Sch527123 (Gonsiorek et al., 2007), [^{3}H]SB265610 has a faster K_{off} and, therefore, an increased K_d. It is noteworthy that the B_{max} value obtained using [^{3}H]SB265610 is higher compared with that when using ^{125}I-CXCL8. [^{3}H]Sch527123 also revealed a higher B_{max} value for CXCR2 compared with the value obtained using ^{125}I-CXCL8 (Gonsiorek et al., 2007). This difference is most probably caused by the fact that ^{125}I-CXCL8 mainly binds to the G protein-coupled state of the receptor, as shown by loss of CXCL8 binding in the presence of GTPγS, whereas [^{3}H]SB265610 can bind to both G protein-coupled and uncoupled receptors (Fig. 5). This explanation for differences of B_{max} values was reported earlier in studies using different radiolabeled chemokines acting at human CXCR3 (Cox et al.,

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2001) or the virally encoded chemokine receptor ORF74 (Verzijl et al., 2006).

CXCL8 is not able to displace [^{3}H]SB265610, providing evidence that CXCL8 binds to another binding site compared with the small CXCR2 antagonists. It is most interesting that we observed that the imidazolylpyrimidine compounds were not able to displace [^{3}H]SB265610, whereas the diarylurea and thiazolopyrimidine compounds inhibited the binding of this radioligand (Fig. 6). Furthermore, the partial agonistic effect of the thiazolopyrimidine VUF10948 was inhibited by the diarylurea SB265610, whereas the imidazolylpyrimidine compound 1 was not able to inhibit this effect (Fig. 3). Thus, we conclude that there are not only distinct binding sites for chemokines and small nonpeptidergic antagonists at human CXCR2 but also for the different CXCR2 antagonists. Although some data are available on the binding site of CXCR2 antagonists of the diarylurea and thiazolopyrimide class (Catusse et al., 2003; Nicholls et al., 2008), more research is required to further explore and define the direct interaction sites of the antagonists with CXCR2. Combining CXCR2 modeling studies, based on the recently reported crystal structure of the human β-adrenergic receptor (Cherezov et al., 2007), with mutagenesis studies and use of radiolabeled low-molecular weight CXCR2 antagonists provides opportunities to investigate the different binding sites of CXCR2 antagonists in more detail.

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