Pharmacological Characterization of Sch527123, a Potent Allosteric CXCR1/CXCR2 Antagonist

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ABSTRACT

In neutrophils, growth-related protein-α (CXCL1) and interleukin-8 (CXCL8), are potent chemoattractants (Cytokine 14:27–36, 2001; Biochemistry 42:2874–2886, 2003) and can stimulate myeloperoxidase release via activation of the G protein-coupled receptors CXCR1 and CXCR2. The role of CXCR1 and CXCR2 in the pathogenesis of inflammatory responses has encouraged the development of small molecule antagonists for these receptors. The data presented herein describe the pharmacology of 2-hydroxy-4-nitrophenyl)-1-(5-methyl-furan-2-yl)-propyl[amino]-3,4-dioxo-cyclobut-1-enylamino}-benzamide; repertaxin, (+)-cyclohexyl(hydroxy)methyl]-2,5-dioxo-1-butyl-3-[[(2,3-dichlorophenyl)urea; SB-468477, N-(3-chloro-4-methylphenyl)piperidine-4-carboxamide.

CXCR1 and CXCR2 are G protein-coupled receptors for a number of chemoattractant cytokines (chemokines). Both CXCR1 and CXCR2 are expressed on human neutrophils and mediate both neutrophil chemotaxis and myeloperoxidase release (Ahuja et al., 1996; Patel et al., 2001; Peniger-Barash et al., 2003). CXCR2 expression has also been demonstrated on monocytes and alveolar macrophages (Traves et al., 2004).

Interleukin-8 (IL-8) (CXCL8) is the most potent CXCR1 ligand, although granulocyte chemotactic protein-2 (CXCL6) binds to the receptor with nanomolar affinity (Wolf et al., 1998). CXCR2, on the other hand, is quite promiscuous, binding growth-related protein-α, -β, and -γ (CXCL1-3), epithelial-derived neutrophil attractant-78 (CXCL5), CXCL6, neutrophil-activating peptide-2 (CXCL7), and CXCL8. The receptors and/or their ligands are known to be elevated in a variety of inflammatory diseases, including rheumatoid arthritis, psoriasis, inflammatory bowel disease, acute respiratory distress syndrome, septic shock, pulmonary emphysema, and chronic obstructive pulmonary disease (Nickoloff et al., 1991; Woods et al., 2000; Kudrowska et al., 2002; Banks et al., 2003; Bech et al., 2003). The putative role of CXCR1 and/or CXCR2 in inflammatory diseases has stimulated

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ABBREVIATIONS: IL-8, interleukin-8 (CXCL8); CXCL5, endothelial cell-derived neutrophil activating protein 78; fMLP, formyl-methionyl-leucyl-phenylalanine; CXCL6, granulocyte chemotactic protein-2; CXCL1-3, growth-related protein-α, -β, and -γ; [35S]GTPγS, guanosine 5’-y-35S-triphosphate, triethylammonium salt; IL-3, interleukin-3; MPO, myeloperoxidase; CXCL7, neutrophil-activating peptide-2; FBS, fetal bovine serum; RLU, relative light intensity unit; WGA-SPA, wheat germ agglutinin bead-scintillation proximity assay; Sch527123, 2-hydroxy-4-nitrophenyl)-1-(5-methyl-furan-2-yl)-propyl[amino]-3,4-dioxo-cyclobut-1-enylamino}-benzamide; repertaxin, (+)-cyclohexyl(hydroxy)methyl]-2,5-dioxo-1-butyl-3-[[(2,3-dichlorophenyl)urea; SB-468477, N-(3-chloro-4-methylphenyl)piperidine-4-carboxamide.

TAK-220, 1-acetyl-N-[3-[4-(4-carbanoylbenzyl)piperidin-1-yl]propyl-N-(3-chloro-4-methylphenyl)piperidine-4-carboxamide.
the development of small molecule antagonists with nanomolar affinity for either CXCR1, such as repertaxin [(R)-(−)-2-(4-isobutylphenyl)propionyl methanesulfonyamide] (Bertini et al., 2004), or CXCR2, such as SB-225002 [N-(2-hydroxy-4-nitrophenyl)-N′-(2-bromophenyl)urea], or SB-332235 [N-(2-hydroxy-3-sulfanyl-4-chlorophenyl)-N′-(2,3-dichlorophenyl)urea] (White et al., 1998). A dual CXCR1/CXCR2 antagonist, SB-468477 [N-(2-hydroxy-3-dimethylsulfonylamido-4-chlorophenyl)-N′-(2-bromophenyl)-N″-cyano guanidinide] (Traves et al., 2004), was reported to inhibit receptor binding with IC_{50} = 67 and 12 nM, respectively. More recently, a small molecule antagonist to murine CXCR2, Sch-N, was shown to inhibit smoke-induced pulmonary neutrophilia in mice (Thatcher et al., 2005). Details of its chemical synthesis (referred to as Compound 4 or Sch527123 [2-hydroxy-N,N-dimethyl-3-[(2-[[1(R)-1-(5-methyl-furan-2-yl)-propylamino]-3,4-dioxo cyclobut-1-enylamino]-benzamide]) was published recently (Owers et al., 2006). The studies presented herein show that Sch527123 (Fig. 1a) is a potent, allosteric antagonist of both CXCR1 and CXCR2. As such, Sch527123 repre- sent a novel small molecule antagonist for the treatment of inflammatory conditions arising primarily (or second- ary) from neutrophil infiltration.

**Materials and Methods**

**Cell Culture and Isolation of Peripheral Mononuclear Cells.** Ba/F3 cells stably transfected to express hCXCR1 and hCXCR2 (Hipkin et al., 2004) were propagated in RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, glutamine, and 1% methyl cellulose (R&D Systems, Minneapolis, MN) under constant selection pressure with 500 U/ml amphotericin, 10% murine IL-3 (R&D Systems, Minneapolis, MN) and 1 ng/ml murine IL-3 (R&D Systems, Minneapolis, MN). The purity is over 95%, and 50 ml of blood normally yields 100 million neutrophils.

**Cell Membranes.** High-throughput screening-hCXCR1 and high-throughput screening-hCXCR2 cell membranes were purchased from Stratagene (La Jolla, CA). Ba/F3-hCXCR1, Ba/F3-hCXCR2, and human neutrophil membranes were prepared as described previously (Hipkin et al., 1997). Cells were pelleted by centrifugation and incubated in homogenization buffer (10 mM Tris-HCl, 5 mM EDTA, and 3 mM EGTA, pH 7.6) and 1 mM phenylmethylsulfonyl fluoride for 30 min on ice. The cells were then lysed with a Dounce homogenizer using a sterier type RZR3 Polytron homogenizer (Caf- rama, Wiarton, ON, Canada) with 12 strokes at 900 rpm. The intact cells and nuclei were removed by centrifugation at 5000 g for 5 min. The cell membranes in the supernatant were then pelleted by cen- trifugation at 100,000 g for 30 min. The membranes were resuspended in glygly buffer (20 mM glycylglycine, 1 mM MgCl2, and 250 mM sucrose, pH 7.2), aliquot, quick-frozen, and stored at −80°C. Protein concentration in membrane preparations was determined using the Bradford method (Bradford, 1976).

**[35S]GTPγS Binding Assay.** The exchange of guanosine 5′- [γ-35S]triphosphate ([35S]GTPγS, triethylaminium salt; specific activity = 1250 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) was measured using a scintillation proximity assay (SPA) as described previously (Gonsiorek et al., 2003). For each assay point, 2 μg of membrane was preincubated for 15 min at room temperature with 200 μg of wheat germ agglutinin-coated SPA beads (WGA-SPA; GE Healthcare, Little Chalfont, Buckinghamshire, UK) in SPA binding buffer (50 mM Tris-HCl, 1 mM CaCl2, 5 mM MgCl2, 50 mM NaCl, 0.002% NaN3, 0.1% bovine serum albumin, and 10 μg/ml saponin, pH 7.6). The beads and membranes were transferred to a 96-well Isoplate (PerkinElmer Wallac, Gaithersburg, MD) and preincubated for 60 min with 10 μM GDP with the indicated concentrations of chemokine and/or Sch527123. The guanosine 5′-3-O-(thiotriphosphate exchange reaction was initiated by the addition of 0.1 nM [35S]GTPγS and was carried out for 60 min at room temperature. Membrane-bound [35S]GTPγS was measured using a 1450 Microbeta Trilux counter (PerkinElmer Wallac).

**Synthesis of [3H]Sch527123.** [3H]Sch527123 was prepared in two steps as shown in Fig. 1b: 3-amino-4,5,6,tri-bromo-2-hydroxy-N,N-dimethylbenzamide (1.08 mg) and 10% palladium on carbon (2.45 mg) were weighed into a 1-ml tritiation vessel and ethyl acetate (400 μl) and diisopropyl ester aty (10 μl) was added. The vessel was transferred to an INUS systems Trisorber (Tampa, FL), frozen in liquid nitrogen, evacuated, and subjected to two freeze-thaw cycles. Carrier-free tritium gas (870 mCi) was then added to the vessel, and the reaction was vigorously stirred for 4 h at room temperature. Upon completion of the reaction, the vessel contents were diluted with ethanol and passed through a Millipore-FH 0.45 μm filter (Fisher Scientific, Pittsburgh, PA). The resulting solution was evaporated to dryness, the residue was redissolved in ethanol, and the evaporation process was repeated. A total of 73 mCi of 3-amino-4,5,6,tri-[3H]2-hydroxy-N,N-dimethylbenzamide at a radiochemical purity of 65% (HPLC System 1) was isolated from the reaction and used directly in the next step.

In a 1-ml "reactivial", the crude [3H] product (75 mCi) and 3-ethoxy 4-[1-(5-methyl-furan-2-yl)-propylamino]-cyclobut-3-ene-1,2-dione (5 mg) were dissolved in ethanol (20 μl) and diisopropyl ester (2 μl). The reaction was heated at 65°C for 72 h, after which analysis by HPLC (HPLC System 2) showed that approximately 20% of the desired product had formed. The reaction was evaporated to dryness and purified by HPLC (HPLC System 3) to yield a batch of 12.6 mCi at a specific activity of 39 Ci/mmol. The radiochemical purity as determined by HPLC (HPLC Systems 2 and 4) was 95.2 and 97.8%.

The following HPLC systems were used: 1) SB Phenyl 3 × 150 mm, water:acetonitrile:formic acid (97:3:0.1) (Agilent Technologies, Palo Alto, CA) for 10 min followed by a step gradient to acetonitrile, 0.5 ml/min, 275 nm (HPLC eluate, Packard FloScint III cocktail (1.3); PerkinElmer Life and Analytical Sciences), 2) SB Phenyl 3 × 150 mm, water:acetonitrile:formic acid (65:35:0.1) (Agilent Technologies) for 15 min followed by a step gradient to acetonitrile, 0.5 ml/min, 295 nm (HPLC eluate, Packard FloScint III cocktail (1.3)); 3) Nomura Develosil RP-AQ 9.4 × 250 mm, water:acetonitrile:formic acid (65:35:0.1), 6 ml/min, 295 nm (Phenomenex, Torrance, CA); and 4) Nomura Develosil RP-AQ 3 × 150 mm, water:acetonitrile:formic acid (65:35:0.1) for 15 min followed by a step gradient to acetonitrile.
0.5 ml/min, 295 nm [HPLC eluate, Packard FloScint III cocktail (1:3)].

Radioligand Binding Assays. 125I-CXCL8 (specific activity = 2200 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences. [3H]Sch527123 was synthesized as described above. Competition binding and saturation bindings assays and binding kinetics were performed using SPA technology (Cox et al., 2001). Membranes (2–6 μg per assay point) in SPA binding buffer were preincubated for 30 min at room temperature with WGA-SPA beads (1 μg of membrane protein/80 μg of beads), transferred to a 96-well Isoplate, and further incubated at room temperature with the radioligand and the indicated concentrations of radioactive competitors for 6 to 20 h. For experiments to determine ligand dissociation constants, membranes prebound to WGA-SPA beads were incubated with the indicated concentrations of [3H]Sch527123 in the absence or presence of excess unlabeled compound (total and nonspecific binding, respectively). The membranes and beads were further incubated with excess unlabeled compound (1–10 μM) or the appropriate diluent and counted sequentially every 30 s for up to 48 h. The dissociation rate(s) of specific binding (total – nonspecific binding) was calculated using Prism software (GraphPad Software, Inc., San Diego, CA). Ligand affinities (Kd) from competition binding experiments were calculated from binding IC50 using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Sch527123 was also tested in a variety of enzymes, receptor, and functional assays at MDS Pharma Services (King of Prussia, PA) according to established protocols.

Myeloperoxidase Assay. Neutrophils were cultured with 5 mM cytoschalasin B and or without Sch527123 at 37°C for 10 min. Agonists were added, and the cells were incubated for an additional 1 h at 37°C. Tetrathymethylbenzidine substrate was added, and OD450 was used to determine myeloperoxidase activity in the supernatant. Myeloperoxidase release was normalized to total release after the addition of 1% Triton X-100.

Chemotaxis Assay. Chemotaxis experiments were performed as described previously (Hipkin et al., 2004). Recombinant cells were resuspended at 1 × 10⁶/ml in assay buffer (phenol red free-RPMI 1640 supplemented with 2% FBS). Human neutrophils were resuspended at 2 × 10⁶/ml in the same assay buffer containing 5% FBS. CXCL1 and CXCL8 used in this study were purchased from R&D Systems. CXCL1 binds only CXCR2 with high affinity, whereas CXCL8 binds both CXCR1 and CXCR2 with high affinity. Chemottractants (30 μl) diluted in assay buffer were dispensed into the bottom wells of disposable microchemotaxis plates (ChemoTx 101-5 for BaF3 cells and ChemoTx 101-3 for neutrophils (PMN); Neuroprobe, Gaithersburg, MD), which were then covered with filter. Cells were preincubated with Sch527123 in a CO2 incubator for 90 min. Cell aliquots (25 μl) were applied to each spot on the filter. After incubation (90 min for BaF3 cells and 30 min for PMN in a CO2 incubator), the filters were removed. Migrated cells in the bottom wells were transferred to a Microplate luminometer plate (Thermo Electron Corporation, Waltham, MA), and 25 μl of ATPiTE one-step (PerkinElmer Life and Analytical Sciences) were added to each well. After incubation at room temperature for 10 min, luminescence intensity was measured using a luminometer (Luminoskan; Thermo Electron Corporation). Data are represented as the mean of duplicate determinations.

Calcium Flux Assay. BaF3-hCXCR1 and BaF3-hCXCR2 cells were incubated at 37°C in a CO2 incubator for 30 to 45 min in culture media (1 × 10⁶ cells/ml) containing 2.2 μM Fluo-3AM and 0.022% pluronic acid (Invitrogen, Carlsbad, CA), mixing briefly after 20 min. The cells were washed twice by centrifugation and suspended in Hanks’ balanced saline solution, 25 mM HEPES, and 0.1% fetal calf serum (buffer Λ) at a density of 6 × 10⁶ cell/ml. Cell suspension (40 μl; 25,000 cells) was added to each well of a 384-well polystyrene-coated clear bottom plate (BD Biosciences, Bedford, MA). Plates were then centrifuged at room temperature at 400g for 1 min and then left undisturbed for 15 min. Compounds or buffer (20 μl at 3 × final desired concentration) was added, and the plates were incubated at 25°C for 20 min before placement into the stage of a fluorometric imaging plate reader (FLIPR200; Molecular Devices, Sunnyvale, CA). At this time, 20 μl of CXCL8 or buffer was added to give the indicated concentrations, and fluorescence measurements (excitation at 488 nm, emission at 510–570 nm) were recorded once per second for the first 60 s and then every 6 s for the next 120 s. Data were calculated as maximal fluorescence minus minimal fluorescence at baseline. Dose-response curves were fitted to the data points by nonlinear regression.

Results

Competition Binding Analyses at hCXCR1 and hCXCR2. Ba/F3-hCXCR1 or HTS-hCXCR2 membranes were incubated with 125I-CXCL8 and the indicated concentrations of CXCL8, SB-332235, or Sch527123 (Fig. 2). Receptor-bound radioligand was measured by scintillation proximity assay technology (as described under Materials and Methods).

The potency of SB-332235 to displace 125I-CXCL8 binding to CXCR2 (IC50 = 5.4 ± 1.2 nM, n = 2) was lower than that measured with Sch527123 (IC50 = 0.97 ± 0.05 nM, n = 2). The very steep Hill coefficient derived from the Sch527123 binding curve at CXCR2 (nH = 2.1 ± 0.16) suggests that the concentration of CXCR2 in the assay is too high. If compound
bound/free > 10%, steep binding curves can occur (Kenakin, 1997). Sch527123 similarly inhibited the binding of 100 to 200 pM 125I-CXCL1 to CXCR2 (data not shown).

As expected, only high concentrations of the CXCR2-selective antagonist SB-332235 inhibited 125I-CXCL8 binding to CXCR1 (IC50 = 10 μM). Sch527123 inhibited 125I-CXCL8 binding at CXCR1 (IC50 = 43 ± 3.6 nM, n = 10), albeit with less potency than was measured with CXCR2. The Hill coefficient for Sch527123 binding inhibition at CXCR1 was significantly less than unity (nH = -0.73 ± 0.03, n = 5) consistent with a noncompetitive interaction at the receptor between the compound and chemokine.

Effect of Sch527123 on hCXCR1 and hCXCR2 Signaling in Transfectants. The effect of Sch527123 on receptor signaling was first measured with [35S]GTPγS exchange assays in hCXCR1 or hCXCR2 membranes. Sch527123 inhibited [35S]GTPγS exchange in response to all relevant chemokines at both CXCR1 and CXCR2 (data not shown). For hCXCR1, [35S]GTPγS exchange was assessed in response to CXCL8 in the presence of 0, 30, 300, or 3000 nM Sch527123 as shown in Fig. 3 (left). CXCL8 stimulated a dose-responsive increase in [35S]GTPγS exchange with an EC50 = 0.5 ± 0.29 nM. Coincubation with Sch527123 decreased both the potency and efficacy of CXCL8 to stimulate [35S]GTPγS exchange. The effect of Sch527123 on CXCR1 signaling increased with drug concentration, although the potency of CXCL8 did not decrease predictably as the concentration of Sch527123 increased. For hCXCR2, [35S]GTPγS exchange was measured in response to CXCL1 in the presence of 0, 0.3, 1.0, or 10 nM Sch527123 as shown. CXCL1 stimulated a dose-responsive stimulation of [35S]GTPγS exchange with an EC50 = 26 ± 4.5 nM. As was the case with hCXCR1, Sch527123 decreased the potency and efficacy of CXCL1 to increase [35S]GTPγS exchange. Again, the effect of the compound on chemokine potency did not increase predictably as the levels of Sch527123 increased. Taken together, these data and the observation that the blunting of CXCR1 and CXCR2 activation by Sch527123 was not (consistently) surmountable imply that Sch527123 is a noncompetitive (allosteric) antagonist at both receptors.

We next assessed the effect of Sch527123 on chemokine stimulation of calcium signaling in cells transfected to express hCXCR1 (Ba/F3-hCXCR1) or hCXCR2 (Ba/F3-hCXCR2). Cells were loaded with Fluo-3AM and treated with CXCL8 in the presence or absence of Sch527123 or the compound SB-225002 (as described under Materials and Methods). As shown in Fig. 4, Sch527123 inhibited calcium signaling upon addition of CXCL8, with slightly more potency at hCXCR2 and then hCXCR1. In contrast, SB-225002 failed to inhibit CXCL8-stimulated calcium flux through hCXCR1 and was significantly less potent than Sch527123 in Ba/F3-hCXCR2 cells. Both of these findings are in agreement with our radioligand competition and GTPγS binding data.

Effect of Sch527123 on Cell Chemotaxis. The effects of Sch527123 on CXCR2- and CXCR1-mediated chemotaxis were examined using both recombinant cells (Ba/F3-hCXCR1 and Ba/F3-hCXCR2) and primary hPMN. Sch527123 inhibited chemotaxis (recombinant lines and neutrophils) to all relevant chemokines at CXCR1 and CXCR2 (data not shown). The data presented in Fig. 5 (left bottom) show that 1 nM Sch527123 reduced CXCL8 potency in stimulating Ba/F3-hCXCR2 chemotaxis. Coincubation with 3 nM Sch527123 both decreased chemokine potency and dramatically inhibited maximal cell chemotaxis. Cell movement to chemokine was essentially ablated with 10 nM Sch527123. For Ba/F3-hCXCR1 chemotaxis (Fig. 5, left top), 30 nM Sch527123 had only a marginal effect on CXCL8 potency. Sch527123 (300 nM) significantly decreased chemokine potency and slightly decreased maximal cell movement. Effects of Sch527123 on CXCL1- and CXCL8-mediated human neutrophil chemotaxis are shown in Fig. 5 (middle). Sch527123 (3 nM) significantly inhibited the potency and efficacy of CXCL1-induced PMN chemotaxis, with almost complete inhibition of cell movement with 10 nM compound. These data are consistent with the pharmacology of the compound at CXCR2, which is selectively activated by CXCL1. Coincubation with 200 nM Sch527123 decreased only CXCL8 potency in stimulating PMN movement, a result that is very similar to the compounds effects in Ba/F3-CXCR1 chemotaxis. Similar data were generated with the CXCR2 antagonists SB-332235 and SB-225002 (data not shown). Taken together, these data suggest that directed neutrophil movement in response to low levels of CXCL8 is mediated by CXCR2, whereas PMN movement is more selectively activated by CXCL1.

![Fig. 3. Inhibition of chemokine-stimulated [35S]GTPγS exchange by Sch527123 in hCXCR1 or hCXCR2 membranes.](image-url)
chemotaxis in response to higher concentrations of the chemokine occurs via activation of CXCR1.

Assessment of Sch527123 Selectivity. To assess Sch527123 selectivity, the effect of the compound on neutrophil chemotaxis to C5a and fMLP was tested. As can be seen in Fig. 5 (right), migration of neutrophils to these agents was unaffected by preincubation with 300 nM Sch527123, a concentration that effectively antagonizes chemotaxis through CXCR1 or CXCR2 in recombinants or human neutrophils. Neutrophil migration to leukotriene B4 was similarly unaffected by Sch527123 (data not shown). In addition, Sch527123 inhibited myeloperoxidase release from human neutrophils in response to CXCL8 without inhibiting the cell response to C5a, fMLP (Fig. 6), or leukotriene B4 (data not shown). The potency of Sch527123 to inhibit the CXCL8 response (~30 nM) is consistent with the activity of the
substrate was added, and OD450 was read to determine myeloperoxidase 
indicated concentrations of [3H]Sch527123 in the absence (total binding) or presence of 1 
from a representative experiment (n = 3). 

cells (4 /H9262 
Fig. 7, left) or CXCR2 (K_i = 0.049 ± 0.004 nM; Fig. 7, right). 
Whereas [3H]Sch527123 binding reached steady state at 6 
h in CXCR1 membranes, binding equilibrium was achieved only after prolonged incubation (~24 h) in CXCR2 membranes. Binding competition with [3H]Sch527123 in membranes expressing CXCR1 and CXCR2 (Fig. 8) measured at 4 and 24 h shows that Sch527123 binds with high affinity to both CXCR1 and CXCR2, although the compound is CXCR2-selective. Because of the relatively low-specific activity of the trace, [3H]Sch527123 was used at concentrations (5–11 nM) in (great) excess of the compound affinities for CXCR1 and CXCR2. Therefore, binding affinities (K_i) generated from binding IC_50 using this approach are suspect (at 24 h; CXCR1, Sch527123 K_i = 8.6 nM, SB-332235 K_i = 550 nM, CXCR2, Sch527123 K_i = 8 pM, SB-332235 K_i = 1.0 nM) due to the large correction in the Cheng-
Prusoff equation (Cheng and Prusoff, 1973). Nonetheless, consistent with the binding assays with ^12^5^I-CXCL8 (Fig. 
SB-332235 binds with lower affinity to both receptors relative to Sch527123. In addition, at either 4 or 24 h, 
CXCL8 does not effectively displace [3H]Sch527123 from 
membranes expressing CXCR1 and CXCR2, consistent with noncompetitive inter-
action at the receptors between chemokine and compound 
(see Fig. 3). 

To determine the kinetic binding constants for 
[3H]Sch527123, membranes were incubated with 3 to 10 
M [3H]Sch527123 in the presence or absence of excess unlabeled compound, and the time course of specific binding was analyzed. The observed K_on values were linearly proportional to the concentration of [3H]Sch527123 for both CXCR1 and CXCR2 (data not shown). In CXCR1 membranes, an association binding constant (k_1) value (1.64 × 10^7 ± 2.70 × 10^6 min^-1 M^-1, n = 3) was derived from an observed K_on = 0.25 min^-1 (Fig. 9, top left). Binding at CXCR1 was reversible and dissociated fairly rapidly with a k_d = 0.141 ± 0.014 min^-1 (t_1/2 ~ 5 min.) (Fig. 9, top right). Using these constants, the Sch527123 K_d for 
CXCR1 was calculated (k_d/K_i = 8–9 nM), a value compa-
able with the K_d derived through saturation analyses (see above). With CXCR2 (Fig. 9, bottom left), the value for Sch527123 k_1 = 2.08 × 10^7 ± 2.00 × 10^6 min^-1 M^-1 (n = 4) derived from the observed association rate constant (K_on 
= 0.12 min^-1) was similar to that measured with CXCR1. 
The binding of [3H]Sch527123 to CXCR2 was also revers-
ible (Fig. 9, bottom right), albeit at a much slower rate ($k_2 = 0.00058 \pm 0.00001 \text{min}^{-1}$) with a $t_{1/2} \sim 22 \text{ h}$. Sch527123 $K_d$ for CXCR2 calculated using its kinetic values ($k_2/k_1 = 0.029 \text{nM}$) was consistent with the affinity constant generated by saturation analyses (49 $\pm$ 4 pM; Fig. 7, left).

Having established the binding constants for Sch527123 with CXCR1 and CXCR2 in transfectants, we initiated similar studies using human neutrophils, which express both receptors. Figure 10 illustrates the time course of $[^3H]$Sch527123 dissociation in neutrophil membranes from two representative individuals ($n = 3$). $[^3H]$Sch527123 dissociates with two very distinct rates. Approximately 60 to 70% total specific binding dissociates fairly rapidly ($k_2 = 0.077 \pm 0.002 \text{min}^{-1}$) (regraphed in panels at right), whereas the remainder dissociates much more slowly ($k_2 = 0.00058 \pm 0.00012 \text{min}^{-1}$). These dissociation rates are indistinguishable from the $k_2$ values derived with recombinant CXCR1 and CXCR2, respectively (see Fig. 9). Using $k_1$ values derived with recombinant membranes (or neutrophils; data not shown), calculation of binding affinities ($k_1/k_2$) generated two distinct binding affinities ($K_d = 5 \text{nM}$ and $\sim 0.04 \text{nM}$), values that are indistinguishable from the CXCR1 and CXCR2 $K_d$ values in recombinantly expressed receptor. Therefore, we conclude that $[^3H]$Sch527123 binds with predicted affinities to CXCR1 and CXCR2 expressed on human neutrophils.

**Discussion**

The data presented herein illustrate that Sch527123 is a potent and specific allosteric antagonist of hCXCR1 and hCXCR2. The extraordinary potency of Sch527123 at hCXCR2 ($49 \text{ pM}$) arises from its exceedingly slow dissociation rate from the receptor (approximately 24 h at room temperature). Although this dissociation will be predictably faster at 37°C, extension of CXCR2 coverage arising from slow receptor dissociation may well be therapeutically beneficial. The pharmacology of Sch527123 at CXCR1 is more "orthodox", i.e., a low nanomolar affinity and more rapid receptor dissociation profile (approximately 5 min at room temperature). Therefore, whereas Sch527123 is a potent CXCR1 antagonist, it is undoubtedly CXCR2-selective. The fact that Sch527123 does not inhibit chemokine binding to CXCR1 and CXCR2 in a competitive manner is not unex-
pected, considering the size of the chemokines (8–10 kDa) and the multifaceted nature of chemokine-receptor interactions (Wu et al., 1996; Suetomi et al., 1999). Allosteric antagonism of chemokine receptors by small molecule compounds is probably the norm. For example, the CXCR1 antagonist repertaxin was described as an allosteric ligand that does not block chemokine binding but potently inhibits human neutrophil migration induced by CXCL8. A panel of structurally dissimilar antagonists were recently shown to interact with CCR5 in an allosteric fashion relative to its chemokine ligands (Watson et al., 2005). Indeed, CCR5 antagonist 873140 does not effectively inhibit 125I-CCL5 binding (Maeda et al., 2004; Watson et al., 2005), although CCR5 activation by the chemokine is blocked by the compound (Watson et al., 2005). Another CCR5 antagonist TAK-220 inhibits the binding of radiolabeled CCL3 (MIP-1α) and CCL5 but not CCL4.

Fig. 9. The kinetics of [3H]Sch527123 binding at hCXCR1 and hCXCR2. To measure the association rate of [3H]Sch527123 binding, membranes from HT8-hCXCR1 (top left) and HT8-hCXCR2 (bottom left) cells (2–4 μg/well) were incubated in SPA buffer at room temperature for the indicated times with 3 nM [3H]Sch527123 in the absence (total binding) or presence of 3 to 10 μM unlabeled compound (nonspecific binding). Data show the mean binding of triplicate determinations measured at the indicated times from a representative experiment (n = 2). To measure the rate of [3H]Sch527123 dissociation, membranes from HT8-hCXCR1 (top right) and HT8-hCXCR2 (bottom right) cells (2–4 μg/well) were incubated in SPA buffer at room temperature for the indicated times with 3 to 10 nM [3H]Sch527123 in the absence (total binding) or presence of 1 to 3 μM unlabeled compound (nonspecific binding) before the addition of either buffer (■) or 1 to 3 μM unlabeled compound (●). Data show the mean binding of triplicate determinations from a representative experiment (n = 2) measured sequentially at the indicated times.

Fig. 10. [3H]Sch527123 dissociation kinetics in human neutrophil membranes. Human neutrophil membranes (6 μg/well) were incubated with 5 nM [3H]Sch527123 in the absence (total binding) or presence of 10 μM unlabeled compound (nonspecific binding) for 60 min. The incubation was then continued after the addition of 10 μM unlabeled compound (●) or diluent (□). Receptor-bound [3H]Sch527123 was measured sequentially at the indicated times (n = 3). Data (mean ± S.E.M. of triplicate determinations) from two representative experiments are shown. After the addition of Sch527123, data were analyzed using a two-phase exponential decay. The data from the initial 60 min were re-plotted in the right panels.
(Takashima et al., 2005). The effect of TAK-220 on CCR5 activation by CCL4 was not reported in this study. In contrast, Sch527123 potently inhibits CXCR1 and CXCR2 activation by all of their cognate ligands (data not shown) and inhibits the binding of 125I-CXL8 to CXCR1 and CXCR2 (Fig. 2) and 125I-CXCL1 to CXCR2 (data not shown).

Sch527123 could have clinical utility in disease processes that may be directly (or indirectly) mediated by cells expressing CXCR1 and/or CXCR2, i.e., rheumatoid arthritis, inflammatory bowel disease, acute respiratory distress syndrome, septic shock, pulmonary emphysema, psoriasis, and chronic obstructive pulmonary disease (Nickoloff et al., 1991; Woods et al., 2000; Kurdowska et al., 2002; Banks et al., 2003; Beeh et al., 2003). Indeed, the first definitive evidence of CXCR2 ligand expression in psoriatic tissue (CXCL7 and CXCL8) was published over 15 years ago (Baggiolini and Walz, 1989; Christophers et al., 1989), with the first evidence of receptor up-regulation published 4 years later (Schulz et al., 1993; Kulke et al., 1998). Studies implicating CXCR2 in mediating various pulmonary inflammations are many (for review, see Mukaida, 2003; De Boer, 2002; Hay and Sarau, 2001). Studies with Sch527123 in a variety of rodent and primate models of pulmonary inflammation (Thatcher et al., 2005; Chapman and Hipkin, 2003) and 125I-CXCL1 to CXCR2 (data not shown).

In conclusion, Sch527123 is a novel allosteric antagonist that binds with high affinity to both CXCR1 and CXCR2. Nonetheless, the concentrations of Sch527123 required to ablate PMN chemotaxis through CXCR1 (≥ 1 μM versus CXCL8; data not shown) are such that, therapeutically, Sch527123 would be predicted to be a de facto CXCR2 antagonist.

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