Elucidation of CXCR7-Mediated Signaling Events and Inhibition of CXCR4-Mediated Tumor Cell Transendothelial Migration by CXCR7 Ligands


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Elucidation of CXCR7-Mediated Signaling Events and Inhibition of CXCR4-Mediated Tumor Cell Transendothelial Migration by CXCR7 Ligands


CXCR7 binds chemokines CXCL11 (I-TAC) and CXCL12 (SDF-1) but does not act as a classical chemoattractant receptor. Using CCX771, a novel small molecule with high affinity and selectivity for CXCR7, we found that, although CXCR7 is dispensable for “bare filter” in vitro chemotaxis, CXCR7 plays an essential role in the CXCL12/CXCR4-mediated transendothelial migration (TEM) of CXCR4⁺/CXCR7⁺ human tumor cells. Importantly, although CXCL11 is unable to stimulate directly the migration of these cells, it acts as a potent antagonist of their CXCL12-induced TEM. Furthermore, even though this TEM is driven by CXCR4, the CXCR7 ligand CCX771 is substantially more potent at inhibiting it than the CXCR4 antagonist AMD3100, which is more than 100 times weaker at inhibiting TEM when compared with its ability to block bare filter chemotaxis. From being a “silent” receptor, we show that CXCR7 displays early hallmark events associated with intracellular signaling. Upon cognate chemokine binding, CXCR7 associates with β-arrestin2, an interaction that can be blocked by CXCR7-specific mAbs. Remarkably, the synthetic CXCR7 ligand CCX771 also potently stimulates β-arrestin2 recruitment to CXCR7, with greater potency and efficacy than the endogenous chemokine ligands. These results indicate that CXCR7 can regulate CXCL12-mediated migratory cues, and thus may play a critical role in driving CXCR4⁺/CXCR7⁺ tumor cell metastasis and tissue invasion. CXCR7 ligands, such as the chemokine CXCL11 and the newly described synthetic molecule CCX771, may represent novel therapeutic opportunities for the control of such cells. The Journal of Immunology, 2009, 183: 3204–3211.

The CXCR7 chemokine receptor is a recently deorphanized receptor for the chemokines CXCL12 (SDF-1) and CXCL11 (I-TAC) and plays a role in cell survival, adhesion, tumor growth and development (1–7). Unlike classical chemokine receptors, CXCR7 does not respond to ligand binding by mobilizing intracellular calcium or by stimulating directly the chemotaxis of CXCR7-expressing cells in transfected, endogenous, or induced expression systems (1, 6, 8). Although CXCR7 does not appear to directly induce cell migration, recent studies suggest alternative mechanisms by which CXCR7 can regulate CXCL12-directed cell movement. CXCR7 may influence cell positioning via enhanced cell adhesion, as observed when CXCR7⁺ tumor cells are incubated with CXCR7⁺ endothelial cells (1). Hartmann et al. (8) recently reported that CXCR7 appears to play a role in regulating CXCR4-dependent rapid integrin activation and adhesion of T cells to endothelial cells, though they did not demonstrate directly the presence of CXCR7 on these cells. Mazzinghi et al. (9) recently described a role for CXCR7 in CXCL12-mediated transendothelial migration (TEM) of human re-

1 Abbreviations used in this paper: TEM, transendothelial migration; β-gal, β-galactosidase; GPCR, G protein-coupled receptor; CHO, Chinese hamster ovary.

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association occurs in the absence of classical GPCR-associated Ca$^{2+}$ mobilization. We further show that such association can also be triggered by CCX771. Moreover, we find that CCXCR7 engagement by appropriate ligands also has a profound effect in regulating the ability of human CCX7 $^*$ CCXCR4 $^*$ tumor cells to migrate across an endothelial cell monolayer. Remarkably, this CCXCR4-mediated behavior is significantly more sensitive to CCXCR7 manipulation than it is to direct CCXCR4 inhibition. Specifically, CCX771 is substantially more potent at inhibiting the TEM of these cells toward CCXCL12 than the CCXCR4 antagonist AMD3100, which is more than 100 times weaker at inhibiting TEM when compared with its ability to block the “bare filter” chemotaxis of the same cells.

Materials and Methods

Cells and reagents

Human B lymphoblastoid cell line NC-37, human T lymphoblastoid cell line MOLT-4, and human leukemic monocyte lymphocyte cell line U937 were obtained from the American Type Culture Collection. HUVECs were obtained from Lonza, cultured per the manufacturer’s specifications, and used at passage 3. Human breast carcinoma cell line MDA-MB-435 cells (designated 435-WT) and 435-CXCR7 transfectants have been previously described (1). A CXCR7Δc-term variant lacking the final 43 aa of CXCR7 (Δ319–362) was generated by PCR and used to transfet MDA-MB-435 cells. The Chinese hamster ovary (CHO) cells expressing modified CCX7 or CCR4 or β-arrestin2 were obtained from Discoverx and cultured in Ham’s F-12 medium (Mediatech) containing 10% FBS (Gemini Bioproducts), penicillin/streptomycin (Mediatech), 0.3 mg/ml hygromycin (Invitrogen), and 0.8 mg/ml G-418 (Mediatech). FLAG-tagged CCX4 and C5aR1 were cloned into a mammalian expression vector (pCI-neo; Promega) and transiently transfected into the CHO-CXCR7-Bgal1-β-arrestin2 line. CXCL12 triggered intracellular calcium mobilization in CXCR4 transient transfectants, indicating that the receptor was functional (data not shown). 125I-CXCL12 tracer was purchased from GE Healthcare and 125I-CXCL11 was purchased from PerkinElmer. Chemokines CXCL12, CXCL11, CCL9, CCL2, CXCL10, CCL28, and CCL2 were purchased from R&D Systems. Anti-CXCR4 (12C5), anti-CXCR3 (49B01), and mouse IgG₂a isotype control were purchased from R&D Systems. Anti-CXCR7 (8F11) and mouse IgG₂a isotype control were purchased from BioLegend. Secondary goat anti-mouse F(ab')²-PE was purchased from Jackson ImmunoResearch Laboratories. 11G8 and isotype control mouse IgG1 were prepared in-house, and CCX771 and CCX704 were synthesized at ChemoCentryx. AMD3100 was obtained from Sigma-Aldrich. Cells were permeabilized for intracellular mAb staining using BD Perm/Wash reagents according to the manufacturer’s recommendations (BD Biosciences). Flow cytometry was performed on a modified FACScan (BD Biosciences).

Radiolabeled ligand binding assay

A total of 2–5 × 10⁶ cells/well were mixed with 4-fold dilutions of unlabeled chemokine or compound competitiors and −1 nM (0.025 μCi) of 125I-CXCL12 or 125I-CXCL11 tracer per well in a total volume of 200 μl, and agitated at 4°C for 3 h. Levels of cell-bound radioligand were determined by harvesting the cells on poly(ethyleneimine)-treated GF/B glass filters (PerkinElmer, Waltham, MA) using a cell harvester (PerkinElmer), washing the filters twice with buffer (25 mM HEPES, 500 mM NaCl, 1 mM CaCl₂, and 5 mM MgCl₂, adjusted to (pH 7.1)) and measuring the amount of 125I-bound to each filter (cpm) with a TopCount scintillation counter (PerkinElmer). For the evaluation of possible intracellular stores of CXCR7 protein, 8–10 × 10⁵ NC-37 cells, 6 × 10⁴ HUVECs, 5 × 10⁴ 435-WT cells, and 5 × 10⁴ 435-CXCR7 cells were sonicated for 10 s, rested 1 min, and sonicated again for 10 s (4 W of output power, 60 Sonic Dismembrator; Fisher Scientific), and the volume divided equally among 18 wells for examination of CXCR7 125I-bound CXCL12 binding signature.

Bare filter chemotaxis assay

Cell migration assays were performed using a 5.0-μm 96-well microchamber (ChemoTx; NeuroProbe). Cells were resuspended at 1 × 10⁶ cells/ml in chemotaxis buffer (HBSS with Ca²⁺ and Mg²⁺), and 0.1% BSA with or without chemokine, mAb, or compound) and 20 μl was added on top of the filter for each well. Chemokines were placed in the bottom chamber and the microchambers were incubated 2 h 15 min at 37°C in a humidified incubator. The filters were then removed, 5 μl of CyQuant (Invitrogen) was added to the lower chamber, and fluorescence was measured at 540 nm using a plate reader (Molecular Devices).

TEM assay

The TEM assay was performed using 24-well plates with microporous (5.0 μm) Transwell membrane inserts (Corning; Costar). HUVEC (passage 3) were resuspended at 1 × 10⁶ cells/ml, and 100 μl was added to the top chamber with 600 μl/well HUVEC medium (Lonza) in the bottom chamber and incubated overnight at 37°C. HUVEC monolayers were washed with PBS (without Ca²⁺ and Mg²⁺). NC-37, MOLT-4, or U937 cells were resuspended at 5 × 10⁶ cells/ml in assay medium (IMDM; Invitrogen) with 0.1% BSA with or without test reagents, incubated at room temperature for 10–15 min, then added to the HUVEC monolayer (100 μl/well). Assay medium (600 μl) with or without 10 nM CXCL12 was added to the bottom chambers. After overnight incubation at 37°C, transmigration was assessed by adding 100 μl of CyQuant (Invitrogen) and measuring fluorescence at 540 nm with a plate reader (Molecular Devices).

β-arrestin assay

A total of 2 × 10⁶ CHO-CXCR7-Bgal1-β-arrestin2-Bgal2 (CHO-CXCR7) or CHO-CCR4-Bgal1-β-arrestin2-Bgal2 (CHO-CCR4) cells (Discoverx) were seeded into 96-well plates and cultured overnight. The next day, the medium was removed by aspiration, 100 μl of PBS containing varying concentrations of ligands was added to the wells, and the plates were incubated in 5% CO₂ at 37°C. After 90 min, 50 μl of β-galactosidase (β-gal) substrate (Discoverx) was added to the wells and the plates were incubated at room temperature. After 1 h, light emission was analyzed in a TopCount scintillation counter (PerkinElmer). For Ab blocking studies, the cells were preincubated with Abs for 30 min in 5% CO₂ at 37°C, and then 5 μl of chemokine agonist was added, and the plates were processed as described.

Internalization assay

CHO-WT, CHO-CXCR7, 435-WT, 435-CXCR7, and 435-CXCR7Δc-term cells (3 × 10⁶ per well) were mixed with ~1 nM (0.025 μCi) 125I-CXCL12 or 125I-CXCL11 per well in a total volume of 200 μl and agitated at 4°C for 3 h. The cells were washed with 200 μl of PBS, and then aliquots were incubated at 4°C or 37°C for 0, 10, 30, or 60 min, pelleted, and washed with 200 μl (pH 2.0) of PBS to remove any surface-bound tracer. The cells were pelleted again, and the amount of cell-associated (internalized) radioactive tracer was quantified by TopCount, as described.

Results

Chemokine receptor expression by NC-37 cells and HUVECs

Because it is well established that CCXCR7 mRNA can be present in many cells that do not display actual CXCR7 protein on their cell surface, authentic expression of CXCR7 protein is defined by: 1) a signature 125I-CXCL12 or 125I-CXCL11 chemokine binding profile, in which CCX7 is distinguished from CCX4 and CCX3 by the competition of CXCL11 and CXCL12 for the same binding site, and 2) anti-CXCR7 mAb staining (1). In Fig. 1A, radioiodinated 125I-CXCL12 binding to NC-37 human B lymphoblastoid cells was inhibited by excess unlabeled CXCL11 (IC₅₀, 2.0 nM) and by CCX771 (IC₅₀, 4.1 nM), a novel highly potent CXCR7-binding small molecule, but not by the irrelevant chemo- kine CXCL9 (which binds CXCR3) or CCX704, an analog of CCX771 with low affinity for CCX7. Homologous competition using unlabeled CXCL12 (IC₅₀, 0.2 nM) is shown for comparison (Fig. 1A). HUVECs (passage 3) displayed a similar binding profile: radioiodinated 125I-CXCL12 binding was inhibited by excess unlabeled CXCL12 (IC₅₀, 0.2 nM), CXCL11 (IC₅₀, 1.2 nM), and CCX771 (IC₅₀, 5.3 nM), but not by CXCL9 or CCX704. Furthermore, both NC-37 cells and HUVECs were positive for CXCR7 by flow cytometry (Fig. 1B), thus displaying authentic CXCR7 protein expression. There was no evidence of intracellular stores of CXCR7 protein in NC-37 cells or HUVECs as determined by intracellular anti-CXCR7 staining of permeabilized cells and by CXCR7-signature 125I-CXCL12 binding on sonicated cells (see
Representative of CXCR7 may modulate CXCL12-mediated responses in cells that migrated across bare filters in standard in vitro chemotaxis assays, inhibitor of CXCL12-induced NC-37 cell TEM (IC50, 49 nM). to our results in the bare filter migration, CCX771 was a potent tested CCX771 in the more physiological TEM assay. In contrast CXCL12-induced chemotaxis is mediated by CXCR4. We next left affinity for CXCR4 (Fig. 2, B). CXCL11 alone had no effect on NC-37 cell TEM when added to CXCL10 and found they had no effect (Fig. 3). In addition, levels of CXCR3, we tested the other CXCR3 ligands CXCL9 and CXCL11 blocks CXCL12-mediated TEM, possibly through desensitization of CXCR4 or disruption of the chemokine gradient (Fig. 3). To assess whether CXCL11 affected TEM through low levels of CXCR3, we tested the other CXCR3 ligands CXCL9 and CXCL10 and found they had no effect (Fig. 3). In addition, CXCL11 alone had no effect on NC-37 cell TEM when added to the top well. Representative of n = 3 independent experiments.

FIGURE 1. CXCR7 and CXCR4 are expressed by NC-37 human B lymphoblastoid cells and by HUVECs. A, 125I-CXCL12 displacement by unlabeled competitors. Binding of 125I-CXCL12 to NC-37 cells (left) or HUVECs (right) was inhibited in a concentration-dependent manner by unlabeled CXCL12, CXCL11, or CXCR7-specific compound CCX771 but not by CXCL9 or control compound CCX704. Radioligand binding to cells incubated in the absence of competitor is indicated as “total bound.” The mean ± SEM of quadruplicate wells is shown. B, CXCR4, CXCR7, and CXCR3 expression by flow cytometry. Open histograms show receptor-specific staining; filled histograms show isotype controls. Relevant controls are matched by grayscale shading. C, CCX771 does not inhibit CXCL12 binding to CXCR4. Binding of 125I-CXCL12 to U937 cells was inhibited in a concentration-dependent manner by unlabeled CXCL12 and AMD3100, but not by CCX771. The mean ± SEM of quadruplicate wells is shown. Representative of n = 3 independent experiments.

supplemental Fig. S1).3 NC-37 cells and HUVECs also expressed CXCR4 by Ab staining, but not CXCR3, the alternate receptor for CXCL11 (Fig. 1B).

In addition to potent antagonism of CXCL12 binding to CXCR7, CCX771 is highly selective for CXCR7 and had no effect on 125I-CXCL12 binding to CXCR4 (which was blocked by the CXCR4 selective inhibitor AMD3100 (IC50, 44 nM), as well as by unlabeled CXCL12 (IC50, 1.2 nM)) (Fig. 1C and data not shown).

Novel CXCR7-binding compound CCX771 blocks CXCL12-mediated NC-37 tumor cell TEM

Although ligand binding to CXCR7 in isolation does not trigger classical chemokine functions (such as Ca2+ mobilization or migration across bare filters in standard in vitro chemotaxis assays), CXCR7 may modulate CXCL12-mediated responses in cells that also express CXCR4 (8, 9). Despite incubating CXCR4+ CXCR7+ NC-37 cells with increasing concentrations of CCX771 to selectively disrupt CXCL12 binding to CXCR7, CCX771 had no effect on bare filter chemotaxis to CXCL12 (Fig. 2, left). The control compound CCX704 was also inactive. In contrast, the CXCR4 inhibitor AMD3100 was a potent inhibitor of CXCL12-mediated chemotaxis (IC50, 5.7 nM) with a potency slightly better than its affinity for CXCR4 (Fig. 2, left). These data suggest that the CXCL12-induced chemotaxis is mediated by CXCR4. We next tested CCX771 in the more physiological TEM assay. In contrast to our results in the bare filter migration, CCX771 was a potent inhibitor of CXCL12-induced NC-37 cell TEM (IC50, 49 nM). Indeed, its potency was ~20 times greater than that of AMD3100 (IC50, 830 nM), which interestingly showed a consistent and marked decrease in its potency to block CXCR4-mediated migration in the TEM vs bare filter assay (Fig. 2, right). The control CCX704 had no effect on TEM (Fig. 2, right).

CXCL11 blocks CXCL12-mediated TEM

Given that a synthetic CXCR7-binding compound blocks CXCL12-mediated TEM, we asked whether an endogenous protein ligand for CXCR7, CXCL11, could play a similar role. Incubating NC-37 cells with 100 nM CXCL11 inhibited CXCL12-mediated TEM, whereas treatment with irrelevant chemokines CCL2 and CCL28 had no effect (Fig. 3). As a positive control, pretreatment with CXCL12 also inhibited CXCL12-mediated TEM, possibly through desensitization of CXCR4 or disruption of the chemokine gradient (Fig. 3). To assess whether CXCL11 affected TEM through low levels of CXCR3, we tested the other CXCR3 ligands CXCL9 and CXCL10 and found they had no effect (Fig. 3). In addition, CXCL11 alone had no effect on NC-37 cell TEM when added to the top well.

FIGURE 2. CCX771 blocks NC-37 cell TEM to CXCL12. NC-37 cells were pretreated with AMD3100, CCX771, or CCX704 control compound and assayed for bare filter (left) or TEM (right) to 1.0 or 10 nM CXCL12, respectively. Cell migration to CXCL12 in the absence of added inhibitor is indicated (max signal). Basal cell migration in the absence of chemokine is indicated (background). The mean ± SEM of either 6-wells (bare filter migration) or triplicate (TEM) is shown. Representative of n = 3 independent experiments.

FIGURE 3. CXCL11 blocks NC-37 cell-mediated TEM to CXCL12. NC-37 cells were pretreated with the indicated chemokines (100 nM) and then assayed for TEM (top) or bare filter migration (bottom) to 10 or 1.0 nM CXCL12, respectively. The mean ± SEM of either triplicate wells (TEM) or 30-wells (bare filter migration) is shown. * p < 0.05 by Student’s t test comparing the indicated treatment vs buffer (no treatment) in the top well. Representative of n = 3 independent experiments.
the top, bottom, or both wells (data not shown). Furthermore, similar to CCX771 or CXCL11 had no effect on bare filter migration of NC-37 cells to CXCL12 (Fig. 3). As a positive control, pretreatment of cells in the top well with CXCL12 inhibited bare filter migration to CXCL12 in the bottom well (Fig. 3). Thus, although irrelevant in bare filter migration, ligands that bind selectively to CXCR7 inhibit CXCL12-mediated TEM, implicating CXCR7 in this process.

**Regulation of CXCL12-mediated TEM requires CXCR7 expression on the migrating cell**

To determine whether the effect of CCX771 and CXCL11 on TEM requires CXCR7 expression by the migrating cell, we next identified human tumor cell lines that express CXCR4 but not CXCR7. MOLT-4 human T lymphoblastoid cells and U937 human myeloid leukemia cells do not display the CXCR7 binding signature (Fig. 4A, NC-37 cells shown as control [IC50, 1.4 nM]), nor do they stain with CXCR7 Abs (Fig. 4B). Both cell lines are positive for CXCR4 expression (Fig. 4B). Treating the cells with CCX771 or CXCL11 had no effect on CXCL12-mediated MOLT-4 (Fig. 4C) or U937 (Fig. 4D) TEM. In contrast, 10 µM AMD3100 inhibited CXCL12-mediated TEM in both cell lines (Fig. 4, C and D). Pretreatment of the cells in the top well with 100 nM CXCL12 also blocked CXCL12-mediated TEM, likely through CXCR4 receptor desensitization or disruption of the chemokine gradient (Fig. 4, C and D). Thus, CXCR7 expression by HUVECs alone is not sufficient to invoke regulation of CXCL12-mediated TEM; CXCR7 expression by the migrating cell and possibly by cells of the endothelial monolayer is required for CXCR7 regulation of CXCL12-mediated TEM.

CXCR7 associates with β-arrestin2 upon ligand binding

It is now well established that ligand binding does not trigger classical Ca2+ mobilization via CXCR7 (1). We tested whether CXCR7 elicits other intracellular responses, such as association with β-arrestins. We used an enzymatic complementation assay to determine whether CXCR7 associates with β-arrestins upon ligand binding. In this assay, complementary fragments from the β-gal active site are fused to the C terminus of CXCR7 and β-arrestin2, such that the spatial association of these molecules leads to enzyme activity detected by fluorescence.

CHO-CXCR7 transfectants displayed the CXCR7 binding signature. 125I-CXCL12 binding was inhibited by excess unlabeled CXCL11 (IC50, 0.6 nM) and CXCL71 (IC50, 11 nM), but not by control chemokine CXCL9 or CXCL70; a competitive binding curve using unlabeled CXCL12 is included for comparison (IC50, 0.07 nM) (Fig. 5A). The cell line was also positive for CXCR7 staining by flow cytometry using two distinct anti-CXCR7 mAbs (clones 11G8 and 8F11), and negative for CXCR4 and CXCR3 (Fig. 5B).

The CHO-CXCR7 cells were cultured in the presence of varying concentrations of chemokines, and β-arrestin2 recruitment was determined by monitoring β-gal activity. Both CXCL11 and CXCL12, but not CXCL9, induced the association of CXCR7 with β-arrestin2 in a concentration-dependent manner (Fig. 5C). The mean EC50 value and SE was 42 ± 11 nM for CXCL11 (n = 14 experiments) and 18 ± 7 nM for CXCL12 (n = 15 experiments). In contrast, an engineered CHO cell line containing a different chemokine receptor, CCR4 (CHO-CCR4), did not recruit β-arrestin2 in response to CXCL11 or CXCL12, but did recruit β-arrestin2 in response to the CCR4 ligand CCL22 (Fig. 5D). To investigate the potential for cross-talk between CXCR4 and CXCR7 in CXCL12-mediated β-arrestin2 association with CXCR7, we generated CXCR4–β-arrestin2 transient CHO transfectants. The presence of CXCR4 did not alter CXCL12-mediated β-arrestin2 association with CXCR7 (see supplemental Fig. S2).

We next asked whether CCX771 could modulate β-arrestin2 association with CXCR7 and discovered that CCX771 alone induced a concentration-dependent association of CXCR7 with β-arrestin2 (Fig. 6). CCX771 was substantially more potent than its agonist CXCL11 (IC50, 0.6 nM) and CCX771 (IC50, 11 nM), but not by control chemokine CXCL9 or CXCL70; a competitive binding curve using unlabeled CXCL12 is included for comparison (IC50, 0.07 nM) (Fig. 5A). The cell line was also positive for CXCR7 staining by flow cytometry using two distinct anti-CXCR7 mAbs (clones 11G8 and 8F11), and negative for CXCR4 and CXCR3 (Fig. 5B).
A–C. 3 experiments performed, each of which gave similar results, is shown for tin2 with CXCR7 in a concentration-dependent manner. The mean/

Binding of 125I-labeled CXCL12 to CHO-CXCR7 transfectants was in -

hibited CXCL12 and CXCL11-induced association of 

exhibited an IC 50 of 7.1 nM against CXCL12 and an IC 50 of 5.4 

against CXCL11, whereas 8F11 for 11G8; IC 50, 24 nM for 8F11) to CXCR7 (Fig. 7) and in-

quadruplicate wells is shown. The mean 

define the maximal level of binding inhibition. The mean 

 SEM of triplicate wells is shown. Data are representative of 

SEM of triplicate wells is shown. A representative data set of 

of intracellular tracer levels was accomplished by stripping the cells of 

incubation at 37°C but not during incubation at 4°C. CHO-WT cells did not 

experiments. 

FIGURE 6. CCX771 triggers β-arrestin2 recruitment. CCX771, but not 

CCX704 compound control, AMD3100, or the CXCR7-specific mAbs 

11G8 or 8F11 (or their isotype controls) triggered association of 

of triplicate wells is shown. Data are representative of 

three independent experiments. 

CXR7 ligands are internalized upon receptor binding

As β-arrestin recruitment is often associated with receptor inter-

nalization (10), we next examined ligand internalization after bind-

ing to CXCR7. CHO-CXCR7 transfected cells were loaded with 125I-

CXCL12 or 125I-CXCL11 by incubating the radioligands with the 

IC50, 61 nM for 8F11) and 125I-CXCL11 binding (IC50, 25 nM 

for 11G8; IC50, 24 nM for 8F11) to CXCR7 (Fig. 7) and in-

hibited CXCL12 and CXCL11-induced association of β-arres-
tin2 with CXCR7 (Fig. 7). 11G8 exhibited an IC50 of 27 nM 

against CXCL12 and 20 nM against CXCL11, whereas 8F11 

exhibited an IC50 of 7.1 nM against CXCL12 and an IC50 of 5.4 

nM against CXCL11 (Fig. 7).

FIGURE 5. CXCL11 or CXCL12 binding to CXCR7 triggers β-arres-
tin2 recruitment. A. 125I-CXCL12 displacement by unlabeled CXCL11. Binding of 125I-labeled CXCL12 to CHO-CXCR7 transfected was in-
hibited in a concentration-dependent manner by unlabeled CXCL11, 

FIGURE 7. CXCR7-specific mAbs that block CXCL12 and CXCL11 binding inhibit chemokine-mediated β-arrestin2 recruitment. A. 125I-CXCL12 and 

125I-CXCL11 displacement by CXCR7-specific mAbs. Binding of 

125I-CXCL12 (left) and 125I-CXCL11 (right) to CHO-CXCR7 transfected was inhibited in a concentration-dependent manner by CXCR7-specific 

mAbs 11G8 and 8F11, but not by isotype controls. Radioligand binding to 
cells incubated in the absence of competitor is indicated (total bound). Unlabeled CXCL12 (100 nM) was included to 
define the maximal level of binding inhibition ( inset). The mean ± SEM of 
quadraplicate wells is shown. B. CXCR4, CXCR7, and CXCR3 expression 

by flow cytometry. Open histograms show receptor-specific staining; filled 
histograms show isotype controls. Relevant controls are matched (gray-
scale shaded histogram). C. CXCL12 and CXCL11, but not CXCL9, trig-
gerred association of 

FIGURE 8. CXCR7-mediated internalization of CXCL12 or CXCL11. Radioligand internalization assay using CHO-CXCR7 cells. Determination of 
intracellular tracer levels was accomplished by stripping the cells of 
surface-bound tracer with a low pH wash. CHO-CXCR7 transfactants 

125I-CXCL12 (left) and 125I-CXCL11 (right) over time during incubation at 37°C but not during incubation at 4°C. CHO-WT cells did not 

internalize radiolabeled CXCR7 ligands during incubation at 37°C. Data are representative of n = 3 independent experiments.
CXCR7-mediated internalization of CXCL12 or CXCL11 requires an intact C-terminal receptor domain. A, Flow cytometry analysis of 435-CXCR7 transfectants. Transfectants 435-WT, 435-CXCR7, and 435-CXCR7ΔC-term (the latter express a variant of CXCR7 lacking the cytoplasmic C-terminal domain, deletion of aa 319–362) were stained for CXCR7 and CXCR4 expression (11G8, open histogram; 12G5, dotted histogram; isotype control, filled histogram). B, Radioligand binding assay. Binding of 125I-CXCL12 to 435-CXCR7 or 435-CXCR7ΔC-term transfectants was inhibited by increasing concentrations of unlabeled CXCL11 (top) or CXCL12 (bottom). Radioligand binding to cells incubated in the absence of competitor is indicated as total bound for each cell type. The mean ± SEM of quadruplicate wells is shown. C, Radioligand internalization assay using 435-CXCR7 transfectants. Determination of intracellular tracer levels was accomplished by stripping the cells of surface-bound tracer with a low pH wash. A representative data set of the two to three experiments performed, each of which gave similar results, is shown for all parts of this figure.

indicating that the radiolabeled ligands were being internalized (Fig. 8). CXCR7-negative CHO-WT cells did not accumulate radioactivity over time when incubated at 37°C, indicating that ligand internalization proceeded via CXCR7 (Fig. 8).

By evaluating the behavior of a series of C-terminal truncations of the CXCR7 receptor, we also confirmed that the C terminus of CXCR7 is essential for ligand internalization (Fig. 9). MDA-MB-435 (435) human breast carcinoma cells transfected with CXCR7 or CXCR7ΔC-term were positive for CXCR7 staining by flow cytometry, and negative for CXCR4 (435-WT cells were negative for both receptors) (Fig. 9A). The transfectants displayed the CXCR7 binding signature: 125I-CXCL12 binding was inhibited by excess unlabeled CXCL11 (IC50 of 0.6 nM for 435-CXCR7 and 6.6 nM for 435-CXCR7ΔC-term). 125I-CXCL12 binding was also inhibited by excess unlabeled CXCL12 (IC50 of 0.1 nM for 435-CXCR7 and 0.9 nM for 435-CXCR7ΔC-term; 435-WT cells did not bind to 125I-CXCL12) (Fig. 9B). The ΔC-term variant was ~10-fold less potent than the intact receptor in our binding assays. In contrast, the maximal level of 125I-CXCL12 binding by 435-CXCR7ΔC-term was consistently 2-fold higher than the maximal binding observed by the 435-CXCR7 (Fig. 9B). Thus, although the ΔC-term mutation may have slightly altered the intrinsic chemokine-binding characteristics of the receptor, it remains a high affinity receptor for CXCL12 and CXCL11. In agreement with our

CHO-CXCR7 internalization data, the 435-CXCR7 cells internalized radioligands at 37°C. In contrast, neither the 435-WT nor the 435-CXCR7ΔC-term transfectants accumulated radioactivity over time when incubated at 37°C, indicating that the C terminus of CXCR7 is essential for ligand internalization (Fig. 9C).

Discussion

We have dissected several elements of CXCR7-mediated signaling and identified novel ways in which CXCR7 regulates CXCL12/CXCL11 chemokine-binding characteristics of the receptor, it remains a high affinity small molecule CXCR7 ligand with allosteric agonist properties. Kalatskaya et al. (11) showed that high concentrations of AMD3100 can modulate the conformation of constitutive CXCR7 homodimers (EC50 46 μM) and directly trigger β-arrestin2 association with CXCR7 (10 and 100 μM). The authors also showed that although AMD3100 did not block 125I-CXCL12 binding to CXCR7, 100-1000 μM AMD3100 enhanced 125I-CXCL12 binding, and conclude that AMD3100 plays a role as an allosteric modulator of CXCL12 binding to CXCR7 (11). These results imply that in studies using high concentrations of AMD3100, the drug may be acting in part through CXCR7, which underscores the utility of CCX771, a high affinity small molecule CCX771 is significantly more effective at inhibiting the CXCL12/CXCL4-mediated TEM of these cells than direct inhibition by the extensively characterized CXCR4 drug AMD3100 (Mozobil/Plerixafor).

It has recently been suggested that AMD3100 may itself be a CXCR7 ligand with allosteric agonist properties. Kalatskaya et al. (11) showed that high concentrations of AMD3100 can modulate the conformation of constitutive CXCR7 homodimers (EC50 46 μM) and directly trigger β-arrestin2 association with CXCR7 (10 and 100 μM). The authors also showed that although AMD3100 did not block 125I-CXCL12 binding to CXCR7, 100-1000 μM AMD3100 enhanced 125I-CXCL12 binding, and conclude that AMD3100 plays a role as an allosteric modulator of CXCL12 binding to CXCR7 (11). These results imply that in studies using high concentrations of AMD3100, the drug may be acting in part through CXCR7, which underscores the utility of CCX771, a high affinity small molecule CCX771 is significantly more effective at inhibiting the CXCL12/CXCL4-mediated TEM of these cells than direct inhibition by the extensively characterized CXCR4 drug AMD3100 (Mozobil/Plerixafor).

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Furthermore, Hartmann et al. (8) have suggested that CXCR7 is required for CXCL12/CXCR4 triggered LFA-1 and αβ integrin activation and subsequent adhesion of T cells to endothelium, although T cell expression of CXCR7 remains controversial (1, 8, 12). Our data indicate that NC-37 TEM toward CXCL12 is mediated by CXCR4, and that CXCR7 ligands interfere with this CXCR4-mediated event. The effect on migration is dependent on endothelial cells, as neither the small molecule CCX771 nor the endogenous CXCR7 protein ligand CXCL11 had an effect on CXCL12-mediated tumor cell chemotaxis in bare filter assays. Yet CXCR7 expression by HUVECs alone is not sufficient to invoke regulation of CXCL12-mediated TEM; CXCR7 expression by the migrating cell is required to enable CCX771 and CXCL11 regulation of CXCL12-mediated TEM. Although our data indicate that HUVECs are required, it still remains to be determined whether CXCR7 expression by the endothelial cells in concert with CXCR7 expression by the migrating cell is required for regulation of CXCL12-mediated TEM by CCX771 and CXCL11. In this context, note that the highest levels of tumor cell adhesion to HUVECs in our previous study correlated with high CXCR7 expression on both cell types (1).

In this study, we demonstrate that binding of CXCL11 or CXCL12 to CXCR7 result in ligand internalization in CHO-CXCR7 cells, 435-CXCR7 cells (which contain unmodified CXCR7 (Fig. 9)), as well as HeLa cells, which endogenously express the native receptor (data not shown). CXCL12 was previously reported to induce the internalization of CXCR7 in receptor transfectants, an effect that was dependent on an intact C-terminal domain (12). Recently, CXCL12 binding to CXCR7 on zebrafish somatic cells was shown to lead to rapid internalization of both molecules (6); thus, ligand sequestration and internalization may be a mechanism by which CXCR7 regulates CXCL12-mediated responses. For example, given its ~10-fold higher affinity for CXCL12, coexpression of CXCR7 in the same cell as CXCR4 may sequester CXCR7 at the cell surface, and reduce the potency of CXCL12 on CXCR4-dependent intracellular calcium mobilization, as observed by Levoye et al. (13).

While this manuscript was under review, Kalatsaya et al. (11) also demonstrated that CXCL12 induced β-arrestin2 association with CXCR7 (EC_{50}, 11.8 nM) using HEK293 transfectants and BRET analysis. Given that CXCL12 triggers β-arrestin2 association with both CXCR4 and CXCR7, β-arrestin2 may represent a discrete intracellular signaling molecule that can contribute to cross-talk between CXCR7 and CXCR4. Altering the interaction of β-arrestin2 with CXCR4 has important functional consequences. CXCL12-induced β-arrestin2 signaling in and chemotaxis of cell lines were reduced via short hairpin RNA knockdown of β-arrestin2 and enhanced by its overexpression, in a manner dependent on p38 MAPK and ERK (14). CXCL12-induced migration was decreased in splenocytes from β-arrestin2 knockout mice (15). We have found that the expression of CXCR4 in the same cell as CXCR7 does not appear to affect CXCL12-mediated β-arrestin2 association with CXCR7 (see supplemental Fig. S2). Recent studies indicate that coexpression of CXCR4 and CXCR7 in the same cells result in more robust CXCL12-induced intracellular calcium signaling (3). If CXCR7 sequesters β-arrestin2 away from CXCR4, this may remove certain signal-dampening properties of the β-arrestins, and allow for enhanced signaling via CXCR4. Although this may be the case for acute signaling events detected by intracellular calcium mobilization, prolonged and continuous β-arrestin2 sequestration by CXCR7 may have different functional consequences in more complex biological processes, such as TEM. Ongoing research in our laboratories is focused on identifying whether CXCR7 association with β-arrestin2 is responsible for modulating the function of CXCR4 in CXCL12-stimulated TEM.

It is well documented that CXCR7 constitutively forms heterodimers with CXCR4, and that this interaction may enable interreceptor regulation (3, 11, 13, 16, and reviewed in Ref. 17). Whether or not CXCL12 stabilizes heterodimer formation is controversial (3, 13, 16). Luker et al. (16) found that early prototypes of CCX771 (CCX733 and CCX754) enhanced CXCR7 homodimerization, but had no effect on heterodimer formation with CXCR4. Levoye et al. (13) reported that heterodimerization of CXCR7 with CXCR4 alters the relative spatial orientation between CXCR4 and G_{i1}, which may contribute to the observed ~4-fold reduction in CXCL12-mediated G protein activation. There was no evidence of cross-regulation of CXCR4 by G protein sequestration by CXCR7, although CXCR7 was shown to bind to inactive G protein G_{i1} (13). In summary, in addition to recent work by others investigating receptor heterodimerization and G_{i1} coupling, in this study, we identify β-arrestin2 as a candidate molecule involved in regulating CXCR4/CXCR7 cross-talk.

It is uncommon to discover small molecule ligands that activate GPCRs whose endogenous ligands are proteins, particularly chemokines (18). We were therefore intrigued by the ability of CCX771 to induce the association of CXCR7 with β-arrestin2. We explored the possibility that CXCR7 might be a “spring-loaded” receptor, poised to rapidly associate with β-arrestin2 and internalize upon any indiscriminate molecular contact. Because neither of the CXCR7-binding Abs triggered β-arrestin2 recruitment, we conclude that specific molecular interactions between CXCR7 and its ligands are required, and that CCX771 might in some way mimic CXCL11 or CXCL12 binding. Recently, novel small molecule agonists for CCR3, CCR5, CCR8, and CXCR3 have been reported (18–21). One of these, the agonist for CCR5, triggered β-arrestin2 recruitment (18), similar to the effect of CCX771 on CXCR7. Ali et al. (22) argue that nonchemotactic chemokine receptor agonists may be useful in interfering with leukocyte recruitment via homologous and heterologous receptor desensitization. Thus CCX771 joins the small but growing class of potential therapeutic agents that may act via targeted cellular desensitization to endogenous chemotactic gradients.

NC-37 is an EBV-transformed human B lymphoblast that serves as a model cell line for lymphoblastic leukemia (23). It has been shown that a fraction of circulating leukemic cells (possibly leukemia stem cells), as well as metastases from certain solid organ tumors, preferentially colonize the bone marrow, a site rich in growth factors and other stimuli that maintain the hematopoietic stem cell niche (reviewed in Ref. 24). In particular, CXCL12 and CXCR4 were recently shown to play a role in homing of prostate cancer metastases to the bone marrow (25). The ability of cancer cells to undergo TEM from the blood to the bone marrow stroma is critical for cancer cell “engraftment” of the bone marrow niche (26). Our data indicate that targeting CXCR7 may inhibit the TEM of circulating tumor cells, thereby preventing access to the rich microenvironment required for neoplastic expansion.

CXCL11 was reported to display antitumor activity in vivo when overexpressed by implanted tumor cells, with speculation that the underlying mechanism relates either to recruitment of tumor-killing CXCR3 lymphocytes (27–29), or to anti-angiogenic effects mediated by CXCR3 endothelial cells (30). Given our recent findings, it is possible that the antitumor activity of CXCL11 observed in these previous studies might in part reflect CXCR7-mediated inhibition of CXCR4-driven effects. The CXCR4 antagonist AMD3100 interferes with CXCL12/CXCR4 mediated retention of hematopoietic stem cells in the bone marrow, and results in their mobilization to the blood where the cells can be easily harvested for subsequent transplantation (reviewed in Ref. 31). In our studies, the selective CXCR7 small
molecule CCX771 is superior to AMD3100 in interfering with CXCL12/CXCR4-mediated TEM. These effects will be limited to cells that also express CXCR7, which may provide greater selectivity of action than indiscriminant CXCR4 blockade. Our results suggest that novel agents such as CCX771 may replicate some of the therapeutic benefits of AMD3100 and provide utility in hematopoietic stem cell mobilization, which we are currently evaluating in our laboratory.

Disclosures

The authors have no financial conflict of interest.

References