Molecular Pathways: Targeting the CXCR4–CXCL12 Axis—Untapped Potential in the Tumor Microenvironment
Stefania Scala

Abstract
Evidence suggests that the CXC–chemokine receptor-4 pathway plays a role in cancer cell homing and metastasis, and thus represents a potential target for cancer therapy. The homeostatic microenvironment chemokine CXCL12 binds the CXCR4 and CXCR7 receptors, activating divergent signals on multiple pathways, such as ERK1/2, p38, SAPK/JNK, AKT, mTOR, and the Bruton tyrosine kinase (BTK). An activating mutation in CXCR4 is responsible for a rare disease, WHIM syndrome—the Bruton tyrosine kinase (BTK). An activating mutation in CXCR4 is responsible for a rare disease, WHIM syndrome. While inhibiting adenyl cyclase, the Gαi subunit dissociates from the receptor, generating diacylglycerol and inositol 1,4,5 trisphosphate (IP3), which controls the release of intracellular Ca2+. In turn, different subtypes of the α subunit impart different signals: Gαi subunits inhibit cAMP formation via inhibition of adenyl cyclase activity, and the αqi subunits activate phospholipase C (PLC)-β, generating diacylglycerol and inositol 1,4,5 triphosphate (IP3), which controls the release of intracellular Ca2+. While inhibiting adenyl cyclase, the Gαi subunits activate the NF-κB, JAK–STAT, and PI3K–AKT pathways as well as mTOR, and the INK/p38 MAPKs regulating cell survival, proliferation, and chemotaxis. Recent studies have shown CXCR4 signaling through mTOR in pancreatic cancer, gastric cancer, and T-cell leukemia cells (10–13). In human renal cell cancers, CXCL12 induces phosphorylation of the specific mTOR targets, P70S6K and 4EBP1 (14), and CXCR4 and mTOR inhibitors have been reported to impair human renal cancer migration (Fig. 1; ref. 14). Unlike the α subunits, βy dimer subunits promote RAS-mediated MAPK signaling, thereby regulating cell proliferation and chemotaxis (6). Finally, in addition to these classic signaling pathways, CXCR4 triggers Bruton tyrosine kinase (BTK) phosphorylation and

Background
Chemokines are small chemoattractant cytokines that are expressed in discrete anatomical locations. In adult vertebrates, chemokines are essential for proper lymphoid organ architecture and for leukocyte trafficking (1). Chemokines are classified according to their conserved N-terminal cysteine residues (C) that form the first disulfide bond. These residues can be adjacent (CC) or separated by amino acids (CXC and CX3C). Forty-seven chemokines have been identified—27 CC and 17 CXC chemokines as well as a single CX3C and two single C chemokines (1, 2). Chemokines act on chemokine receptors (CKR), members of the seven-transmembrane domain G-protein–coupled receptor (GPCR) superfamily. Classically, one of the CKR intracellular loops interacts with heterotrimetric, pertussis toxin–sensitive G proteins called Gαi, initiating a cascade of signal transduction events in response to ligand binding. In addition, CKRs can signal through non–G-protein–mediated pathways or even through other G-protein subtypes (3). There are also atypical CKRs (ACKR), which do not mediate conventional signaling and do not elicit directional migration (4, 5). The CXCL12–CXCR4 axis is the focus of this Molecular Pathways review.

Encoded on chromosome 2q21, CXCR4 is an evolutionarily highly conserved GPCR expressed on monocytes, B cells, and naïve T cells in the peripheral blood. Human CXCR4 was originally identified as a receptor for CXCL12 by screening CKR orphan genes for codons for their ability to induce intracellular Ca2+ in response to human CXCL12. Its ligand, CXCL12, is a homeostatic chemokine, which controls hematopoietic cell trafficking, adhesion, immune surveillance, and development. The amino-termini of CXCL12 binds the second extracellular loop of CXCR4 and activates downstream signaling pathways. The third intracellular loop of CXCR4 is necessary for Gαi-dependent signaling, and intracellular loops 2 and 3 as well as the C-terminus of CXCR4 are required for chemotaxis (6, 7). CXCL12 binding to CXCR4 triggers multiple signal transduction pathways that are able to regulate intracellular calcium flux, chemotaxis, transcription, and cell survival (8). CXCL12 binding promotes a three-dimensional CXCR4 conformation favoring Gαi protein dissociation into α and βy subunits (8, 9). In turn, different subtypes of the α subunit impart different signals: Gαi subunits inhibit cAMP formation via inhibition of adenyl cyclase activity, and the αqi subunits activate phospholipase C (PLC)-β, generating diacylglycerol and inositol 1,4,5 triphosphate (IP3), which controls the release of intracellular Ca2+. While inhibiting adenyl cyclase, the Gαi subunits activate the NF-κB, JAK–STAT, and PI3K–AKT pathways as well as mTOR, and the INK/p38 MAPKs regulating cell survival, proliferation, and chemotaxis. Recent studies have shown CXCR4 signaling through mTOR in pancreatic cancer, gastric cancer, and T-cell leukemia cells (10–13). In human renal cell cancers, CXCL12 induces phosphorylation of the specific mTOR targets, P70S6K and 4EBP1 (14), and CXCR4 and mTOR inhibitors have been reported to impair human renal cancer migration (Fig. 1; ref. 14). Unlike the α subunits, βy dimer subunits promote RAS-mediated MAPK signaling, thereby regulating cell proliferation and chemotaxis (6).
downstream MAPK in mantle cell lymphoma and primary acute myeloid leukemia (AML) blasts, suggesting a potential interaction of CXCR4 on BTK and a potential for concomitant CXCR4 and BTK inhibition, the latter possibility raised by the availability of a recently approved inhibitor of BTK, ibrutinib (15).

CXCL12 binding to CXCR4 also causes CXCR4 desensitization, manifested as uncoupling from G proteins by GPCR kinase (GRK)-dependent phosphorylation and subsequent interaction of CXCR4 with β-arrestin, which mediates internalization of the receptor (Fig. 1; ref. 16). Upon internalization, CXCR4 is targeted for lysosomal degradation (9, 16). This requires agonist-induced ubiquitination of the carboxyl terminal (C-terminal) tail lysine residues (16).

Recently, CXCR7 was also found to be a high-affinity receptor for CXCL12 and CXCL11 (4, 17, 18). CXCR7, renamed as ACKR3, belongs to the atypical CKR group (ACKR) of proteins, which do not mediate conventional signaling and do not elicit directional migration. The highly conserved DRYLAIV domain, which controls G-protein binding and activation in CXCR4, is replaced by DRYLSIT in the CXCR7 protein. Thus, typical chemokine...
responses mediated by G-protein activity are not generated after ligand binding. However, evidence suggests that CXCR7 activates intracellular signaling pathways, including the AKT, MAPK, and JAK–STAT3 pathways (18). CXCR7 is also regulated by ubiquitination, but in contrast with CXCR4, CXCR7 is constitutively ubiquitinated, and agonist activation induces its deubiquitination. Upon agonist binding, CXCR7 is rapidly internalized via a β-arrestin–dependent pathway and recycled to the cell surface. CXCR7 itself is not degraded, but rather it delivers bound chemokine to lysosomes for degradation (16). β-arrestin recruitment to the CXCR4–CXCR7 complex enhances downstream β-arrestin–dependent cell signaling (ERK1/2, p38, and SAPK/JNK), which induces cell migration in response to CXCL12 (11, 13, 18, 19).

Clinical–Translational Advances

The CXCR4 antagonist AMD3100 is the most studied among the agents that inhibit CXCL12/CXCR4 signaling. AMD3100 was initially studied as an anti-HIV agent, and then it was discovered that this compound increases white blood cell counts in the blood and is able to mobilize stem cells from the bone marrow (20, 21). Cell migration in and out of the bone marrow follows opposite chemokine signals, which implies that chemokines regulate the numbers of circulating granulocytes and monocytes. CXCL12 is the predominant signal that retains CXCR4+ hematopoietic stem cells (HSC) inside the bone marrow (21). Plerixafor (previously AMD3100) is available clinically, having been developed as a mobilizing agent for hematopoietic precursors. The FDA approved plerixafor in 2008 for "use in combination with granulocyte-colony stimulating factor (G-CSF) to mobilize hematopoietic stem cells to the peripheral blood for collection and subsequent autologous transplantation in patients with non-Hodgkin lymphoma (NHL) and multiple myeloma" (20, 22).

A clinical indication for CXCR4 antagonists has emerged recently in the rare human WHIM syndrome (warts, hypogammaglobulinemia, infections, and myelokathexis). WHIM syndrome is associated with germline-dominant mutations in the C-terminus of CXCR4, which result in a truncation of the receptor (23). This blocks receptor internalization, determining persistent CXCR4 activation and bone marrow myeloid cell retention, an outcome known as myelokathexis, the retention and apoptosis of mature neutrophils in the bone marrow (24). Two phase I trials showed that plerixafor was able to safely and rapidly increase absolute lymphocyte, monocyte, and neutrophil counts in the peripheral blood of WHIM syndrome patients in a dose-dependent manner for 1 to 2 weeks (25). Another phase I study demonstrated a durable increase in circulating leukocytes, fewer infections, and improvement in warts in combination with topical imiquimod in 3 patients with WHIM syndrome who self-injected a low dose of plerixafor (4% to 8% of the FDA-approved dose) s.c. twice daily for 6 months (26). However, despite the fact that B cells and T cells were mobilized to blood, and that naïve B cells underwent class switching in vitro, no improvement was seen in Ig levels or vaccine responses (26). Thus, it appears that alternate dosing regimens or a more long-lasting CXCR4 antagonist will be required to achieve more durable activity that may in turn provide more stable levels of immune cells in the blood to enhance adaptive immune function.

CXCR4-activating mutations have also been recently found in Waldenstrom macroglobulinemia. Waldenstrom macroglobulinemia is an incurable B-cell neoplasm characterized by accumulation of malignant lymphoplasmacytic cells in the bone marrow, lymph nodes, and spleen, with excess production of serum IgM producing hyperviscosity, tissue infiltration, and autoimmune-related pathology. Activating somatic mutations have been found by whole-genome sequencing in Waldenstrom macroglobulinemia, including a locus in CXCR4 (27). In addition, approximately 90% to 95% of Waldenstrom macroglobulinemia patients harbor an activating mutation in MYD88 (MYD88L265P) that promotes both the interleukin-1 receptor–associated kinase (IRAK) and BTK, which in turn activate NF-κB–p65-dependent nuclear translocation and malignant cell growth. The location of the CXCR4 somatic mutations in the C-terminal domain of Waldenstrom macroglobulinemia patients is similar to the location of the mutations observed in the germline of patients with WHIM syndrome. Somatic mutations in MYD88 and CXCR4 are thus felt to be important and to affect overall survival in Waldenstrom macroglobulinemia (27), opening the way for possible combination therapy directed against MYD88 and CXCR4 signaling in Waldenstrom macroglobulinemia (27).

The CXCR4–CXCL12 axis as a potential target in cancer therapy

Although there has been great enthusiasm for exploiting the CXCR4–CXCL12 axis as a target in cancer therapy, to date the promise has yet to be fulfilled. Initial interest in pursuing the CXCR4–CXCL12 axis as a target for cancer therapy was fueled by observations implicating CXCR4 in promoting metastasis (2, 28). CXCR4 is expressed in at least 20 different human cancers (8, 29–33); CXCR7 is also expressed in tumors and found to be involved in cell growth, survival, and metastasis (34, 35). Like CXCR4, it is expressed on tumor-associated vessels and on neovascularature (36). On the other hand, CXCL12 is secreted in the tumor microenvironment by stromal cells (21, 28). On the basis of the data such as these, it has been thought that CXCR4 antagonism could prevent the development of metastases by targeting multiple steps in the process of dissemination. Restricted by the tumor type, inhibiting CXCR4 should interfere with tumor cell growth, migration and chemotaxis, and homing toward secondary organs.

In addition to a potential role for agents targeting the CXCR4–CXCL12 axis in preventing metastasis, other indications have been suggested. Some researchers have proposed that inhibition of the CXCR4–CXCL12 axis could be used to mobilize leukemic cells from the marrow, rendering them more amenable to cytotoxic chemotherapy by removing them from the prosurvival stem cell niche (8, 21, 37–39).

Others have suggested that modulation of the CXCR4–CXCL12 axis could revert the tolerogenic polarization of the microenvironment rich of immunosuppressive cells such as regulatory T cells (Treg), M2, and N2 neutrophils (40–42). Recent data show that blocking the interaction of T cells expressing CXCR4 with cells in the microenvironment secreting CXCL12 may modulate immunotherapy with anti–CTLA-4 or anti–PD-1 (41, 42). Although inhibition checkpoints have been shown to induce immune-mediated tumor shrinkage, major responses have been reported in only a subset of patients following PD-1 blockade (43). The resistance to immunotherapy reported in colon cancer, ovarian cancer, and in the model of pancreatic ductal adenocarcinoma (PDA)
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is based on multiple mechanisms: spatial distribution of effector T cells relatively to tumor, recruitment of tumor-specific T cells from the vessel, and T cells’ proliferation activity. In the PDA model, cancer-associated fibroblasts (CAF) cells may regulate the T-cell access to the tumor through release of CXCL12, which is bound by the PDA cancer cells. Plerixafor, inhibiting CXCR4, increases accumulation of T cells among cancer cells, impairing tumor growth and increasing tumor sensitivity to anti–PD-L1. The related mechanism must involve either T cells or myelomonocytic cells, CXCR4-expressing cells. Not of note, in this model, neither cancer cells nor CAF cells, including immunosuppressive cells. Because the recruitment of immunosuppressive infiltrates (F4/80, tumor-associated macrophages, myeloid-derived suppressive cells, and Tregs) and increased PD-L1 expression in multiple hematopoietic cells, including immunosuppressive cells. The breach the immunosuppressive tumor population was partially mediated by CXCL12-induced hypoxia, the efficacy of CXCR4 inhibition was evaluated. A combined treatment (sorafenib/plerixafor/anti–PD-1) showed the most pronounced delay in tumor growth, probably mediated by increased intratumoral penetration and activation of CD8 T lymphocytes in HCC (42). Moreover, in a mouse model of intraperitoneal papillary epithelial ovarian cancer, CXCR4 antagonism increased tumor cell apoptosis and necrosis, reduced intraperitoneal dissemination, and selectively reduced intratumoral FoxP3+ Tregs (40). Consistent with these data, plerixafor and the antagonistic CXCR4 peptide R (45, 46) inhibit Treg-suppressive activity in primary renal cancer specimens in which higher numbers of activated Tregs (expressing CTIL-4/CXCR4/PD-1/ICOS) were detected. A possible mechanism may involve the Treg–FOXP3 promoter. Treg–FOXP3 activity is regulated postranslationally by histone/protein acetyltransferases and histone/protein deacetylases (HDAC). Pan-histone/protein deacetylase inhibitors (pan-HDAC) have been shown to increase FOXP3 acetylation and DNA binding, enhance Treg production and suppressive activity, and have beneficial effects in the prevention and treatment of autoimmune disease and transplant rejection (47). Because CXCR4 signaling transduces also on FOXP3 and HDACs upregulated CXCR4 mRNA expression (48), it is possible that CXCR4 modulation affects the acetylation status of FOXP3 promoter.

**CXCR4 antagonists in development**

Plerixafor has many positive attributes, including demonstrated antimetastatic potential in preclinical studies; however, there is room for the development of additional agents targeting the CXCR4–CXCL12 axis. CXCR4 inhibition was originally conceived as a strategy to block infection of CD4+ T cells by HIV because CXCR4 functions as a coreceptor for T-tropic (X4) HIV virus entry (20). During its development as an anti-HIV drug, plerixafor displayed a lack of oral bioavailability and cardiotoxicity. In addition, its toxicity profile is such that it limits long-term administration, as might be required for metastasis prevention therapy (49). Although a 6-month administration was tolerated in WHIM patients, doses used were 4% to 8% of the FDA-approved dose (26). Finally, plerixafor lacks CXCR4 specificity because it also binds the other CXCL12 receptor, CXCR7, as an allosteric agonist (50). The ability of CXCR4 antagonists to induce stem cell mobilization raised questions about whether the same treatment might mobilize cells from solid tumors that express CXCR4. In a recent phase I study, the efficacy of the peptidic CXCR4 antagonist LY2510924 was concomitantly evaluated on CD34+ mobilization versus count of circulating tumor cells (CTC). Although there was significant mobilization of CD34+ cells upon treatment with LY2510924, no apparent effect on CTC count was observed (51).

Table 1 lists CXCR4 antagonists in clinical development. The majority of clinical trials have focused on the mobilizing activity of CXCR4 antagonists and were conducted in multiple myeloma, AML, and chronic lymphatic leukemia (Table 1). Among peptidic inhibitors, 4F-benzoyl-TN14003 (BKT-140) is a 14-residue biostable synthetic peptide that binds CXCR4 with a greater affinity compared with plerixafor. Studies in mice demonstrated the efficient and superior mobilization and transplantation of stem cells collected with G-CSF-BKT140, compared with the single agent alone (52, 53). Additional studies demonstrated anticanter activity (54) and an ability of BKT140 to directly induce cell death of chronic myeloid leukemia (CML) cells and to revert resistance to imatinib mediated by increasing BCL6 (37). A Phase I trial conducted in multiple myeloma patients showed that BKT140 was well tolerated and produced a robust mobilization that resulted in the collection of functional CD34+ cells that rapidly engrafted (55). However, to date, anticanter activity has not been demonstrated.

Evaluation of antimetastatic activity in solid tumors is in phase I and II clinical development, and a limited number of results are available. LY2510924 (Eli Lilly and Company) is a potent selective peptide CXCR4 antagonist; a phase I trial showed that its most common drug-related adverse events were fatigue, injection-site reaction, and nausea. However, its potential as an anticaner agent has yet to be established. In the phase I trial, the best response achieved was stable disease in 9 patients (20%; ref. 51). In two phase II studies, one in renal cell carcinoma (NCT01391130) and one in small cell lung cancer (SCLC; NCT01439568) that evaluated the efficacy of LY2510924 in combination with sunitinib and carboplatin/etoposide,
The question at hand is how best to design trials that will circumvent the problem that CXCR4 antagonists may not reduce primary tumor growth in such cancers as ovarian and colorectal cancer, where both local and distant metastasis prevention would be important and where CXCR4 likely plays a role. One proposed model of clinical development would be to add it to chemotherapy, such as 5-fluorouracil and oxaliplatin, which are used in neoadjuvant treatment of locally advanced rectal cancer. This type of setting would identify a time-limited treatment and a rapid evaluation of results. Another ideal setting would be for treatment of glioblastoma following primary surgery, in which it is possible to hypothesize that CXCR4 inhibition coupled to radiotherapy may have an additional effect on vasculogenesis ameliorating tumor control, as shown in preclinical studies (61).

Conclusions

Extensive preclinical data indicate that targeting the CXCR4–CXCL12 axis may have several beneficial actions, including (i) affecting CXCR4-expressing primary tumor cells; (ii) synergizing with other cancer-targeted therapies; and (iii) modulating the immune response. Although any or all of these could be valuable anticancer strategies, clinical results to date have been disappointing. Additional studies and studies with new agents will be required to determine whether inhibition of the CXCR4–CXCL12 axis may produce enough anticancer activity to be effective therapy in the complex setting of human cancer.

Disclosure of Potential Conflicts of Interest

S. Scala is listed as a co-inventor on a patent, which is co-owned by Istituto Nazionale per lo Studio e la Cura del Tumori and Consiglio Nazionale delle Ricerche, related to cyclic peptides binding CXCR4 receptor and relative medical and diagnostic uses. No other potential conflicts of interest were disclosed.

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