CXCR4 Receptor as a Promising Target for Oncolytic Drugs

Yan Lavrovsky[1], Yan A. Ivanenkov[1], Konstantin V. Balakin[1,2,*], Darya A. Medvedeva[1] and Alexandre V. Ivachtchenko[1]

[1]*Small Molecule Drug Discovery, ChemDiv, Inc., San Diego, California, 92121 USA;* [2]*Institute of Physiologically Active Compounds, Russian Academy of Sciences, 142432, Severnyi Proezd 1, Chernogolovka, Moscow Reg., Russia*

Abstract: There has been considerable *in vivo* evidence that chemokine receptor CXCR4 and its endogenous ligand CXCL12 modulate some important physiological and pathophysiological processes, including cancer metastasis, angiogenesis, invasion, growth and progression. In this review we elucidate key aspects of CXCL12-CXCR4 signaling system with emphasis on peptide-based and small-molecule CXCR4 inhibitors.

Key Words: Chemokine, chemokine receptor, CXCR4, CXCL12, SDF-1, tumor, cancer, drugs, inhibitors, antagonists, design.

1. BIOLOGICAL ASPECT OF CXCR4 MEDIATED CANCER GROWTH AND PROGRESSION

Chemokines (chemotactic/chemoattractant cytokines) are highly basic small secreted proteins consisting on average of 70-125 amino acids with molecular masses ranging from 6 to 14 kDa which mediate their effects through binding to seven transmembrane domain (7-TM) of the specific family of G-protein-coupled receptors (GPCR) located on target cell membrane. Initially, chemokines were recognized as chemoattractants and activators of specific types of leucocytes in a variety of immune and inflammatory responses. Over the past few years there has been a "chemokine revolution" in anti-cancer drug discovery, which elucidated their crucial role at all stages of neoplastic transformation and progression [1]. Thus, tumor cells extensively express functional chemokine receptors, which can sustain proliferation, angiogenesis, survival and promote organ specific localization of distant cancer metastases [2].

The chemokine receptor CXCR4 possesses multiple fundamental functions in both normal and pathologic physiology. CXCR4 is a GPCR receptor that transduces signals of its endogenous ligand, the chemokine CXCL12 (stromal cell-derived factor-1, SDF-1, previously SDF1-α). The interaction between CXCL12 and CXCR4 plays a critical role in the migration of progenitors during embryologic development of the cardiovascular, hemopoietic, central nervous systems, and so on. This interaction is also known to be involved in several intractable disease processes, including HIV infection, cancer cell metastasis, leukemia cell progression, rheumatoid arthritis (RA), asthma and pulmonary fibrosis.

1.1. The Pivotal Role of CXCR4 Chemokine Receptor in Cancer Pathology

Unlike other chemokine receptors, CXCR4 is expressed in many normal tissues, including those of the central nervous system, while it is also commonly expressed by over 25 different tumor cells including cancers of epithelial, mesenchymal, haematopoeitic origin, etc. [3]. For example, tumor cells from breast, prostate, pancreatic, lung and ovarian carcinomas, neuroblastoma and glioblastoma, all express CXCR4 [4,5]. This receptor was also found in human acute lymphoblastic, myeloblastic and myelogenous leukemias, in non-Hodgkin`s lymphoma, in tumors derived from kidney, as well as in melanoma and rhabdomyosarcoma [5-7]. In other cancer cells studied, CXCR4 may be co-expressed with other chemokine receptors or less commonly, other receptors are present without expression of CXCR4. Several lines of evidence show that the CXCL12-CXCR4 chemokine system may also be involved in promoting tumor cell survival and growth. For instance, in cells from adult glioblastoma and pediatric medulloblastoma, CXCR4-CXCL12 signalling network induced chemotaxis and enhanced proliferation and survival [8]. In several types of cancer, including glioma, melanoma, NSCLC, renal and thyroid cancers, CXCL12 can stimulate tumor proliferation and/or survival of CXCR4-expressing tumor cells [9]. Production of CXCL12, at both mRNA and protein level, has been detected in several CXCR4-expressing tumors, thus suggesting a possible autocrine or paracrine loop of growth. It has recently been reported that CXCR4 is frequently expressed in human pancreatic cancer cells and along with its ligand, CXCL12, in addition to enhancing motility and invasion, promotes their proliferation and excites anti-apoptotic effects [10]. Furthermore, Wang and Ma have suggested that β-catenin is a vital key intracellular factor in the CXCR4-CXCL12 axis promoting metastatic events of pancreatic cancer [11]. It was recently reported that CXCR4 is expressed in several types of malignant brain tumors [12]. Human breast cancer cells express CXCR4 and CCR7 [13,14], and CXCR4 repression was found to effectively inhibit breast cancer metastasis in experimental animal models [6]. The specific ligands for these receptors CXCL12 and CCL21 are found at elevated levels in lymph nodes, lung, liver and bone marrow; organs to which breast tumours generally metastasize. In addition, as communicated in recent studies, melanoma cells generally expressing CCR7 and CCR10 chemokine receptors [13] also significantly co-express CXCR4 and CXCR3 that play a critical role in tumor growth, metastasis and tissue invasion

*Address correspondence to this author at the Small Molecule Drug Discovery, ChemDiv, Inc., San Diego, California, 92121 USA; Tel: (858) 794-4860; Fax: (858) 794-4931; E-mail: kb@chemdiv.com

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[15]. Leukaemic and lymphoma cells also express a wide variety of chemokine receptors, including CXCR4. Recently, CXCR4 has been reported to be a key effector in the formation of peritoneal carcinomatosis in gastric cancer in proliferation, migration and invasion of epithelial ovarian cancer cells [16,17]. Furthermore, there is a growing *in vitro* and *in vivo* evidence that CXCR4 expression by leukaemia cells allows for homing and their retention within the marrow. As such, leukaemia cells appear to utilize CXCR4 to access niches that are normally restricted to progenitor cells, and thereby reside in a microenvironment that favours their growth and survival [18]. It has also been recently reported that CXCR4-CXCL12 signaling activates phosphorylation of extracellular-signal regulated kinase 1/2 (ERK1/2) and stimulates meningioma cell proliferation [19]. Considering high diversity and complexity of chemokine-related signal propagation we discuss here the basic features of chemokine CXCR4-CXCL12 signalling network and disclose the main principles of how this pair works in the field of cancer growth and progression.

It should be noted that CXCR4 is not a tumor specific marker and not all cancers express this receptor. Both the receptor and its specific ligand are widely expressed in normal tissues and play a fundamental role in a variety of physiological processes including fetal development, joint mobilization of haemopoietic stem cells and specific trafficking of a majority of leukocyte types [20].

1.2. Signaling *via* CXCR4-CXCL12 Chemokine System

The chemokine receptor CXCR4 is directly involved in a number of biological processes including organogenesis, hematopoiesis, and immune response. Several recent reports highlight the high complexity of intra/extracellular signal transduction initiated by chemokine receptors, especially by CXCR4 [21,22]. In general, chemokines activate 7-TM GPCR chemokine receptors which are coupled to heterotrimeric Gαβγ protein subunit. Heterotrimeric G-proteins associate with the intracellular domains of GPCRs when in their inactive, or guanosine diphosphate (GDP)-bound, state. Upon chemokine ligand binding, the GDP is readily exchanged for guanosine triphosphate (GTP) resulting in instantaneous activation of corresponding G-protein. The active G-protein subsequently initiates dissociation of Gαβγ into its Gα and Gβγ subunits to stimulate many intracellular mediators. In contrast to other chemokine receptors, stimulation of CXCR4 can lead to prolonged activation of both mentioned subunits [23]. Signaling *via* CXCR4 also enhances tyrosine phosphorylation, association of components of focal adhesion complexes such as paxillin and NF-κB activity in nuclear extracts [24]. In a metastatic breast cancer cell line, NF-κB directly regulates the CXCR4 promoter and can upregulate expression of CXCR4, facilitating increased responses to CXCL12 [25]. In breast cancer cell lines, CXCL12 also induces phosphorylation of FAK, Pyk2, the cytoskeletal proteins paxillin and Crk, the tyrosine phosphatase SHP2 and the adaptor protein Cbl [26]. There is one interesting report of cross talk between the BCR/ABL oncogenic tyrosine kinase and CXCR4 signalling [27]. In CML, BCR/ABL kinase phosphorylates, activates and disregulates proliferation and survival pathways of progenitor cells in the bone marrow. Immature leukaemic cells leave the marrow and migrate in large numbers to the blood and spleen. BCR/ABL strongly activates a CXCR4-dependent signalling component through the *Src* family tyrosine kinase, Lyn. Cross-talk between BCR/ABL and CXCR4 signalling may allow the oncoprotein to couple to PI3-kinase, MAPK cascades and 'take over' the chemokine pathway. This could lead to disruption of chemotaxis and hence release of the transformed cells into the periphery. Undoubtly, the precise signaling mechanisms by which all chemokine receptors regulate cellular function will become evident in time, but it has proven to be difficult to link specific CCR or CXCR signalling pathways right through to a biological response such as chemotaxis, cell growth and progression.

1.3. The Complicity of CXCR4 in Cancer Metastasis, Angiogenesis, Adhesion and Invasion

Cancer metastasis results from a non-random process, in which organ selectivity by the tumor cells is highly dependent on interactions between tumour and host stromal cells as well as between cancer cell and several essential molecular factors expressed at the remote organs that eventually turn into preferred sites of metastasis formation [28,29]. In aggregate, these factors support the consecutive steps required for metastasis formation, including tumor cell adhesion to microvessel walls, extravasation into target tissue and migration. For instance, chemokine CXCL12, lysophosphatidic acid (LPA) and thrombin promote the migration and invasion of cancer cells through their cognate receptors, CXCR4, LPA1 and PAR1, respectively, enabling the cancer cells to escape from the location of the primary tumour [30]. Metastasis is a major cause of morbidity and mortality in breast cancer patients. To prevent these lethal outcomes, improved strategies to treat metastatic neoplasms are strongly needed. Blood flow and other mechanical factors influence the delivery of cancer cells to specific organs, whereas molecular interactions between the cancer cells and the new organ influence the probability that the cells will grow there. Inhibition of the growth of metastases in secondary sites offers a promising approach for effective cancer therapy. Metastases arise following the spread of cancer from a primary site and the formation of new tumor nidus in distant organs. When a cancer is detected at an early stage, before it has spread, it can often be treated successfully by small molecule inhibitors of tumor growth or surgery/local irradiation, and the patient will be cured. However, when cancer is detected after it is known to have metastasized, treatments are much less successful.

Many chemokines play multiple roles in tumor growth, invasion and metastasis by inducing cellular transformation, angiogenesis, secretion of proteinases, and organ specific metastasis [31]. As mentioned above, chemokines control the directional migration of leukocytes and it seems that mechanisms utilised for leukocyte trafficking may also be used by tumor cells. Studies on contribution of chemokine receptors to organ specific metastasis provide important clues about why some cancers metastasize to specific organs. Recent elegant studies have shown that tumour cells express patterns of chemokine receptors, including CXCR4, that 'match' chemokines that are specifically expressed in organs to which these cancers commonly metastasize [32].

Cancer cells migrate towards the chemoattractant gradient until reaching the site for secondary colonization. For chemokine receptor expression by a cancer cell to be advantageous, a chemokine gradient is required to be established and in breast, prostate and ovarian cancer, neuroblastoma, melanoma and some forms of leukaemia, the respective ligand is strongly expressed at sites of tumor spread. Tumour cell migration in response to CXCR4 stimulation requires the polarization of intracellular signalling molecules that results in a leading edge that protrudes outward, coupled with contractile forces at the back and sides of the cell to propel the cell towards a chemoattractant. For example, melanoma cells express functional CCR7, CCR10 (and lower levels of CXCR4) (Fig. (**1**)). Melanoma cells also express specific ligands for these receptors at the two major sites of metastasis, skin and lymph nodes [13]. Both breast cancer cells and primary breast tumours were found to express the chemokine receptors CXCR4 and CCR7 at high levels. The specific soluble ligands for these receptors CXCL12 and CCL21 are found at elevated levels in lymph nodes, lung, liver and bone marrow – organs to which breast tumours often metastasize, whereas skin tissue expresses high levels of CCL27, a soluble ligand for the CCR10 receptor [33]. Therefore, breast cancer cells that are taken to the lung by the blood flow would find a strong chemokine-receptor 'match', which would lead to chemokine mediated signal activation. By contrast, breast cancer cells taken to skin would not find such a match. Melanoma cells, however, taken to skin by the circulation (or by local invasion) would find a CCL27-CCR10 chemokine-receptor 'match' that would lead to the activation of chemokine-mediated pathways. These results are further supported by experimental tumor models: transduction of tumor cells with CCR7 conferred improved ability to metastasize to regional lymph nodes [34], while CXCR4-transfected cells preferentially migrated to the lung [35].

Cyclooxygenase 2 (COX2) expressed in tumour and stromal cells generates prostaglandin E2 (PGE2), which binds to EP2 (pro-angiogenic factor) receptors on cancer cells and promotes tumour cell proliferation and extracellular matrix (ECM) degradation through the expression of matrix metalloproteinase 2 (MMP2) and MMP9 [36], a response also elicited by thrombin and CXCL12. Stimulation of mentioned GPCR receptors (CXCR4, LPA1, PAR1 and EP2) also causes increased release of vascular endothelial growth factor (VEGF), thereby promoting vascular permeability, which is important for tumour cell extravasation and tumour angiogenesis. Specifically for solid tumours, as they grow, the hypoxic condition in the tumour microenvironment results in the stabilization of hypoxia-inducible factor-1 (HIF1), which upregulates CXCL12 [37] and VEGF. Cancer cells also produce several CC and CXC chemokines, such as CCL2, CCL5, CXCL8 (interleukin 8 (IL8)) to recruit tumor associated macrophages (TAMs) and leukocytes to the tumour. These immune cells then help to promote blood vessel growth by releasing VEGF and other angiogenic factors (AF). Concomitantly, tumour or stromal inflammatory mediators that act on GPCRs, such as IL8, prostaglandin E2 (PGE2) and sphingosine-1-phosphate (S1P), can also regulate the activity of MMPs that degrade the ECM, which clears a path, at the same time as endothelial cell chemotaxis, often involving the coordinated activation of a network of small GTPases such as Rho and Rac and their downstream targets by $G\alpha_{13}$ or $G\beta\gamma$ when released from $G\alpha_i$, paves the way for new blood vessel growth. Finally, S1P is released following the activation of sphingosine kinase activity, and functions in an autocrine and paracrine manner to cause tumour and endothelial cell proliferation and migration. Inflammatory cytokines that accumulate in the tumour milieu also stimulate the nuclear factor kappa B (NFkB)-dependent increased expression and release of IL8 from stromal and cancer cells, which promotes endothelial cell migration towards the growing tumour. Ultimately, pro-angiogenic GPCRs activate a network of small GTPases, Akt and mitogen-activated protein kinase (MAPK) signalling that promotes the migration, survival and growth of endothelial cells. Several other important mediators are also implicated in cancer growth, metastasis, invasion and angiogenesis, these include HIF1α, hypoxia-inducible factor-1; IL8, interleukin 8; NFkB, nuclear factor kappa B, etc.

Clinical importance and therapeutic implications of the pivotal CXCL12-CXCR4 interaction in cancer cell migration was recently exhaustively discussed by Arya *et al.* [38]. In most *in vitro* studies with CXCR4 expressing cancer cell lines, activation of the receptor with CXCL12 stimulates specific migration of cancer cells or invasion through matrigel or monolayers of endothelial cells, fibroblasts and/or bone marrow stromal cells [39]. Furthermore, blocking CXCR4 was found to inhibit metastasis of breast cancer cells [40]. CXCR4 antagonists also blocked both growth of primary tumor and organ-specific metastasis of head and neck cancer in xenograft mouse models [41]. Thus, using the orthotopic SCCHN animal model, it was shown that anti-CXCR4 treatment suppressed primary tumor growth by inhibiting tumor angiogenesis and prevented lung metastasis. Furthermore, collected data from 600 prostate cancer patients revealed that CXCR4 protein expression was significantly elevated in localized and metastatic prostate cancer compared to normal or benign prostate tissue and CXCL12 protein levels were higher in metastatic, compared to normal, prostate tissue [42].

Overall these results support the concept that chemokines could direct tumor cell migration *in vivo*: malignant cells bearing chemokine receptors on the cell surface would be endowed with the capability to respond to chemokine gradient and selectively migrate to specific organs where the chemokine is present. However, metastasis is complex multistep process and there are several stages at which the interaction between tumor cell chemokine receptors and their ligands could be important. It has been suggested that, in addition to tumor cell movement to a gradient, chemokines play a critical role in tumor cell adhesion, invasion, survival, growth and angiogenesis.

As mentioned above, the chemokine signaling pathway *via* chemokine receptors can modulate many intracellular functions including expression of integrins by a tumor cell, which can then facilitate adhesion of cancer cells to and/or invasion through the extracellular matrix. Thus, it was demonstrated that CXCL12 stimulation of different ovarian cancer cell lines upregulated the expression of β1 integrin [43]. For one's turn integrin modulation correlates with highly increased tumor cell adhesion. This migration can be abro-

gated by a broad spectrum of MMP inhibitors [44]. Additionally, β1 integrins have also been reported to regulate both the formation of and adhesion within, ovarian cancer spheroids [45]. In small cell lung cancer cells (SCLC) CXCL12 stimulation induced firm adhesion to marrow stromal cells *via* activation of α4β1 integrin and also induced SCLC cell invasion into the extracellular matrix [46]. In lymphocytes, the chemokines CXCL12 and CCL21 activate adhesion, mediated by LFA-1 and VLA-4, and also transendothelial migration where the small GTPase, RAP1, serves to increase the adhesive capacity of these adhesion molecules [47]. Adhesion mechanisms can also impact chemokine receptor expression, e.g. in non-transformed lymphocytes, activation of L-selectin (by antibody crosslinking or specific ligands) mobilises intracellular stores of CXCR4 to increase cell surface expression [48].

It has long been known that chemokines induce production of proteases, such as matrix metalloproteases and urokinase-type plasminogen activator (u-PA) in tumor cells and TAM. Tumor-derived proteases can cleave the extracellular matrix molecules and lead to the dissolution of the basement membrane. Thus, they are important for invasion and it has been suggested that monocytes infiltrating the tumor tissue provide cancer cells with a ready-made path for invasion (countercurrent invasion theory) [49]. A variety of proteolytic enzymes, in particular the tissue type plasminogen activator (t-PA), u-PA and the large family of matrix-metalloproteinases (MMPs) have been implicated in this degradation [50]. The activity of these enzymes has been associated with more aggressive neoplastic behaviour. For example, t-PA and u-PA and their respective receptors, annexin II and u-PAR, were demonstrated to contribute to the invasive behaviour of pancreatic cancer [51]. MMP-2 expression is increased in several tumors and strongly correlates with nodal status and tumor stage [52]. Chemokines are potent inducers of enzymes and receptors which degrade the extracellular matrix and favour tumor invasion. In a gene expression analysis, the chemokine CCL5 specifically induced gene expression of various MMPs, especially MMP9, along with the u-PA receptor [53]. Macrophages can produce proteases and a strong evidence demonstrates that chemokines activate TAM to release MMPs in the tumor microenvironment (Fig. (**1**)). In particular, MMP9 derived from hematopoietic cells of a host origin, has been shown to contribute to skin carcinogenesis. In addition, MMP9 has complex effects beyond matrix degradation, including promotion of angiogenesis and release of growth factors [54]. Moreover, several chemokines and chemokine receptors, like CXCL16-CXCR6 and more common CCR5 and CXCL12-CXCR4, connected to $CD4^+$ T-cells were reported to enhance invasion and disease progression in an experimental model of skin carcinogenesis [55]. Even if CXCL12 is a non-ELR chemokine, its activity has been implicated in neoangiogenesis [56]. There are also links between CXCR4 and

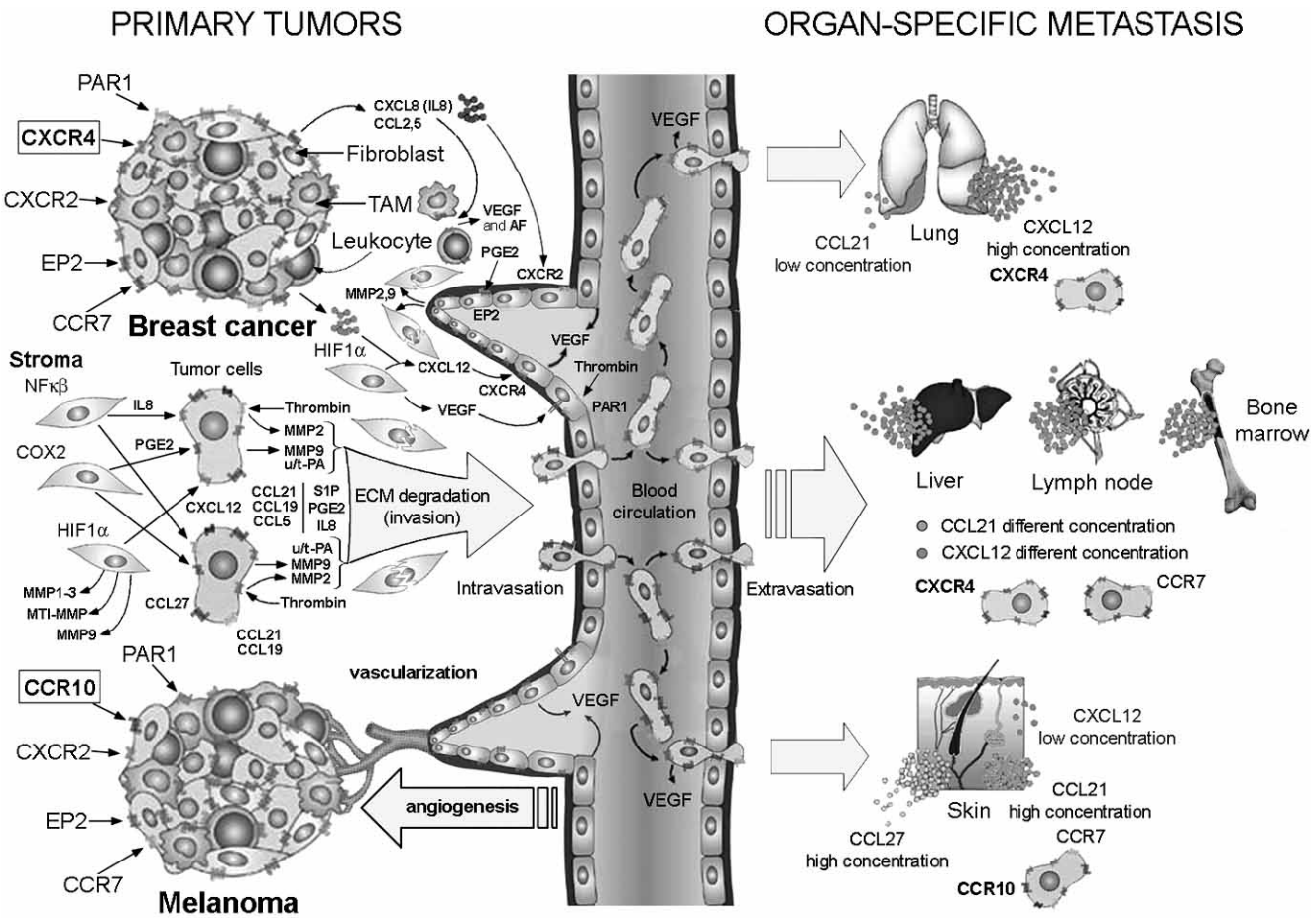


Fig. (1). Chemokines and chemokine receptors can promote organ-specific metastasis, invasion and angiogenesis of primary tumors.

vascular endothelial growth factor (VEGF). Thus, it was recently shown that vascularization of the gastrointestinal tract is defective in mice lacking either CXCR4 or its ligand, CXCL12 [57]. In breast cancer cell lines, VEGF was demonstrated to have an autocrine action and induce expression of CXCR4 that promoted migration and invasion towards CXCL12 [58]. Several other chemokines and chemokine receptors are strongly associated with key stages of tumor growth and progression, angiogenesis and metastasis, invasion and adhesion. Some of them were found to regulate the activity of many different cellular factors. For example, it was recently found that chemokines regulate MEK1/2 and AKT-related intracellular pathways playing a critical role in cholangiocarcinoma cell invasion [59]. As reported by Yang *et al.*, inhibition of CXCR4-mediated cyclic AMP suppression effectively supress brain tumor growth *in vivo* [60].

2. ANTAGONISTS OF CXCR4 CHEMOKINE RECEPTOR

2.1. Peptide-Based Inhibitors of CXCR4 Activity

CXCR4 is the most actively studied chemokine receptor implicated in a wide variety of critical vital intra/extracellular signaling pathways in normal and pathologic physiology. Basically, the CXCR4-CXCL12 signaling network was found to strongly regulate tumor cells migration in mammals and organ-specific metastatic events in different *in vivo* models. In addition, this ligand-receptor system manipulate cancer growth and progression, angiogenesis, adhesion and tissue invasion. For instance, interaction between CXCL12 and CXCR4 plays an important role in the migration of progenitors during embryologic development of the cardiovascular, hemopoietic, central nervous systems, and so on. This specific action was also found to be involved in a number of intractable diseases, including HIV infection, rheumatoid arthritis and pulmonary fibrosis. It was suggested that this interaction may be a critical therapeutic target in numerous pathologic conditions, therefore CXCR4 antagonists have been proposed as potential drug candidates. These findings had implications in the field of cancer therapy, and several peptide-based and small molecule CXCR4 antagonists have been developed, which may provide clinical benefits in the therapy of several cancers.

The majority of CXCR4 antagonists which entered preclinical or clinical trials are the peptide-based agents (Fig. (**2**)). They represent promising lead-like compounds and drug candidates initially targeted for the treatment of HIV infection, but there are the strong evidence that they also can be effectivelly used in anticancer drugs. It was recently shown that immunodeficient mice innoculated with CXCR4-positive human NSCLC had significantly lower organ-specific metastasis while they were treated with antibodies against CXCL12 in non-small-cell lung cancer *in vivo* models [61-63]. Several CXCR4 inhibitors and/or CXCL12 blockers were found to delay tumor growth and reduce tumor mass in NOD/SCID mice infected by non-Hodgkin's lymphoma [64]. It was also shown that a neutralization of interactions CXCL12-CXCR4 *in vivo* significantly impaired metastasis of breast cancer cells to regional lymph nodes and

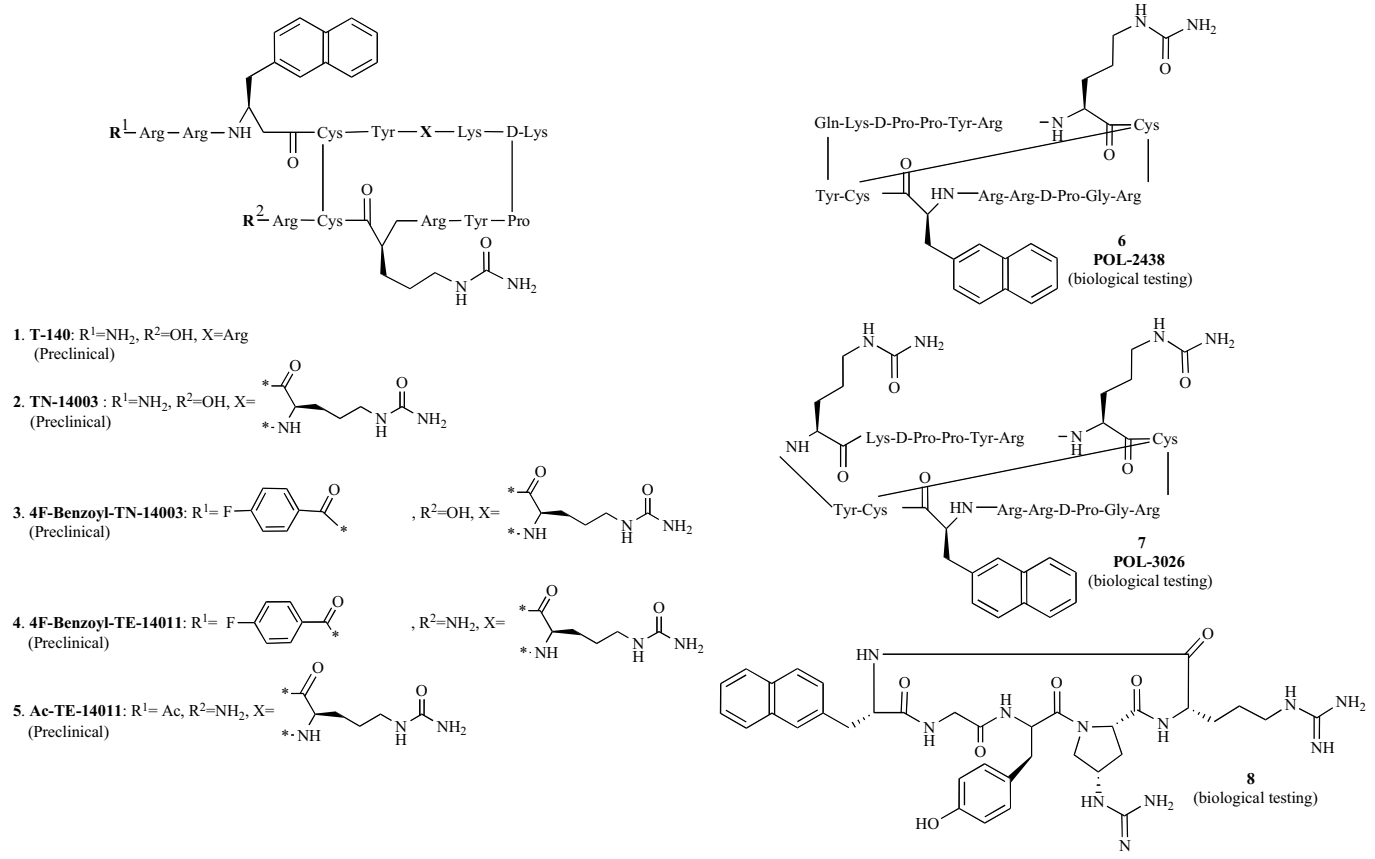


Fig. (2). Cyclic peptide-based inhibitors of CXCR4 with anticancer activity.

lung [6,13]. CXCR4 was also found to be highly expressed in several types of malignant brain tumors, including malignant melanoma [12,65].

Several 14-mer peptides, T-140 [[L-3-(2-naphthyl)alanine3]-T134] (**1**) and its structural analogues TN-14003 (**2**), 4F-Benzoyl-TN-14003 (**3**), 4F-Benzoyl-TE-14011 (**4**) and Ac-TE-14011 (**5**) (Fig. (**2**)), were previously developed as specific CXCR4 antagonists. These compounds were initially identified as potential HIV-entry inhibitors and anti-RA agents [66,67]. They were also identified as anti-metastatic and anti-apoptotic agents targeted for the treatment of chronic lymphocytic leukemia and breast cancer [66,68-70]. These compounds effectively inhibited CXCL12-induced migration of human breast cancer cells (MDA-MB-231), human leukemia T-cells (Sup-T1) and human umbilical vein endothelial cells at concentrations of 10-100 nM *in vitro* [71]. These results demonstrate that T-140 analogs can be efficiently utilized for effective anticancer therapy as anti-metastatic agents. Furthermore, several highly potent small molecule antagonists of CXCR4 were recently developed based on topological pharmacophore of T-140 analogs [72].

Based on T-140 3D-pharmacophore, several other peptide-based analogues were also developed and tested for their *in vitro* activity against several tumor cultures, such as lymphoblastic leukemia cells [73]. It was recently shown, that growth and viability of chronic lymphocytic leukemia (CLL) B-cells are favored by interactions between CLL and nontumoral accessory cells. In turn, CLL cells were also found to express a high level of CXCR4 chemokine receptor that manage leukemia cell chemotaxis and metastasis [74]. Marrow stromal cells or nurselike cells constitutively secrete CXCL12 thereby attracting and rescuing CLL B-cells from apoptosis in a contact-dependent fashion. It was recently reported, that CXCR4-specific antagonists T-140 (**1**) and TN-14003 (**2**) strongly inhibit CXCR4-CXCL12 signaling cascades in CLL cells [67]. Thus, T-140 and its analogues strongly inhibit chemotaxis and migration of CLL cells beneath stromal cells as well as actin polymerization. These findings have demonstrated that CXCR4 antagonists effectively inhibit CXCL12-induced cell migration, CXCR4-CXCL12 signaling pathway and stromal protection of CLL cells from spontaneous or fludarabine-induced apoptosis. Therefore, the CXCR4-CXCL12 signalling system represents a potentially attractive biological target in CLL drug discovery.

As recently reported by Mori *et al.* [75], several CXCR4 antagonists can effectively inhibit CXCL12-induced migration and tissue invasion of human pancreatic cancer cells. The authors have primarily investigated the role of CXCL12-CXCR4 network in the pancreatic cancer metastasis *via* cell migration and invasion, and the inhibitory effect of novel highly potent CXCR4 antagonist, TN-14003 (**3**), on pancreatic cancer cell metastasis. The overall expression of CXCR4 receptors was tentatively detected in six pancreatic cancer cell lines using Western blotting and immunocytochemistry assays. In these experiments CXCL12 stimulated both migration and invasion of cancer cells in a dose-dependent manner. The pernicious effect of CXCL12-induced cancer metastasis was observed at ligand concentrations of ~100 ng/ml. Observed effect was completely blocked by TN-14003 at a nanomolar concentration (~100 nM). The stimulatory effect of CXCL12 on cancer cell migration and the inhibitory action of TN-14003 were mediated *via* the alteration in phosphorylation of MAPK kinases in the same way as T-140 related compounds repress the growth and migration of CLL cells. Thus, actin polymerization initiated by CXCL12 (100 ng/ml) resulted in significant increase of cancer metastatic events, which could be effectively reduced by TN-14003 (100 nM). Interestingly, CXCL12 enhanced cancer cell adhesion to laminin was not reversed by TN-14003. Based on these results, it was concluded that CXCL12-CXCR4 signalling pathway is deeply involved in pancreatic cancer metastasis through migration and invasion. Therefore, antagonists of CXCR4 receptor based on TN-14003 topological organization might be effective anti-metastatic agents targeting pancreatic cancer.

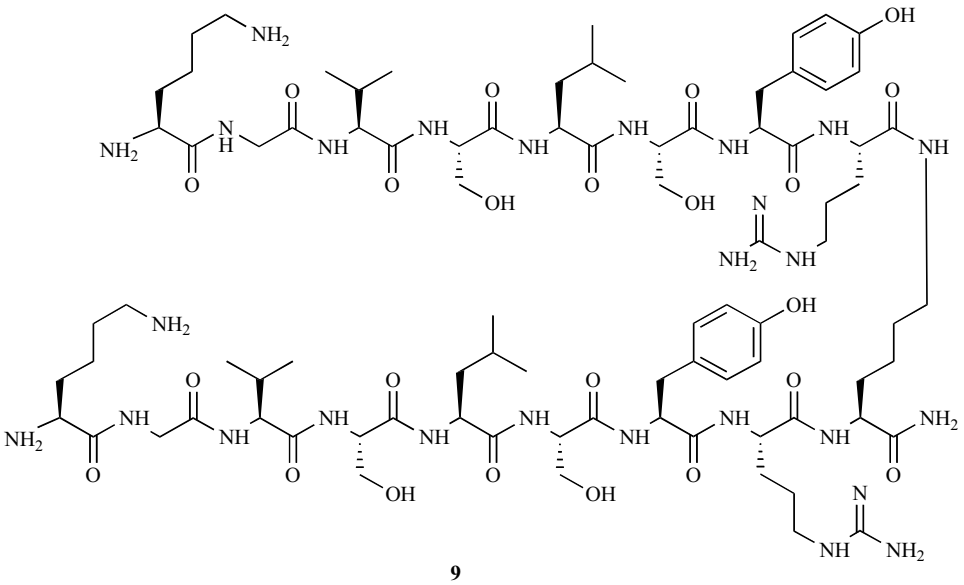
Pharmacophore identification of a specific CXCR4 inhibitor, T-140, contributed to development of novel effective anti-HIV agents with very high selectivity indexes [76]. A polyphemusin peptide analogue, T-22 ([Tyr(5,12), Lys7]-polyphemusin II), and its shortened potent analogue, T-134 (des-[Cys(8,13), Tyr(9,12)]-[D-Lys10, Pro11, L-citrulline16]-T22 without C-terminal amide bond) have demonstrated specific binding ability and high activity toward CXCR4 chemokine receptor. Although these compounds were initially identified as anti-HIV agents, they have also been tested for their inhibitory potency against several cancer types. For instance, inhibition of CXCR4 activity by peptide antagonist, T-22, blocks metastatic implantation of CXCR4-transduced B16 (CXCR4-luc-B16) melanoma cells in lung [77]. Thus, CXCR4 inhibition caused by T-22 renders B16 cells susceptible to killing by antigen-specific T-cells. T-22 synergizes with cyclophosphamide or anti-CTLA4 mAb in the treatment of established lung metastases, suggesting a novel strategy for augmenting the efficacy of immunotherapy.

Several novel highly potent inhibitors of CXCR4 receptor with promising pharmacokinetic profile were recently designed based on the naturally occurring β-hairpin peptide polyphemusin-II and optimized using classical medicinal chemistry approach [78]. The design method involved incorporating important residues from polyphemusin II into a macrocyclic template-bound β-hairpin mimetic. The potency and ADME properties of the tested peptidomimetics were optimized in iterative cycles using a parallel synthesis technique, resulting in the CXCR4 inhibitors POL-2438 (**6**) and POL-3026 (**7**) (Fig. (**2**)). Initially, the inhibitory abilities of these compounds were unambiguously confirmed *in vitro* in a series of HIV-1 invasion biological assays. Thus, POL-3026 showed excellent plasma stability, high selectivity for CXCR4 chemokine receptor, favorable pharmacokinetic properties in dog. Therefore this compound has a potential to become a promising drug candidate for the treatment of HIV infections, cancer (for angiogenesis suppression and inhibition of metastasis), inflammation, and also can be beneficially applied in stem cell transplant therapy. Furthermore, novel CXCR4 antagonists **8** and FC-131 [cyclo(D-Tyr-Arg-Arg-L-3-(2-naphthyl)alanine-Gly)] were recently found by the efficient utilization of cyclic pentapeptide libraries using a structural tuning of core tetrapeptide scaffolds. These com-

pounds have been already tested *in vitro* for their activity in several tumor models [79,80]. It was also found that compound **8** is the most potent inhibitor from the synthesized pentapeptide libraries and may be particularly useful for the treatment of some cancer types.

Complex application of ligand- and mechanism-based design led to novel competitive chemokine CXCR4 antagonist, CTCE-9908 (**9**), promoted by Chemokine Therapeutics (Fig. (**3**)) [81]. This compound represents a new generation of drugs that promise more targeted therapies to treat the underlying cancer while keeping healthy cells intact. CTCE-9908 is currently in phase I/II trials for the treatment of advanced breast and ovarian, metastatic lung and metastatic prostate cancers. Furthermore, phase I trials in localized osteosarcoma and bone cancer are also under way.

CTCE-9908 directly inhibits the basic function of chemokine CXCL12-CXCR4 signaling pathway *in vitro*. In preclinical studies, this compound has been shown to drastically reduce cancer metastases by 50-70% and to have promising anti-angiogenic features [82]. This compound has also been shown to dramatically inhibit spontaneous formation and progression of lung metastases by a 67% decrease in the number of visible lung nodules in mice infected by osteosarcoma [83]. Although CTCE-9908 was primarily designed to selectively block the CXCR4 activity, there are strong evidences that this compound can also inhibit CXCR7 receptor,



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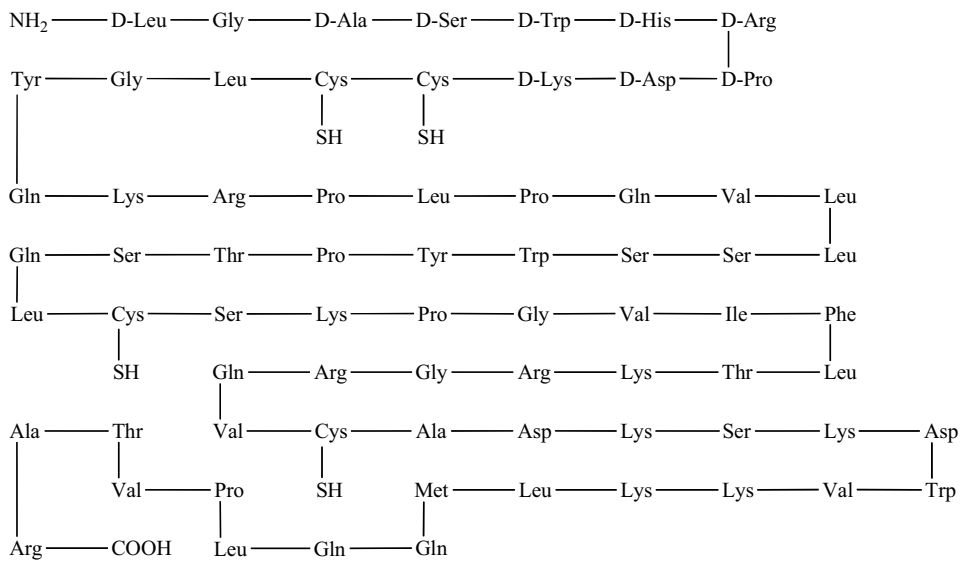
CTCE-9908
(phase I/II)



10
RCP-168
(Biological testing)

Fig. (3). Peptide inhibitors of CXCR4 with anticancer activity.

because chemokine ligand CXCL12 is believed to activate at least two sets of receptors, CXCR4 and CXCR7, which have been deeply implicated in cancer growth and metastasis. Of note, the results of phase I dose-escalation trial in 24 healthy patients revealed no significant toxicities associated with CTCE-9908 injections in the dosage of 0.5, 2, and 5 mg/kg of body weight. Since CTCE-9908 is not primary cytotoxic, it can be successfully utilized in combination and synergistically with chemotherapy and surgery. In 2005, the FDA assigned orphan drug designation to CTCE-9908 for the treatment of osteogenic sarcoma. Moreover, this compound is being designed to address cardiovascular and infectious diseases.

RCP-168 (**10**) is a novel peptide-based inhibitor of CXCR4 chemokine receptor (Fig. (**3**)). This compound overcomes stroma-mediated chemoresistance in chronic and acute leukemias [84-86]. As reported by Zeng *et al.* [86], polypeptide RCP-168 possesses the strongest antagonistic activity against CXCL12- or stromal cell-induced chemotaxis of leukemic cells. Furthermore, RCP-168 was found to significantly reduce the binding affinity of anti-CXCR4 monoclonal antibody 12G5 to surface of CXCR4 in a concentration-dependent manner and inhibit CXCL12 induced AKT activation as well as extracellular signal-regulated kinase phosphorylation. Finally, RCP-168 greatly enhanced chemotherapy-induced apoptosis in stroma-cocultured Jurkat, primary chronic lymphocytic leukemia, and in a subset of acute myelogenous leukemia cells harboring Flt3 mutation. The same results were also obtained using the small molecule CXCR4 inhibitor AMD-3465. The combined data suggest that the CXCL12-CXCR4 interaction contributes to the resistance of leukemia cells to chemotherapy-induced apoptosis. Therefore, inhibition of these interactions by RCP-168 represents a promising and feasible strategy for targeting leukemic cells within the bone marrow microenvironment.

Protein transduction domains (PTDs), such as the TAT PTD, have been shown to deliver a wide variety of cargo in cell cultures and to treat cancer and cerebral ischemia in several preclinical models [87]. The TAT PTD penetrate the cell membrane by a common lipid raft-dependent macropinocytosis mechanism. Consequently, PTDs resemble small-molecule therapeutics in their lack of pharmacologic tissue specificity *in vivo*. Two peptides, a p53-activating peptide (DV3-TATp53C') and a cyclin-dependent kinase 2 antagonist peptide (DV3-TAT-RxL), were recently targeted against CXCR4 in multiple malignancies. Treatment of tumor cells expressing these peptides resulted in an enhancement of tumor cell-killing compared to the treatment with nontargeted parental peptides. These observations clearly show that a multidomain approach can be effectively used to further refine and enhance the tumor selectivity of biologically active, transducible macromolecules for treatment of cancer.

2.2. Small-Molecule Inhibitors of CXCR4 Activity

A wealth of data on small-molecule CXCR4 receptor antagonists has been generated over the last few years, as a great variety of molecules have been tested, and the understanding of structure activity relationships has improved [88,89]. In addition to a wide variety of peptide-based CXCR4 antagonists, several small molecule agents are currently in active development as promising next-generation anticancer therapeutics. A well-known small molecule inhibitor of CXCR4 activity is AMD-3100 (**11**) (Fig. (**4**)) and its bismacrocyclic analogs [90]. The bicyclam AMD-3100 (originally called JM-3100) in which the two cyclam rings are connected *via* aromatic bridge was designed from JM-2763 and was initially identified as a promising anti-HIV agent. Now, this compound is under active development for the treatment of several cancer types.

Systemic utilization of the selective CXCR4 inhibitor AMD-3100 effectively blocked the heightened metastatic potential of CXCR4-expressing pancreatic cancer cells [91] and strongly inhibited growth and progression of intracranial medulloblastoma and glioblastoma in xenograft models by increasing the cellular apoptosis and decreasing the excessive proliferation of tumor cells [92]. Recently it was found that CXCR4 chemokine receptor is overexpressed in various glioma cell lines including glioblastoma. In cells from adult glioblastoma and pediatric medulloblastoma, CXCR4-CXCL12 signalling pathway may induce intracellular chemotaxis and enhance tumor cell proliferation, progression and survival [92]. AMD-3100 was shown to obstinately resist these effects *in vitro*. When AMD-3100 was used for the treatment of mice bearing intracranial glioblastoma or medulloblastoma, tumor burden was sigificantly smaller in AMD-3100-treated animals. The similar effect was also obtained using the combination of AMD-3100 with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Treatment of glioblastoma multiforme cells with BCNU followed by AMD-3100 resulted in synergistic antitumor efficacy in all cells tested as well as treatment using subtherapeutic doses of BCNU in combination with AMD-3100 resulted in significant tumor regression *in vivo*, and this reflects both increased apoptosis and decreased proliferation following combination drug therapy [93].

CXCL12 factor is known to selectively increase the expression level of membrane type-2 matrix metalloproteinase (MT2-MMP), as well as against any other types, including MT-MMPs, MMP-2 or MMP-9. As communicated by Zhang *et al.* [94], the CXCL12 enhanced MT2-MMP expression was effectively blocked by AMD-3100. Obtained results highlight the promising potential of AMD-3100 as an effective agent blocking the tumor tissue invasion and metastasis. Recent studies have clearly demonstrated the high ability of AMD-3100 to reduce the activation of extracellular signal-regulated kinases 1 and 2 as well as Akt kinase. These specific intracellular mediators implicated in the CXCR4-related signalling downstream pathways promote tumor cells survival, proliferation and migration.

It was recently found that CXCR4 chemokine receptor is one of the key intracellular regulators promoting the growth and progression of primary melanoma [95]. Thus, CXCL12-CXCR4 signaling system was tested towards induction of phosphorylation, proliferation, apoptosis, and migration capabilities of extracellular signal-regulated kinase-1 and -2 (Erk-1 and Erk-2). It was found that CXCL12 activated induction of both Erk-1 and Erk-2 kinases was specifically inhibited by AMD-3100 *in vitro* [96]. Furthermore, AMD-3100 effectively reduced tumor growth and ascitic fluid for-

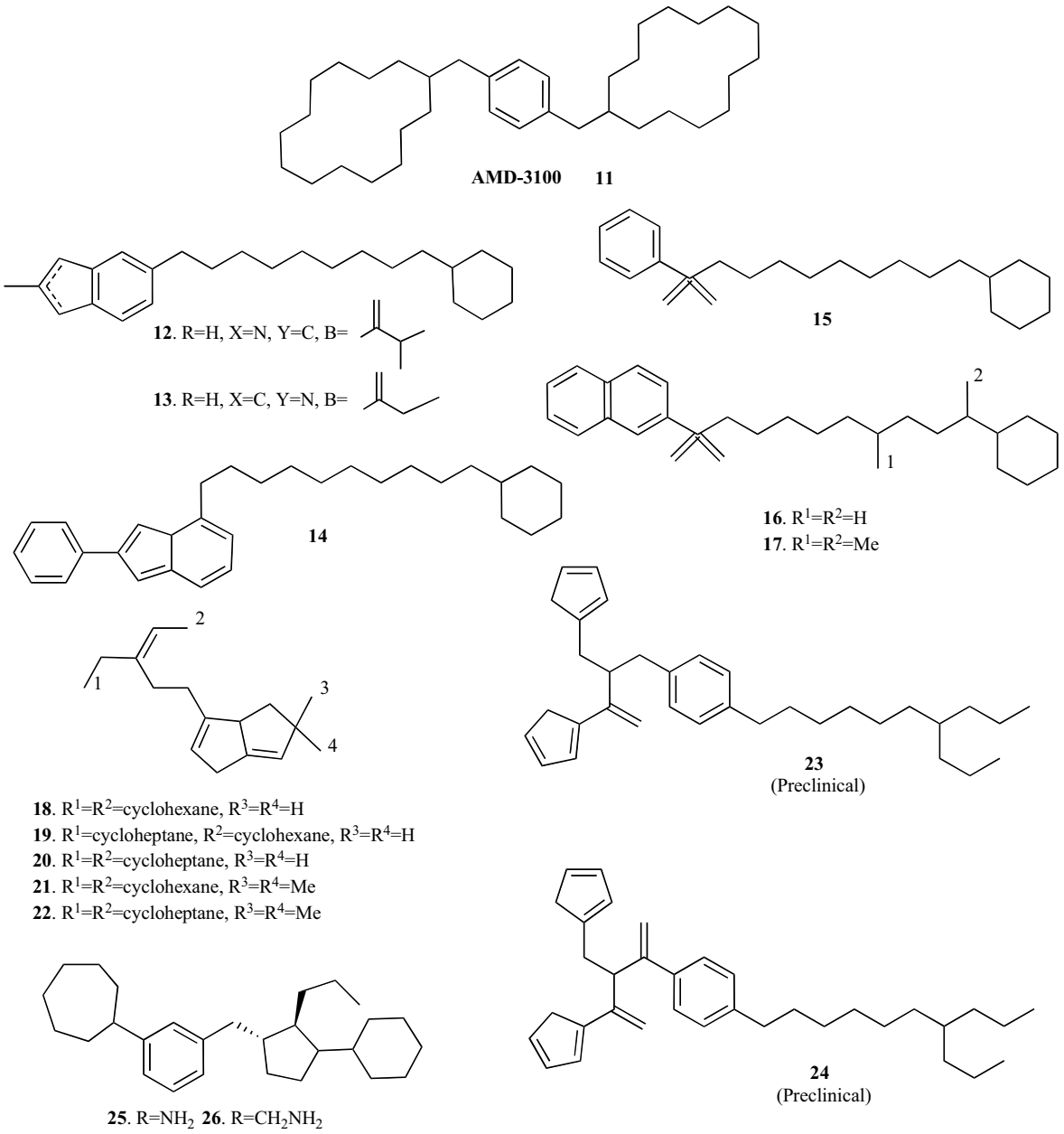


Fig. (4). Small-molecule inhibitors of CXCR4 with anticancer activity.

mation in nude mice inoculated with Human gastric carcinoma cell lines (NUGC4 cells) [15]. It was also recently shown that administration of CXCL12 and TNF-alpha increased synergistically ICC cell migration, which could be effectively suppressed by AMD-3100 [97]. Finally, bicyclam AMD-3100 is actively pursued as a stem cell mobilizer in patients with multiple myeloma and non-Hodgkin's lymphoma, therefore it can be effectivelly used in transplantation [98,99].

Several novel antagonists of CXCR4 chemokine receptor were recently disclosed in patent applications. Thus, a series of novel potent inhibitors of CXCR4 activity containing the common cyclohexylamino ring as well as indole (**12** and **13**) and imidazole **14** fragments were recently developed by Takeda Chemical Industries as promising anticancer agents (Fig. (**4**)) [100,101]. Bioisosteric analogues **15**-**17** in which indole and imidazole fragments were replaced by phenyl and naphthalene, while carboxamide moiety was replaced by sulfonamide fragment were also synthesized and tested for their activity against several tumor cell lines [88]. 5,6-Dihydroimidazo[2,1-*b*][1,3]thiazoles **18**-**22** developed by Novartis were described as agents targeted for the treatment of transplant rejection, inflammatory and autoimmune diseases, cancer and other proliferative disorders including HIV infection [102].

Diimidazoles **23** and **24** (Fig. (**4**)) which are topological analogs of compounds **12**-**17** were recently discovered by Ono Corp. as promising agents for the treatment of inflammatory and immune disorders such as rheumatoid arthritis, transplant rejection, allergic diseases, HIV infection, neuro-

logical, cardiovascular and metabolic diseases [103]. These compounds are currently entered in early phase of biological evaluation for the treatment of several cancer types. Furthermore, pyrimidine-based compounds **25** and **26** (Fig. (**4**)) were also recently developed by Ono as potential CXCR4 antagonists [104,105]. Thus, the exemplified compound **25** displayed an IC_{50} value of 1.6 nM towards the inhibition of CXCL12-CXCR4 signaling pathway in human CEM cells.

Structures of several CXCR4 antagonists with anticancer activity are still not disclosed. These include novel small molecule inhibitors WZ-811, WZ-40 and WZ-811S which are currently entered in the early stages of preclinical studies promoted by Emory University for the treatment of different cancer types [106]. Compound OPL-CXCL12-LPM developed by Osprey Pharmaceuticals represents a highly potent inhibitor of CXCL12-CXCR4 signaling pathway which can be effectively used for the treatment of several tumors and arthritic diseases. As communicated by the originator, this compound was extensively tested in different tissue cultures and in mouse xenograft models. Surprisingly, OPL-CXCL12-LPM has not exhibited detectable systemic toxicity in preliminary animal toxicology studies and the immediate goal of Osprey Pharmaceuticals is to capitalize upon proof of principle in animals and validate this compound in advanced phases of clinical trial especially for anticancer therapy. A novel small molecule antagonist of CXCR4 chemokine receptor, BKT-140, was recently developed by Biokine Therapeutics as an effective therapeutic agent. Particularly, this compound is currently entered in preclinical trials for the treatment of some cancer types [82].

2.3. Strategies to the Rational *In Silico* Design of Novel CXCR4 Antagonists

Undoubtedly, high-throughput screening (HTS) of large diversity-based libraries is still a common strategy within many pharmaceutical companies for the discovery of novel chemokine receptor ligands. For example, design, synthesis, and discovery of novel CCR1, CCR4, CCR5 and CCR8 antagonists were recently described in several recent publications [107-110]. HTS technique was also successfully applied for the identification of novel small molecule antagonists of other chemokine receptors, including CXCR1 and CXCR2 [111]. Several recent studies discussed the utilization of high-throughput technology for identification of novel highly potent antagonists of CXCR4 chemokine receptor [112,113].

Although pharmacophore hypothesis for small molecule antagonists of whole receptors belonging to CXCR family remains elusive mainly as a result of conformational flexibility inherent to the majority of identified ligands, several specific pharmacophore models were recently described and validated. For example, pharmacophore identification of a specific CXCR4 inhibitor, T-140, led to development of novel effective anti-HIV agents with very high selectivity indexes [76]. It was clearly identified that a common pharmacophore frame contains four indispensable amino acid residues (Arg2, Nal3, Tyr5, and Arg14). Based on this result, a series of L-citrulline (Cit)-substituted analogues of T-140 with decreased net positive charges have been synthesized and evaluated in terms of anti-HIV activity and cytotoxicity. As a result, novel effective inhibitors, TC-14003 and TC-14005, possessing higher selectivity indexes as against of T-140 have been developed. Pharmacophore hypothesis was also generated for T-140 analogue T-22, which specifically blocks T cell-line-tropic HIV-1 infection [114].

A minimalistic 3D-pharmacophore model was recently developed for several other cyclopentapeptide CXCR4 antagonists [115]. For instance, an exhaustive systematic exploration of the conformational space for a series of analogs of FC-131, a cyclopentapeptide CXCR4 antagonist, has been recently performed. By comparing the resulting low-energy conformations using different sets of atoms, specific conformational features of high/medium affinity compounds were identified. These features included the spatial arrangement of three pharmacophoric side chains as well as the orientation of a specific backbone amide bond. Together these features represent a minimalistic 3D pharmacophore model for binding of the cyclopentapeptide antagonists to CXCR4 receptor. The model enables rationalization of the experimental affinity data for this class of compounds as well as for the peptidomimetic KRH-1636.

In addition, several docking and crystallographic studies were recently performed to *in silico* design of novel CXCR4 antagonists. Initially, a particularly helpful strategy for determining the critical ligand binding motifs towards targeted receptors using low temperature NMR structures of peptides was developed [116]; and it has been immediately applied successfully to determine a binding motif for the chemokine receptor CXCR4. In further study the possible binding modes for cyclopentapeptide CXCR4 antagonists have been thoroughly investigated by the molecular docking of several different cyclopentapeptides into the developed 3D-binding site of CXCR4 [117].

It was also recently demonstrated in [118] that soluble heparin and heparan sulfate have a negative affect on CXCL12-mediated chemotaxis *in vitro*. Based on NMR spectroscopic and X-ray crystallographic data the incontrovertible structural evidence for binding of an unsaturated heparin disaccharide to CXCL12 have clearly shown that a cluster of basic residues in the dimer interface is strongly required for chemotaxis and is a target for inhibition by heparin. Thus, the first cluster was directly associated to β-strands in the dimer interface, while the second one included the amino-terminal loop and the α-helix. As elucidated by X-ray crystallography, two unsaturated disaccharides were presented within the whole binding pocket. One is in the dimer interface with direct contacts between residues His(25), Lys(27), and Arg(41) of CXCL12 and the heparin disaccharide. The second disaccharide contacts Ala(20), Arg(21), Asn(30), and Lys(64). It should be especially noted that this study has provided the first X-ray structure of a CXC class chemokine in complex with glycosaminoglycans. Based on the observed results a unique mechanism of action in which GAGs bind around CXCL12 dimers as they sequester and present CXCL12 to CXCR4 was originally suggested.

CONCLUSION

In the last decade chemokines and chemokine receptors received a great attention as promising targets for the treat-

ment of many solid and hematological cancers in addition to atherosclerosis, psoriasis, Alzheimer's disease, allergy, diabetes and HIV infection, etc. In the normal tissue they play key role in several vital processes including chemotaxis of immune cells within inflammatory hearth. On the othe hand, in cancer, they are implicated in a majority of pathological events controlled tumor growth and progression. Among a variety of chemokine receptors, CXCR4 and its specific endogenous ligand CXCL12 were found to be highly expressed in many types of solid tumors. This ligand-receptor pair remains one of the critical regulatory system promoting uncontrolled tumor cell growth, organ-specific metastasis, angiogenesis and tissue-invasion. Design of peptide-based and small-molecule antagonists of tumor associated chemokine receptors, especially against CXCR4, and their clinical utilization attract growing number of industrial and academic scientists. These agents are represented by derivatives of various peptide-based antagonists, including cyclic tetrapeptides and their structurally-related or topological analogues, as well as by small molecule inhibitors containing different structural patterns. Biochemically, they induce growth arrest and apoptosis and/or terminal differentiation as well as the blockage of metastasis and angiogenesis in a variety of solid and hematological neoplasms in patients with advanced disease. Unfortunately, relatively modest progress in understanding pharmacology and clinical role of chemokine receptors antagonists has been made since their discovery. From this point of view, specific natural and synthetic inhibitors of chemokine activity are promising tools for dissecting role of chemokines and their receptors in both normal and aberrant biological processes. Further optimization of these molecules into clinical candidates may yield drugs with enhanced efficacy against cancers, neurodegenerative and inflammatory diseases. As outlined in this paper, successful discovery of novel CXCR4 antagonist leads relies on a combination of techniques from a wide range of disciplines, including high-throughput screening, 2D/3D-pharmacophore-based design and traditional medicinal chemistry approaches.

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