Journal of Medicinal Chemistry

Perspective

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# Drug Discovery Research Targeting the CXC Chemokine Receptor 4 (CXCR4)

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## INTRODUCTION: CHEMOKINES AND CHEMOKINE RECEPTORS

Chemokines are small 70 amino acid long soluble proteins that chemoattract a variety of mononuclear cell types to sites of inflammation or secondary lymphoid organs by interacting with chemokine receptors.[1] They are divided into four subfamilies of CC, CXC, CX3C, and C chemokines based on the positions of two conserved cysteine residues in their amino (N)-termini.[2,3] The CXC chemokine receptor 4 (CXCR4) is an attractive target for therapeutic intervention because of its involvement in the pathogeneses of a wide range of infectious, inflammatory, and other diseases. CXCR4 is a transmembrane (TM) protein that belongs to the superfamily of G-protein-coupled receptors (GPCRs).[2,4,5] It possesses seven TM helices and transmits signals from its natural chemokine ligand, stromal cell-derived factor (SDF)-1$\alpha$/CXCL12, to intracellular biological pathways via heterotrimeric G-proteins.

The activation of CXCR4 by SDF-1$\alpha$ can trigger different downstream signaling pathways that result in a variety of physiological responses, such as chemotaxis, cell survival and proliferation, intracellular calcium flux, and gene transcription (Figure 1).[6−15] These normal physiological responses also share several downstream effectors with multiple pathological processes, including tumor cell metastasis, and autoimmune and inflammatory diseases. For instance, CXCR4-mediated chemotaxis and cell survival involve PI3 kinase (PI3K) which also plays a major role in cancer cell survival, proliferation, and metastasis.[10] Whereas cancer cell proliferation requires the activation of Akt (serine/threonine protein kinase) via the PI3K pathway, physiologically occurring cell survival can activate Bcl-2-associated death promoter (BAD) via both MEK (MAP kinase kinase) and PI3K pathways, which leads to the inhibition of the proapoptotic protein Bcl-2.[6] Similarly, although the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway allows a G-protein independent signaling pathway via CXCR4, the receptor phosphorylation by JAK2 and JAK3 leads to the activation and nuclear translocation of a variety of STAT proteins, which lead to cancer cell survival and proliferation.[16]

The structures of multiple chemokines have been determined by NMR or X-ray crystallography, including those of SDF-1$\alpha$,[17,18] viral macrophage inflammatory protein (vMIP)-II,[19,20] macrophage inflammatory protein (MIP)-1$\beta$,[21] and "regulated on activation, normal T-cell expressed and secreted" (RANTES).[22] These structures demonstrate the highly conserved three-dimensional structures of all chemokines, including a flexible N-terminus, a three-stranded antiparallel $\beta$-sheet, and a C-terminal $\alpha$-helix.[23] In the typical structure, the first two cysteine residues are situated close together near the amino N-terminus, with the third cysteine residue residing in the center of the molecule and the fourth cysteine residue located close to the carboxyl C-terminal end.[24] An "N-loop" of approximately 10 amino acids follows the first two cysteine residues. Following the N-loop, there is a single-turn "$3_{10}$-helix", a $\beta$-sheet with three $\beta$-strands, and a C-terminal $\alpha$-helix, connected by turns called "30s," "40s," and "50s" loops. The third and fourth cysteine residues are located in the 30s and 50s loops, respectively.

Because of its involvement in a wide range of physiological and pathologic processes, there has been intensive biological, chemical, and pharmaceutical research to understand the molecular mechanisms of chemokine−receptor interactions and the modulation of chemokine−receptor functions. The ultimate goal is to translate these discoveries into novel treatment strategies for clinical applications. This review describes and discusses some of the recent advances in medicinal chemistry and drug discovery that involve CXCR4, which is implicated in human immunodeficiency virus (HIV) 1 infection, normal hematopoietic and neural stem cell migration, cancer−stromal cell interaction, solid tumors, and inflammation and autoimmune diseases such as rheumatoid arthritis and allergic asthma.

## CXCR4 ANTAGONISTS AGAINST HIV-1 ENTRY

HIV-1 enters target cells through a fusion process in which the HIV-1 gp120 envelope glycoprotein binds to CD4, the main receptor for HIV-1 on the target cell surface.[25−28] However, CD4 alone is not sufficient for HIV-1 fusion, and the chemokine receptors CXCR4 and CCR5 act as coreceptors for syncytium-inducing and nonsyncytium-inducing HIV-1 strains, respectively (Figure 2). The initial binding of HIV-1 gp120 to CD4 results in conformational changes in gp120 and CD4.[29−31] The gp120−CD4 complex then interacts with a chemokine coreceptor such as CXCR4 or CCR5 to form a heterotrimeric complex of gp120−CD4−coreceptor.[32−34] During the asymptomatic stage of disease, macrophage-tropic (M-tropic) strains of HIV-1 (also known as R5-tropic) primarily use CCR5 as the entry coreceptor.[26−28] However, in 40−50% of HIV-1-infected individuals, T-cell-tropic (T-tropic) strains (also known as X4-tropic), which predominantly use CXCR4, eventually replace M-tropic strains, resulting in rapid disease progression.[35−37] Dual-tropic strains are those

Received: May 5, 2011
Published: November 15, 2011





dx.doi.org/10.1021/jm200568c | J. Med. Chem. 2012, 55, 977−994

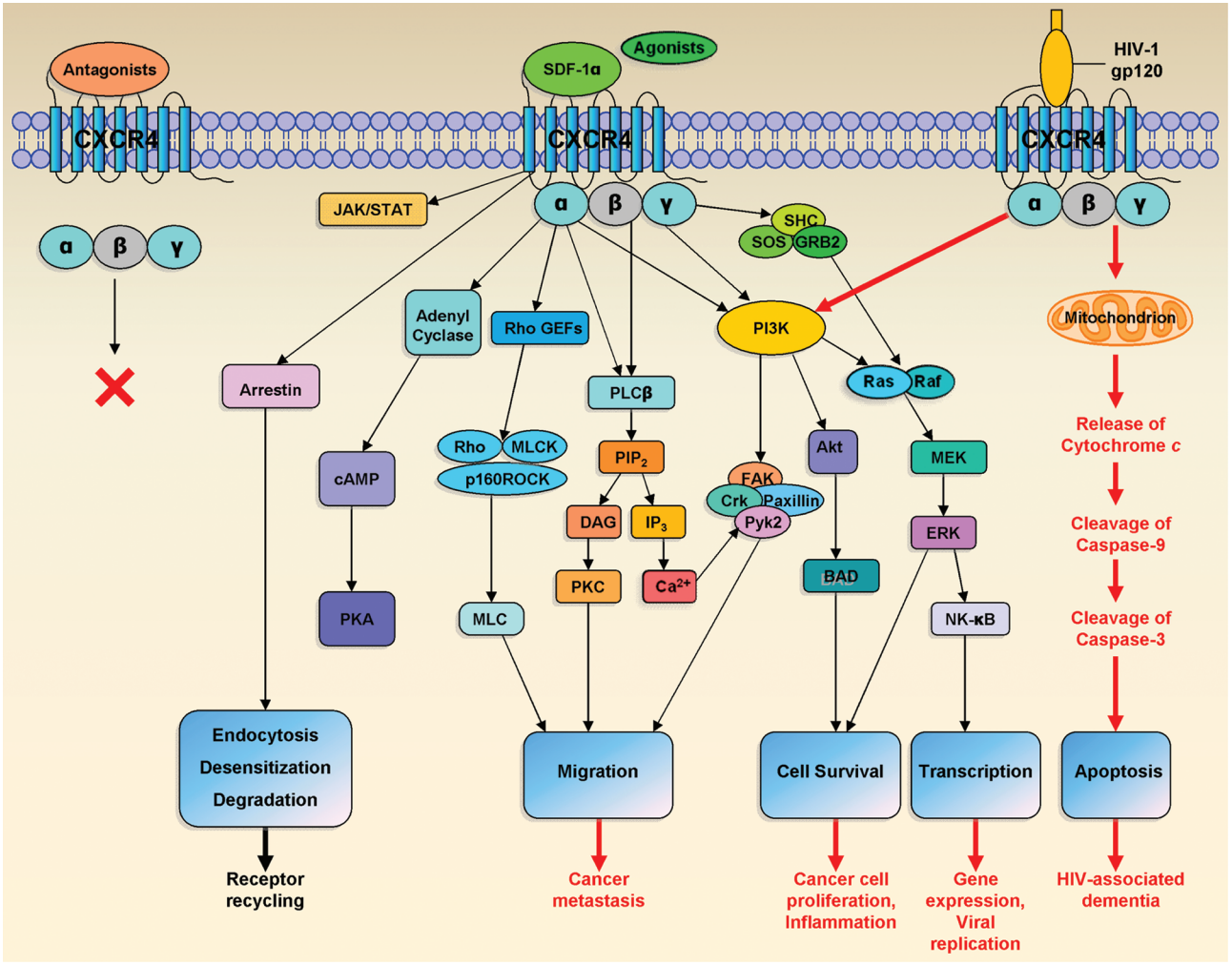



**Figure 1.** CXCR4 intracellular signaling pathways. CXCR4 activation by SDF-1$\alpha$ can trigger a variety of physiological responses, such as chemotaxis, cell survival and proliferation, intracellular calcium flux, and gene transcription, whereas CXCR4 antagonists fail to do so. These normal physiological responses also share several downstream effectors with multiple pathological processes, including tumor cell metastasis, HIV-associated dementia (induced by HIV-1 gp120), and autoimmune and inflammatory diseases.[6−15]

HIV-1 isolates that are capable of using both CXCR4 and CCR5. CXCR4-using variants are believed to evolve from CCR5-using variants by way of transitional intermediate variants. Natural chemokines that bind CXCR4 or CCR5 can inhibit HIV-1 infection,[38,39] most likely by blocking common binding sites on the chemokine receptors that are also required for gp120 interaction and/or by inducing receptor internalization.[40,41]

Viral resistance to CCR5 inhibitors is well documented. Most often, it involves selection of pre-existing minor HIV-1 variants that can use CXCR4 for entry.[42−44] Also, selection of mutations that change the coreceptor use from CCR5 to CXCR4 has been demonstrated in vitro.[45] More recently, Mosier's group has demonstrated that coreceptor switching may be the only route to resistance for macromolecular CCR5 inhibitors and that this pathway requires more mutations and encounters more fitness obstacles than development of resistance to small molecule CCR5 inhibitors.[46] Although the use of CXCR4 inhibitors may not have a wide window of opportunity for the treatment of HIV-infected individuals in comparison to CCR5 inhibitors because the X4-tropic virus appears at the late stage of HIV infection, the development of CXCR4 inhibitors is still urgently needed to overcome the viral drug resistance against CCR5 inhibitors and to reduce the occurrence of transitional intermediate variants that can successfully switch to CXCR4-using variants.

The discovery of distinct chemokine coreceptors for different HIV-1 strains has important clinical implications for developing new therapeutic strategies, and it has presented many new directions for acquired immune deficiency syndrome (AIDS) research. One of these new important areas of research is the biochemical and biophysical characterization of the interactions of chemokine receptors with HIV-1, natural ligands, and de novo designed inhibitors.[47−55] A combined approach that integrates structural biology, molecular biology, and synthetic chemistry has been used to define precise regions involved in the molecular interactions of chemokine receptors with their natural ligands and with HIV-1 gp120. More recently, crystal structures of CXCR4 bound to an antagonist small molecule (IT1t) and to a cyclic peptide (CVX15) have been reported at a resolution of 2.5−3.2 Å.[56] Compared with previous GPCR structures, these recent structures indicate a more open and larger binding pocket of CXCR4 that is located closer to the extracellular surface. Taken together, these studies have provided structural and chemical insights into receptor−ligand


dx.doi.org/10.1021/jm200568c | J. Med. Chem. 2012, 55, 977−994

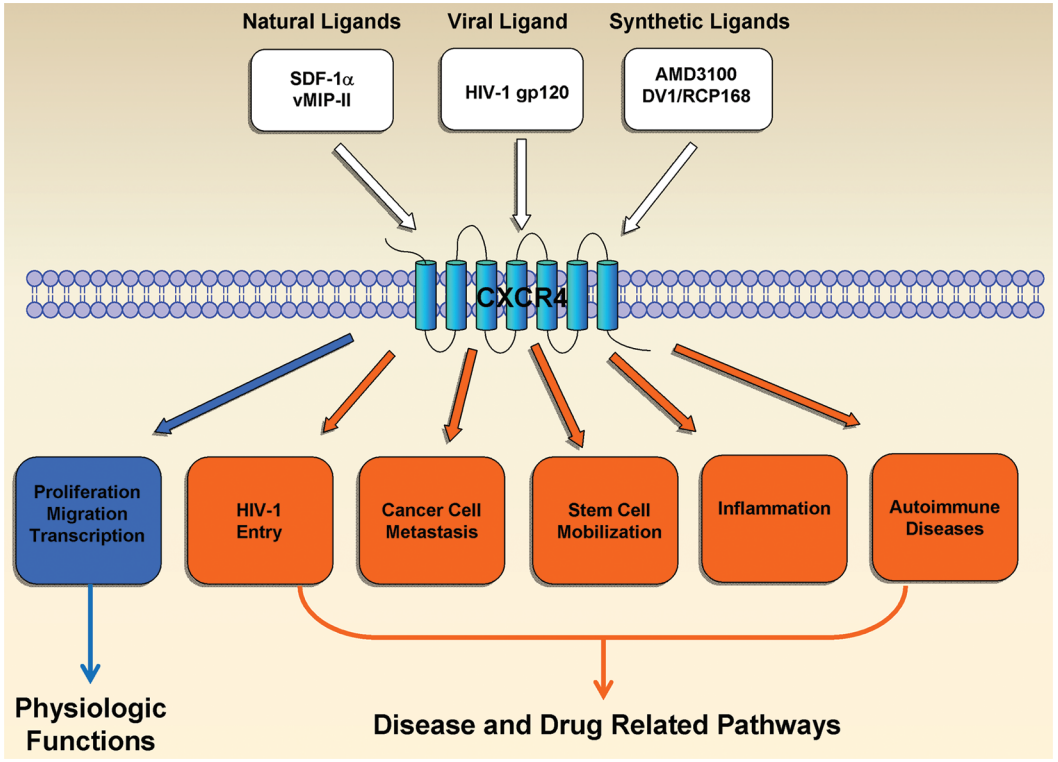



**Figure 2.** Representative CXCR4 interactions with several different classes of CXCR4 ligands, including SDF-1$\alpha$ (natural agonist), vMIP-II (natural antagonist), HIV-1 gp120 (viral ligand), AMD3100 (small synthetic organic molecule), and DV1/**9** (modified peptides). Orange arrows represent disease and drug related pathways, and blue arrows indicate physiologic functions of CXCR4.

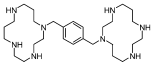
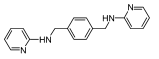
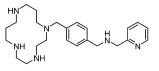
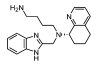
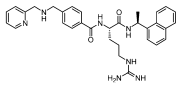
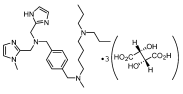
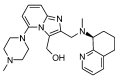
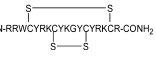
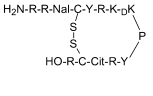
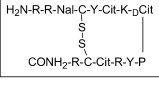
interactions and a foundation for the design of therapeutic agents that can prevent HIV-1 entry.

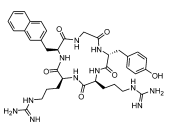
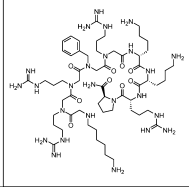
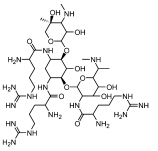
When compared to other anti-HIV-1 agents targeting components of the rapidly mutating virus population (such as reverse transcriptase and protease inhibitors), strategies based on HIV-1 coreceptors, CXCR4 and CCR5, have appeal because they target invariant host determinants.[40,53,57−69] The fact that individuals with CCR5 mutations are highly resistant to HIV-1 infection also demonstrates the feasibility of this approach for inhibiting actual viral infection,[3,70] although it has been recently found that CCR5 deficiency is a potential risk factor for both early and late clinical manifestations after West Nile virus infection.[71] Early chemical screening and medicinal chemistry efforts identified several CXCR4 antagonistic peptides and organic compounds that are structurally unrelated to natural chemokines.[53,63−66,68] Among these, *N*-$\alpha$-acetylnona-D-arginine amide (**1** (ALX40-4C)) and plerixafor (AMD3100) were the first CXCR4 antagonists to enter clinical trials (Table 1).[53,72,73] Compound **1** is a highly basic peptide designed originally as an arginine mimic of the HIV Tat protein. It inhibits HIV-1 infection exclusively by blocking direct virus−CXCR4 interactions.[72,73] With a net charge of +9, **1** can bind to the negatively charged domains of CXCR4, where it competes with the V3 loop of HIV-1 gp120 to prevent virus entry.[53,72] AMD3100 is a bicyclam containing two cyclam rings connected by a *p*-phenylenebis(methylene) linker. It is a highly potent and selective anti-HIV-1 inhibitor that acts by binding to CXCR4 (Figure 2).[65] Mutation of $Asp^{171}$ or $Asp^{262}$ (located in TM domains IV and VI of CXCR4, respectively) to alanine also suggested that the negatively charged aspartate residues at positions 171 and 262 of CXCR4 may represent crucial sites for electrostatic interaction of the positively charged bicyclam rings.[74] Similarly, molecular modeling suggested that one cyclam ring of AMD3100 interacts with $Asp^{171}$ in the TM domain IV of CXCR4, whereas the other ring is sandwiched between the carboxylate groups of $Asp^{262}$ and $Glu^{288}$ from TM helices VI and VII, respectively.[75] More recently, using homology modeling and molecular docking, Kawatkar et al. demonstrated that several small CXCR4 antagonists, including AMD3100, have a distinct binding site that is different from the region occupied by the N-terminus of SDF-1$\alpha$. This suggested the coexistence of an antagonistic ligand-binding site with a SDF-1$\alpha$ signaling site in CXCR4.[76]

On the basis of this proposed molecular mechanism for AMD3100, Zhan et al. designed and synthesized a series of candidate compounds by replacing the cyclam moieties of AMD3100 with N-containing basic centers that are hypothesized to bind to the acidic residues in CXCR4.[77] The synthesis of a series of small molecular CXCR4 antagonists with two aromatic amine moieties connected by a para-xylylene group ultimately led to the discovery of *N*1,*N*4-di-2-pyridinyl-1,4-benzenedimethanamine (WZ811), a potent compound that can block CXCR4 at subnanomolar concentrations (Table 1). **2** (WZ811) was also more effective than AMD3100 in inhibiting CXCR4/SDF-1$\alpha$-mediated modulation of cyclic adenosine monophosphate (cAMP) levels and SDF-1$\alpha$-induced Matrigel invasion. However, clinical applications of AMD3100 and its derivatives are limited because of their lack of oral bioavailability, which is related to their high positive charge at physiological pH. To improve these characteristics, analogous compounds with fewer basic amine groups have been developed, including monocyclam derivatives (AMD3465)[78,79] and non-cyclam derivatives (**3** (AMD070))[80] (Table 1). AMD3465, a monocyclam with a


dx.doi.org/10.1021/jm200568c | *J. Med. Chem.* 2012, 55, 977−994

## Table 1. List of CXCR4 Inhibitors[a]

| Name of CXCR4 Inhibitor and Analogue Designation | Molecular Type | Amino Acid Sequences, Modification Diagrams, or Chemical Structures | Biological Activities | Stage of Development | References |
|---|---|---|---|---|---|
| 1 (ALX40-4C) | D-Peptide | Ac-NH-(D-Arg)$_9$-COOH | Block HIV-1 entry | Phage I | 53,72,73 |
| AMD3100 | Small Orgnic Molecule |  | (1) Block HIV-1 entry; (2) promote stem cell migration; (3) inhibit stromal–leukemia cell interactions; and (4) anti-RA and allergic asthma activities | Approved in 2008 for patients with lymphoma and multiple myeloma as a source of hematopoietic stem cells for transplantation | 57, 200,111, 117,113,125 |
| 2 (WZ811) | Small Orgnic Molecule |  | CXCR4 Antagonist | Pre-clinical | 77 |
| AMD3465 | Small Orgnic Molecule |  | Block HIV-1 entry | Pre-clinical | 78 |
| 3 (AMD070) | Small Orgnic Molecule |  | Block HIV-1 entry | Phase I | 80 |
| 4 (KRH-1636) | Small Orgnic Molecule |  | Block HIV-1 entry | Phase I | 84 |
| 5 (KRH-3955) | Small Orgnic Molecule |  | Block HIV-1 entry | Pre-clinical | 85,86 |
| 6 (GSK812397) | Small Orgnic Molecule |  | Block HIV-1 entry | Pre-clinical | 87 |
| T22 | Peptide |  | (1) Block HIV-1 entry; and (2) anti-RA activities | Pre-clinical | 58 |
| T140 | Peptide |  | (1) Block HIV-1 entry; (2) promote stem cell migration; and (3) anti-RA activities | Pre-clinical | 88,89,201 |
| TC14012 | Peptide |  | Block HIV-1 entry | Pre-clinical | 88 |

| Name of CXCR4 Inhibitor and Analogue Designation | Molecular Type | Amino Acid Sequences, Modification Diagrams, or Chemical Structures | Biological Activities | Stage of Development | References |
|---|---|---|---|---|---|
| 7 (FC131) | Peptide |  | (1) Block HIV-1 entry; and (2) anti-RA activities | Pre-clinical | 89 |
| 8 (CGP64222) | Small Orgnic Molecule |  | Block HIV-1 entry | Pre-clinical | 93 |
| R3G | Small Orgnic Molecule |  | Block HIV-1 entry | Pre-clinical | 203 |
| SDF-1α | Peptide | 1-8 NT \| 9-68 SDF-1α Core<br>KPVSLSYRCPCRFFESHVARANVK<br>HLKILNTPNCALQIVARLKNNNRQ<br>VCIDPKLKWIQEYLEKALNK | Block HIV-1 entry | Pre-clinical | 59,60,67,69 |
| CTCE-9908 | Peptide | KGVSLSYRKRYSLSVGK | Inhibit cancer growth and metastases | Phase II | 128-130 |
| vMIP-II | Peptide | 1-10 NT \| 11-71 vMIP-II Core<br>LGASWHRPDKCCLGYQKRPLPQV<br>LLSSWYPTSQLCSKPGVIFLTKRGR<br>QVCADKSKDWVKKLMQQLPVTA<br>R | Block HIV-1 entry | Pre-clinical | 19,20 |
| V1 | Peptide | 1-21 NT vMIP-II<br>LGASWHRPDKCCLGYQ<br>KRPLP | Block HIV-1 entry | Pre-clinical | 61 |
| DV1 | D-Peptide | 1-21 NT vMIP-II D-Amino Acids<br>*LGASWHRPDKCCLGYQ*<br>*KRPLP* | Block HIV-1 entry | Pre-clinical | 64 |
| 9 (RCP168) | D-Amino Acid Containing Peptide | 1-10 NT \| 11-71 vMIP-II Core D-Amino Acids<br>*LGASWHRPDK*CCLGYQKRPLPQVL<br>LSSWYPTSQLCSKPGVIFLTKRGR<br>QVCADKSKDWVKKLMQQLPVTA<br>R | (1) Block HIV-1 entry; and (2) inhibit stromal–leukemia cell interactions | Pre-clinical | 98,99,124 |

[a] D-Amino acids are shown in italic.

pyridinylmethylene amine moiety, is comparable to AMD3100 in its antiviral activity, and it is capable of inhibiting the replication of virus strains that are resistant to other anti-HIV drugs such as zidovudine.[78] It targets the side chains of Asp$^{171}$ and Asp$^{262}$ as well as Glu$^{288}$ on CXCR4. It also binds to His$^{281}$ of the target protein. Compound **3** is a potent orally available CXCR4 antagonist that strongly inhibits virus infectivity via CXCR4.[80,81] It works in an additive or synergistic fashion when combined with other HIV inhibitors such as reverse transcriptase and protease inhibitors. More recently, Schols' group further modified **3** by generating novel heterocyclic analogues of **3**[82] and also by (1) sequentially replacing the benzimidazole ring with a substituted pyridine ring, (2) opening up the tetrahydroquinoline ring, and (3) combining both of these modifications to afford open chain analogues while still maintaining potent HIV-1 inhibition.[83]

Oral availability still remains one of the key limitations to be overcome for these compounds to be effective as anti-HIV drugs because retroviral infection continues throughout the life of the infected person. More recent advances in this area of research have led to the development of two additional orally active CXCR4 antagonists. *N*-{(*S*)-4-Guanidino-1-[(*S*)-1-naph-


dx.doi.org/10.1021/jm200568c | J. Med. Chem. 2012, 55, 977–994

thalen-1-ylethylcarbamoyl]butyl}-4-{[(pyridin-2-yl-methyl)-amino]methyl}benzamide (KRH-1636, Table 1) is a potent small non-peptide CXCR4 inhibitor derived from a small cyclopentapeptide, cyclo(-Nal-Gly-D-Tyr-Arg-Arg-) (**7** (FC131)).[84] Although this compound was efficiently absorbed from the rat duodenum, it could not be further developed as an anti-HIV-1 agent because of its poor oral bioavailability. Continuous efforts to find more potent CXCR4 antagonists that are bioavailable when administered orally led to the development of KRH-3955 (Table 1), a **4** (KRH-1636) derivative, which has a much more potent anti-HIV-1 activity than either AMD3100 or **4**.[85,86] **5** (KRH-3955) showed oral bioavailability of 25.6% in rats, and its oral administration blocked T-tropic HIV-1 replication in human peripheral blood lymphocytes and in a severe combined immunodeficiency mouse model. It was also active against recombinant T-tropic HIV-1 containing resistant mutations in reverse transcriptase, protease, and envelope with enfuvirtide resistance mutations. Experiments that examined the effects of **5** on the binding of several anti-CXCR4 monoclonal antibodies (MAbs) suggested that the binding sites of **5** are located in all three extracellular domains of CXCR4. Similarly, Jenkinson et al. recently reported an orally bioavailable and highly selective CXCR4 antagonist ([5-(4-methyl-1-piperazinyl)-2-({methyl[(8S)-5,6,7,8-tetrahydro-8-quinolinyl]amino}methyl)imidazo[1,2-*a*]-pyridin-3-yl]methanol) (GSK812397, Table 1), that was derived from a chemical class distinct from either **3** or **5**.[87] The pharmacokinetic properties of **6** (GSK812397) compare favorably with those of **5**, as its bioavailability was found to be similar across several species, including rat (21%), dog (21%), and monkey (17%).

An 18 amino acid synthetic polyphemusin (T22)[58] and its downsized analogues (T140 and TC14012 (14 amino acid residues)) also inhibit CXCR4-mediated HIV-1 entry[88] (Table 1). On the basis of the finding that the critical pharmacophore of T140 comprises $Arg^2$, L-3-(2-naphthyl)alanine $(Nal)^3$, $Tyr^5$, and $Arg^{14}$, screening of pentapeptide libraries containing these critical residues resulted in the identification of the cyclic pentapeptide **7**, which has anti-HIV activity similar to that T140 (Table 1).[89] More recently, smaller cyclic peptides based on the structure of T140[90,91] as well as non-peptide analogues[92] have been reported. The non-peptide analogues have 50% inhibitory concentrations that are at least 2 orders of magnitude higher than that of T140. Other studies have reported a hybrid peptoid/peptide oligomer of nine residues (CGP64222), triarginine derivative of gentamicin C (R3G), and neomycin B-hexaarginine conjugate (NeoR) as Arg mimic and cationic CXCR4 antagonists[93] (Table 1). **8** (CGP64222), a basic peptide oligomer of nine residues, inhibits the replication of a wide range of laboratory HIV strains through selective interaction with CXCR4. It specifically binds to the second extracellular loop (ECL2) of CXCR4, and it retains its full activity against the azidothymidine (AZT)-resistant HIV-1 strain.[94]

An alternative route for designing specific CXCR4 inhibitors is to use natural chemokine ligands as design templates and to mimic specific regions of these chemokine ligands.[59−64,67−69,95] First, when the SDF-1$\alpha$ sequence is used as the design template[59,60,67,69] (Table 1), a series of peptides derived from the N-terminus of SDF-1$\alpha$ were generated. The studies of these peptides demonstrated that the N-terminus of SDF-1$\alpha$ is essential for CXCR4 recognition, signal transduction, and antiviral activity. However, these peptides were less potent than the native SDF-1$\alpha$.[67,69] Noting the earlier observation that peptides and organic compound inhibitors of CXCR4, including **1**,[53] AMD3100,[57] and T22,[58] have highly positive charges, the attachment of positively charged residues to the N-terminal peptide sequence of SDF-1$\alpha$ enhanced its ability to bind to CXCR4.[59]

CXCR4 can also be recognized by vMIP-II which recognizes a variety of CC and CXC chemokine receptors, including CXCR4, CCR5, and CCR2[96,97] (Table 1, Figure 2). Studies of synthetic peptides derived from the N-terminus of vMIP-II demonstrated that the N-terminus of vMIP-II is the major determinant for CXCR4 recognition.[61] Only the 1−21 residue peptide of vMIP-II (V1) showed CXCR4 binding, and it selectively prevented CXCR4 signal transduction and its coreceptor function in mediating the entry of T- and dual-tropic HIV-1 isolates. Despite having a dramatically different conformation of side chain groups, an all-D-amino acid analogue (DV1) of V1 peptide displayed higher binding affinity for CXCR4 and significant antiviral activity as an inhibitor of the replication of CXCR4-dependent HIV-1 strains[64] (Figure 2). The use of unnatural D-peptides can be advantageous for therapeutic development, as such peptides are highly stable and resistant to proteolytic degradation.

In addition to short peptides derived from natural chemokines, a systematic strategy based on the full length chemokine sequences was developed for the synthesis of a new family of unnatural chemokines called SMM (synthetically and modularly modified) chemokines that, unlike natural chemokines, have high receptor binding selectivity.[98] This strategy was applied successfully to transform vMIP-II, a very nonselective chemokine, into a series of new analogues with significantly enhanced selectivity and potency for CXCR4 or CCR5, through modification of only a small N-terminal module of 10 residues. Two representative SMM-chemokines, RCP168 and RCP188, selective for CXCR4 and CCR5, respectively, showed similar or significantly enhanced binding affinities for their corresponding target receptors, whereas they have decreased or even completely abolished cross-binding activities for other receptors (Table 1). **9** (RCP168) was also more effective in inhibiting HIV-1 infection than SDF-1$\alpha$, and its anti-HIV activity was comparable to that of Fuzeon (T-20, also known as enfuvirtide), a currently marketed drug targeting HIV-1 entry (Figure 2). More interestingly, **9** did not interfere with the $Ca^{2+}$ signaling induced by SDF-1$\alpha$ at its effective CXCR4 binding concentration and only showed its anti-$Ca^{2+}$ signaling effects at concentrations over 20 times higher than the CXCR4 binding concentration. Moreover, **9** significantly inhibited HIV-1 entry, in contrast to its much weaker activity in interfering with SDF-1$\alpha$ signaling. Thus, these disparate inhibitory activity profiles of **9** in differentiating HIV-1 coreceptor function versus the normal function of CXCR4 may prove to be advantageous in clinical applications, as **9** may not induce unwanted $Ca^{2+}$ signaling or interfere with SDF-1$\alpha$ signaling important for the normal physiological functions at the concentrations used for inhibiting HIV-1 infection. The mechanistic basis for the disparate activities of **9** was further demonstrated by mutational mapping analysis of the binding sites of **9**, which revealed that **9** binding sites on CXCR4 overlap significantly with HIV-1 but differ from SDF-1$\alpha$.[99] These novel and potent CXCR4 peptide antagonists will serve as leads for the development of new therapeutic agents for HIV-1 infection and other diseases affecting the immune system.


dx.doi.org/10.1021/jm200568c | J. Med. Chem. 2012, 55, 977−994

It remains to be further studied whether the blocking of the normal CXCR4–SDF-1$\alpha$ functions in HIV-1 therapy may cause potential undesirable side effects, given that CXCR4[100,101] or SDF-1$\alpha$[102] knockout mice die during embryogenesis with evidence of hematopoietic, cardiac, vascular, and cerebellar defects,[103] which indicates the essential role of CXCR4 in development. However, since CXCR4 antagonists are given to adults, not neonates, it is unlikely that these potential side effects will be a clinical issue, unless given to pregnant women. To avoid affecting the normal function of CXCR4, Sachpatzidis et al. took the approach of allosteric agonists and identified two CXCR4 peptide ligands (RSVM and ASLW) that are insensitive to the CXCR4 antagonists, AMD3100 and T140, or the monoclonal antibodies, 12G5 and 44717.111. This was achieved by screening a semirandomized 17-mer library in a yeast strain that expressed a functional CXCR4 receptor.[68] In chemotaxis assays, **10** (RSVM) behaves as a partial agonist, while **11** (ASLW) behaves as a superagonist that displays a chemotactic index greater than the maximum observed in SDF-1$\alpha$. Allosteric agonists that have different binding sites from CXCR4 antagonists may be therapeutically useful when combined with small molecule antagonists for anti-HIV therapy, since they could allow maintenance of essential receptor functions. The data also illustrated that other binding sites may exist for nonphysiological agonists.

## CXCR4 ANTAGONISTS AND AGONISTS THAT MODULATE STEM CELL AND CANCER CELL MIGRATION AND THEIR POTENTIAL APPLICATIONS IN THE TREATMENT OF HEMATOPOIETIC AND SOLID CANCERS

Adult bone marrow (BM) is the primary source of hematopoietic stem cells (HSCs) that initiate hematopoiesis.[104] The elevation of intermediates of HSCs, namely, hematopoietic progenitor cells (HPCs), in blood of patients recovering from chemotherapy or radiation[105,106] led to the realization that the HSCs and HPCs are able to exit the bone marrow and that these mobilized cells could be collected and would rehome to marrow and restore hematopoiesis. Currently, mobilized peripheral blood is the primary source of hematopoietic cells used for hematopoietic transplantation. However, HPCs and HSCs circulate in blood at frequencies too low to allow efficient collection of sufficient numbers for transplantation. Therefore, an increase in the number of HSCs in blood following mobilization would improve the efficiency of transplantation and would have the potential to shorten recovery from cytopenia, to enhance immune reconstitution, and to reduce morbidity and mortality.

Granulocyte colony-stimulating factor (G-CSF) is the predominant mobilizer of hematopoietic cells used clinically, but it results in a broad individual variability in mobilization responsiveness,[107] frequently requiring repeated dosing, often with side effects. Although the longer acting G-CSF, pegfilgrastim, may address the multidosing requirement of conventional G-CSF, improved methods to mobilize and collect HSCs and HPCs for hematopoietic rescue are still warranted. Recent studies have indicated that the interactions between CXCR4 and SDF-1$\alpha$ modulate hematopoietic cell mobilization and engraftment.[108,109] CXCR4 is extensively expressed in HSCs and HPCs, and its interaction with SDF-1$\alpha$ promotes the retention of CXCR4 expressing hematopoietic cells in the BM microenvironment (Figure 2). Consequently, the disruption of CXCR4–SDF-1$\alpha$ interactions has been hypothesized to result in the mobilization of hematopoietic cells.[110] Indeed, the blockade of CXCR4 with an antagonist, such as AMD3100[111] or 4F-benzoyl-TN14003 (T140),[112] results in the mobilization of hematopoietic cells (Table 1, Figure 2). AMD 3100 (also known as Mozobil (plerixafor)) was finally approved in 2008 for patients with lymphoma, non-Hodgkin's lymphoma (NHL), multiple myeloma as a source of HSCs for transplantation.[113–115]

Another approach involves the use of the lipopeptide GPCR modulator, called pepducin, which is composed of a peptide motif derived from the amino acid sequence of one of the intracellular (IC) loops of a target GPCR coupled to a lipid tether.[116] Acting as a CXCR4 functional antagonist when administered systemically by iv bolus, ATI-2341 was identified as a potent mobilizer of bone marrow polymorphonuclear neutrophils (PMNs), HSCs, and HPCs. The successful generation of inhibitory CXCR4 nanobodies (VHH-based single variable domains) has also been reported, and these can induce HSC mobilization as rapidly and as nearly as effectively as AMD3100.[117] More recently, Polyphor (POL)6326, a selective and reversible CXCR4 inhibitor designed based on Polyphor's proprietary protein epitope mimetics (PEM) technology, has been reported to induce HSC mobilization as well.[118,119] In fact, **12** (POL6326) has successfully completed phase I clinical trials and is currently being investigated as a stand-alone therapy in a phase II clinical trial for its efficacy in autologous transplantation of HSCs in multiple myeloma patients. Taken together, these results suggest that antagonizing the interactions of SDF-1$\alpha$ produced by BM stromal cells with CXCR4 expressed on hematopoietic cells could be an effective strategy for mobilizing HSC.

In addition to mediating the release and homing of CXCR4 expressing hematopoietic cells from and to the SDF-1$\alpha$-producing BM microenvironment, CXCR4–SDF-1$\alpha$ interaction has also emerged as a critical mediator of stromal–leukemia cell interactions (Figure 2). CXCR4–SDF-1$\alpha$ interaction not only protects chronic lymphocytic leukemia (CLL) cells from apoptosis[120] but also allows the spontaneous migration of malignant cells beneath bone marrow stromal cells. This suggests that CLL cells might use this mechanism to infiltrate the marrow.[121] In fact, the BM microenvironment is known to provide a sanctuary for subpopulations of acute myelogenous leukemia (AML),[122] chronic lymphocytic leukemia (CLL),[120] and acute lymphoblastic leukemia (ALL)[123] to evade chemotherapy-induced apoptosis and acquire a drug-resistant phonotype. As such, CXCR4 inhibition has been hypothesized to interfere with stromal–leukemia cell interactions, thereby increasing the sensitivity of CLL, AML, or ALL cells to chemotherapy. Indeed, inhibition of CXCR4 by **9**, a potent CXCR4 antagonist, enhanced the sensitivity of CLL and AML cells to chemotherapy by reducing the protection conferred by stromal cells[124] (Table 1, Figure 2). Similar results were obtained with small molecule CXCR4 inhibitors, such as AMD3100[125] and AMD3465,[124] further suggesting that CXCR4–SDF-1$\alpha$ interaction contributes to the resistance of leukemia cells to chemotherapy-induced apoptosis. Thus, disruption of this interaction by CXCR4 inhibitors represents a novel strategy for targeting leukemia cells within the BM microenvironment.

Besides hematopoietic cell lines, CXCR4 is widely expressed on multiple solid tumors, including breast, lung, colon, ovarian, bone, brain, and skin cancer cells. The CXCR4–SDF-1$\alpha$ axis is

involved in cancer cell progression, angiogenesis, metastasis, and survival. For instance, Zlotnik's group has demonstrated that CXCR4 is highly expressed in human breast cancer cells, malignant breast tumors, and metastasis and that malignant melanoma, which has a similar metastatic pattern as breast cancer, also shows high expression level of CXCR4.[126] In fact, the overexpression of CXCR4/SDF-1$\alpha$ in breast cancer has been correlated with increased metastasis to brain, lymph node, lung, liver, and bone marrow.[127] In this regard, Chemokine Therapeutics Corp.'s (CTC)E-9908, a small peptide analogue derived from the N-terminus of SDF-1$\alpha$, emerged as a new generation of anticancer drug candidates (Table 1), as it was found to inhibit cancer growth and metastasis in several animal models by targeting CXCR4 that is present on most of these solid tumors.[128−130]

Similar to hematopoietic cells, neural stem cells (NSCs) also have the capacity to migrate, in this case, into distant areas of neurodegeneration. This has been shown in a number of experimental models of central nervous system (CNS) disease, including at ages where extensive migration has conventionally been deemed to be limited.[131−135] Because stem cell engagement with a degenerating environment is the first critical step in regeneration,[95,135] understanding the mechanisms underlying stem cell mobilization during injury is crucial for the full realization of the therapeutic promise of NSCs. In this regard, CXCR4 expressing human NSCs (hNSCs) were previously shown to migrate in vivo toward an infarcted area (a representative CNS injury), where local astrocytes and endothelium up-regulate SDF-1$\alpha$[131−135] (Figure 2). Exposure of hNSCs to SDF-1$\alpha$ and subsequent induction of CXCR4-mediated signaling trigger a series of intracellular processes that lead to the survival, proliferation, and most importantly migration of neural stem cells for injury repair. These steps include rapid and sustained phosphorylation of $p38^{MAPK}$ kinase (implicated in the regulation of cytokine-induced cell migration[136−138]), phosphorylation of the ribosomal S6 kinase ($p90^{RSK}$, implicated in the phosphorylation of cytoskeletal molecules involved in neurite outgrowth[139,140]), phosphorylation of c-Jun (a kinase involved in migratory responses[141]), rapid activation of extracellular response kinase (involved in proliferative responses), and rapid phosphorylation of paxilin (a scaffold molecule critical for migration[142]). As such, CXCR4−SDF-1$\alpha$ interactions implicated in the promotion of stem cell migration may serve as important targets for development of novel stem cell-based therapeutic for CNS diseases.

The extensive exploration of CXCR4−HIV-1 antagonism facilitated the investigation of highly potent and selective CXCR4 agonists capable of mediating NSC migration for injury repair. Recently, we developed a panel of "dual-moiety" chemokine analogues by linking a highly potent and selective CXCR4 binding moiety to a critical CXCR4-activating moiety of SDF-1$\alpha$ (unpublished results), based on the rationale that the newly created analogues may have signaling activities and capability to mobilize CXCR4-expressing hNSCs. Indeed, these "dual-moiety" chemokine analogues were found to promote the migration of NSCs in vitro and in vivo. As such, the effective mobilization of hNSCs with de novo designed agonists may lead to new translational therapeutics for the clinical repair of CNS injuries and other neurodegenerative conditions.

Since SDF-1$\alpha$ was identified as a potential stem cell homing factor, a large number of other studies have focused on CXCR4−SDF-1$\alpha$ interaction as a potential therapeutic target. For instance, many of these studies confirmed the significance of a CXCR4−SDF-1$\alpha$ axis in mobilization of BM-derived stem cells to sites of myocardial infarction and ischemic injury to induce cardiac regeneration.[143,144] Different approaches have been described to modify CXCR4−SDF-1$\alpha$ interaction. An indirect approach by using G-CSF in a rabbit ischemic reperfusion model demonstrated that G-CSF can increase SDF-1$\alpha$ levels and induce rapid mobilization of CXCR4-positive cells toward infarcted areas, thus leading to decreased scar size.[145] Also, acute blockade of the interaction between CXCR4 and SDF-1$\alpha$ by a single dose of AMD3100[146] was found to induce a rapid and transient hematopoietic cell mobilization. In fact, short-term administration of AMD3100 showed significant improvement of blood restoration and angiogenesis during the acute phase of ischemia in mouse models.[147]

## CXCR4 ANTAGONISTS AGAINST INFLAMMATION AND AUTOIMMUNE DISEASES: RHEMATOID ARTHRITIS AND ALLERGIC ASTHMA

Rheumatoid arthritis (RA), an autoimmune disease affecting about 1% of the world population, is characterized by chronic inflammation of multiple joints, proliferation of the synovial cells, massive infiltration of leukocytes into inflamed joints, and destruction of the cartilage and bone of the affected joints.[148−150] A large number of $CD4^+$ memory T-cells infiltrate the inflamed synovia,[151−157] and the pathogenesis of RA is then invoked by the action of inflammatory cytokines, including interleukin (IL)-1, IL-6, interferon (IFN)-$\gamma$, and tumor necrosis factor (TNF)-$\alpha$ and -$\beta$.[151,158] Accumulating evidence also suggests the involvement of CXCR4−SDF-1$\alpha$ interaction in RA pathogenesis. Nanki et al. reported that CD4+ T-cells express high levels of CXCR4 and that the concentration of SDF-1$\alpha$ is also highly elevated in the synovia of RA patients,[152] suggesting that CXCR4 is important for T-cell retention in RA-affected synovial tissues (Figure 2). This is further supported by the observation that T helper 1 (Th1) cells, which are believed to be at least partly involved in RA pathogenesis, are attracted by RA synovial fluid and that chemotaxis can be inhibited in vitro by anti-SDF-1$\alpha$ antibody.[159] Moreover, SDF-1$\alpha$ inhibits T-cell apoptosis in addition to promoting the migration of memory T-cells. Taken together, these results indicate that the development of inhibitors that block CXCR4−SDF-1$\alpha$ interaction might lead to promising RA therapies. Indeed, several CXCR4 antagonists, including AMD3100,[160,161] T22 (an 18-mer peptide),[162] T140 (a 14-mer),[162] and 7 (a cyclic pentapeptide),[89,163] are known to have anti-RA activities (Table 1, Figure 2).

CXCR4 is also associated with the pathophysiology of allergic asthma. Allergic asthma has a number of key characteristics, including airway inflammation of different cell types, airway hyper-responsiveness (AHR), mucus production, and variable airflow.[164,165] The initiating phase is characterized by the appearance of IgE and mast cells. In the propagation phase, Th2-polarized T-lymphocytes and eosinophils infiltrate the airways, initiating a chronic inflammatory state. The effector phase is characterized by the production of spasmogenic substances, AHR, and mucus hypersecretion. Steroid drugs have been the standard treatment, but development of resistance and potent side effects that are attributed to the long-term steroid use have promoted a search for alternatives for reducing the allergic lung inflammation and airway hyperreactivity seen in allergic asthma. One approach has been to explore anti-cytokine therapy for the treatment of


dx.doi.org/10.1021/jm200568c | J. Med. Chem. 2012, 55, 977−994

allergic asthma, as interleukin (IL)-4, IL-5, and IL-13 are fundamentally important in the pathophysiology of allergic asthma.[166] Treatment of human asthmatics with MAbs against IL-5 demonstrated a decrease in the number of eosinophils, although no major change in the AHR was observed.[166] Considerable efforts have also been made to reduce the Th2-type responses that are detrimental to the asthmatic condition. The Th2 subsets express CCR3, CCR4, CCR8, and CXCR4, but only CXCR4 and its ligand SDF-1$\alpha$ have been found to be relevant during Th2-type allergic airway responses[167] (Figure 2). The finding that Th2-type lymphocytes express CXCR4 on their cell surface, whereas Th1-type lymphocytes have very little or no expression of CXCR4,[168,169] adds further support. A cockroach allergen-induced animal model of asthma was used to show that blocking CXCR4 by AMD3100 significantly reduced airway hyperreactivity, peribronchial eosinophilia, and the overall inflammatory responses (Table 1, Figure 2).[170] In fact, analysis of total leukocytes indicated an 80% reduction in recruited leukocytes over background numbers in AMD3100-treated animals compared to control animals treated with saline. Similarly, AMD3465 (Table 1) inhibited eosinophil recruitment during type-2 pulmonary granuloma formation after both *Mycobacteria bovis* purified protein derivative and *Schistosoma mansoni* egg antigen challenge, suggesting potential therapeutic application for chronic hypersensitivity diseases.[171] The blockade of CXCR4 as a means of controlling allergic asthma may lead to effective new therapeutics for down-regulating the inflammation and pathophysiology of allergen-induced response.

## ■ FUTURE DIRECTIONS AND PERSEPECTIVES

Drug discovery research targeting CXCR4, a GPCR, has made tremendous progress in the past 15 years since the 1996 report of CXCR4 as an important coreceptor for HIV-1 entry[25] and gone well beyond the focus of this receptor on HIV-1 infection to other human diseases and treatments including cancer, stem cell mobilization, and inflammation and autoimmune diseases. Given the diverse functions of this important GPCR and many potential therapeutic molecules discovered and reported thus far, some of which are described in this review, research on CXCR4 will continue to be intensive in the future. Some of the future research questions and areas of further investigations, which are not meant to be comprehensive but only reflect those familiar to us, are outlined below in order to highlight some selected examples of research that may be directly or indirectly relevant to the advancement of therapies targeting this important receptor.

**Structure-Based Drug Discovery Using the CXCR4 Crystal Structures.** The determination of the crystal structures of CXCR4 bound to an antagonistic small molecule[56] provides the first high-resolution structure of a chemokine receptor bound to a synthetic ligand. This result is significant, since (1) it allows the verification of previous structure–function studies of CXC4–ligand interactions and drug design work (i.e., checking on the accuracy of the predicted mechanisms of CXCR4 interactions with HIV-1, natural ligands, and de novo designed inhibitors) and, more importantly, (2) it provides a new foundation for future drug design and discovery based on these crystal structures, such as the use of a receptor-based virtual screening approach (RBVS).[172] Prior to the publication of the CXCR4 crystal structures, drug discovery research that targets CXCR4 (and other GPCRs) mostly involved chemical screening and medicinal chemistry efforts[53,63−66,68] or use of the natural ligands as design templates.[98,99,173,174] With the new knowledge of the crystal structures of CXCR4, RBVS approaches can be used to exploit the molecular recognition between a ligand and CXCR4 to select out chemical entities that bind strongly to the active sites of CXCR4. This strategy has been successfully employed for identifying hits within a wide range of target proteins with experimentally determined or homology built structures, such Bcl-2,[175] acetylcholinesterase,[176] metalloenzymes,[177] phosphotriesterase,[177] and GPCRs.[178] As such, these crystal structures of CXCR4 will provide a more reliable basis for receptor structure-based drug design.

**Drug Discovery Research That Will Be More Specific to Different Functions of CXCR4.** In addition to its roles in a wide range of human disease pathologies (i.e., HIV-1 entry, cancer metastasis, and inflammation and autoimmune diseases), the CXCR4–SDF-1$\alpha$ interaction has essential physiological functions in immunomodulation, organogenesis, hematopoiesis, and cerebellar neuron migration[100,102,179] (Figures 1 and 2). This is further demonstrated by the death of CXCR4 or SDF-1$\alpha$ knockout mice, which succumb to hematopoietic, cardiac, vascular, and cerebellar defects during embryogenesis.[100−102] This highlights the importance of selective disruption of a particular targeted disease function of this important receptor without compromising its other functions. In this regard, some evidence shows that HIV-1 gp120 and SDF-1$\alpha$ interact with functionally distinct sites in CXCR4,[55] and that certain synthetic CXCR4 inhibitors such as SMM-chemokines interact with many residues on CXCR4 TM and extracellular domains that are important for HIV-1 entry, but they do not affect SDF-1$\alpha$ binding or signaling.[85] More research will be needed to determine the mechanistic and structural basis for the development of new inhibitory agents that may affect only the pathological responses of CXCR4 but not the physiological ones. In addition, specific functions of CXCR4 may be modulated by different signaling pathways, as studies showed that different signaling pathways are involved in neural stem cell migration versus inflammation.[180] Using SMM-chemokines as probes, we also found seemingly distinct signaling pathways of neuronal apoptosis involved in HIV-associated dementia (HAD) (Figure 1), where these pathways were activated by different agonistic or antagonistic ligands of CXCR4 or CCR5.[181] In this regard, future research should focus on the dissection of distinct pathways responsible for different functions (pathological and physiological) of CXCR4 and the discovery of novel therapeutic agents that impact only the selective pathways, thereby leading to desirable effects on the targeted functions or responses. For example, it will be a question of significant clinical interest whether one may develop novel synthetic agonists that promote CXCR4-mediated stem cell movement toward injury sites for regenerative repairs without provoking unwanted inflammation that also involves the same receptor. The development of such disease function and pathway specific drugs may be conceivable once we understand more of the cellular, molecular, and structural basis underlying the various functions of CXCR4.

**Design and Discovery of CXCR4 Agonists.** Most of the drug discovery targeting CXCR4 has focused on the development of CXCR4 antagonists for HIV-1 infection and cancer. Nevertheless, recent studies suggest that the activation of CXCR4 signaling may represent a new avenue for therapeutic development, for example, to mobilize hematopoietic stem cells or to direct stem cell migration toward brain or cardiac injury

sites for regenerative repairs.[131−135,180,182] Exposure of NSCs to SDF-1$\alpha$ and subsequent induction of CXCR4-mediated signaling trigger a series of intracellular processes that lead to survival, proliferation, and migration of neural stem cells for injury repair. Indeed, we recently developed a panel of "dual-moiety" selective CXCR4 agonists that are capable of mediating NSC migration for injury repair in the brain, which points to the feasibility of this approach (unpublished results). It has also been reported that CXCR4 agonists, such as pepducin[116] and CTCE-0021,[182] can mobilize bone marrow hematopoietic cells. In addition to synthetic agonists, the natural agonist SDF-1$\alpha$ can be delivered by adenovirus to activate CXCR4. For instance, adenovirus-mediated SDF-1$\alpha$ gene transfer has been utilized to improve cardiac structure and function after experimental cardiac infarction through the combined effects of angiogenesis of CD133+ stem cells and antiapoptosis.[183,184] More recently, Lefrancois et al. synthesized two CXCR4 agonists with chemotactic potencies only 1 log unit less than that of SDF-1$\alpha$ by linking the agonistic N-terminus of SDF-1$\alpha$ to the inverse agonist cyclopeptide T140.[185] As such, more interesting discoveries in the development of CXCR4 agonists are anticipated in the near future.

**Structure−Function Studies of CXCR4: Ligand Binding and Signaling Mechanisms.** The mechanism of ligand binding and activation of CXCR4 has been of immense basic importance and practical value for designing both antagonistic and agonistic ligands of CXCR4. Prior to the recent publication of the crystal structures of CXCR4 by Stevens' group,[56] several groups sought to characterize CXCR4 interactions with HIV-1, natural ligands, and de novo designed inhibitors using molecular modeling, receptor chimeras, and site-specific mutagenesis. These studies have been exploited to validate the accuracy of a two-site model that was initially developed for the C5a chemoattractant and its receptor.[186] This model has the chemokine core domain as the "site 1" docking domain and the chemokine N-terminus as the "site 2" signaling trigger. According to this model, the motif composed of amino acids 12−17 of SDF-1$\alpha$, RFFESH loop, first docks onto the N-terminal domain of CXCR4, and this contact allows the subsequent interaction of the flexible N-terminus of SDF-1$\alpha$ with the receptor groove formed by TM domains, thereby triggering the receptor function, whereas the globular portion of the chemokine binds to the extracellular loops.[17,186,187] The N-terminus of SDF-1$\alpha$, being relatively flexible and unstructured in solution, has been confirmed as essential for CXCR4 recognition and signal transduction.[17,18,67] We incorporated this concept and further hypothesized a two-pocket model of CXCR4.[55,99] According to the model shown in Figure 3, the natural CXCR4 chemokine antagonist vMIP-II and its synthetic modified analogues DV1 and **9** (Table 1) are speculated to recognize a binding pocket in CXCR4, whereas the natural CXCR4 chemokine agonist SDF-1$\alpha$ reaches more deeply into another different signaling pocket.[188] The recently published CXCR4 crystal structures in complex with small molecule antagonists may allow consideration of more structure-based experiments to examine these hypotheses. In particular, new crystal structures of CXCR4 in complex with agonists, either natural or synthetic, and with natural antagonists and/or synthetic analogues derived from these natural templates (such as DV1) should shed more insight into CXCR4−ligand binding and signaling mechanisms and allow the testing of the above-described two-site or two-pocket model for CXCR−ligand interaction.

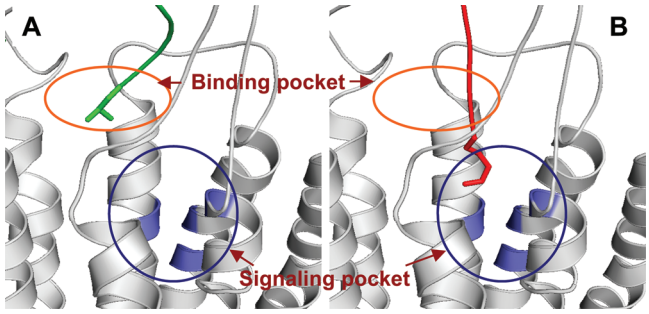



**Figure 3.** Two-pocket model as demonstrated by distinct binding and signaling pockets in CXCR4. As described in our earlier publication,[188] these structural models show the superimposition of the average structures obtained from computational molecular dynamics simulations of **9**−CXCR4 (A) and SDF-1$\alpha$−CXCR4 complexes (B). The superimposition displays the difference in the binding of the N-termini of ligands. In the case of **9** (green), the N-terminus populates site A (red circle, denoted as binding pocket), whereas in the case of SDF-1$\alpha$ (red), the N-terminus populates a hydrophobic region in site B (blue circle, denoted as signaling pocket) in CXCR4. The first N-terminal residue for either ligand is displayed in a stick model.

**Structure−Function Studies of CXCR4 Dimerization.** Structure−function studies of CXCR4 also need to include the study of the role of CXCR4 dimerization, which can provide new opportunities for drug design. Five independent crystal structures of CXCR4[56] have each revealed consistent homodimers with an interface, including TM helices V and VI, which are consistent with previous biological studies that suggested the importance of CXCR4 homodimerization or heterodimerization (with other GPCRs) in CXCR4 function.[189−192] Another piece of evidence for CXCR4 dimerization involves the "warts, hypogammaglobulinemia, infections, and myelokathexis" (WHIM) syndrome.[191] WHIM is primarily a heterozygous disease in which truncated CXCR4 is coexpressed with the wild-type receptor. Dimerization has been proposed as the most likely mechanism to explain the dominance of mutant CXCR4 over the wild-type receptor. Whether synthetic bivalent or multivalent ligands can be used to probe CXCR4 dimerization or polymerization represents an interesting area of research. Since the early 1980s, bivalent ligands that combine two identical pharmacophores, linked by a spacer, into a single molecule have been synthesized for many different GPCRs as specific pharmacological tools. Some of these molecules that contain two identical pharmacophores showed higher binding affinity, potency, or selectivity over their respective monomeric counterparts.[193−197] Our recent work on a synthetic novel bivalent ligand designated as DV1 dimer also suggests the feasibility of this approach for studying CXCR4 dimerization (unpublished results). Similarly, Tanaka et al. recently designed and synthesized novel CXCR4 bivalent ligands consisting of two molecules of a **7** analogue, [cyclo(-D-Tyr-Arg-Arg-Nal-D-Cys-)][Nal) L-3-(2-naphthyl)alanine], connected by a poly(L-proline) or a PEGylated poly(L-proline) linker, and they determined the distance between two binding sites of ligands consisting of CXCR4 dimers.[197] The findings suggest that this type of "molecular ruler" approach could be utilized in the design of bivalent ligands for any GPCR. This line of research will certainly be expanded in the future not only to study CXCR4 dimerization or oligomerization but also to understand how CXCR4 interacts with other GPCRs. Indeed, Sohy et al. used a combination of luminescence complementation and bioluminescence resonance energy transfer assays to demon-


dx.doi.org/10.1021/jm200568c | J. Med. Chem. 2012, 55, 977−994

strate the existence of hetero-oligomeric complexes composed of at least three chemokine receptors (CCR2, CCR5, and CXCR4).[198] Similarly, Sierro et al. also demonstrated that CXCR7 plays a specialized role in endothelial biology and heart valve development by forming functional heterodimers with CXCR4 and enhancing SDF-1$\alpha$-induced signaling.[199] More recently, Demmer et al. constructed symmetric dimers with excellent antagonistic activity using a derivative of a cyclic pentapeptide as monomer.[202]

**The Flexible Nature of CXCR4−Ligand Interface.** The structural and stereochemical flexibility of CXCR4 is one interesting feature of CXCR4−ligand interactions that deserves further investigation. We previously reported a number of D-amino acid containing compounds such as DV1 and **9** (Table 1). They represent various short peptides and full length protein analogues of vMIP-II that can bind to CXCR4 with potency and selectivity comparable to or higher than those of their L-peptide and protein counterparts (such as V1 and vMIP-II) (Table 1).[98,99,173,188] This was surprising because of the profoundly different side chain topologies between D- and L-enantiomers, as they have mirror image conformations. This finding also suggested a marked stereochemical or conformational flexibility of the CXCR4−peptide or CXCR4−protein interface, which appears to be distinctive from other known GPCR−ligand interactions that require a stricter stereochemistry. For instance, from the same studies of vMIP-II, it has been demonstrated that CCR5 binding has a strict structural requirement and involves multiple sites other than the N-terminus of vMIP-II. The recently reported CXCR4 crystal structures show a larger, more flexible, and open ligand binding pocket located closer to the extracellular surface, when compared to the binding sites of other GPCR structures noted in the past.[56] This supports our original findings and might explain the stereochemical flexibility seen in our studies of D-peptide ligands. One may further speculate that the flexibility of the CXCR4−ligand interface might be a feature of CXCR4 that HIV-1 capitalizes on by allowing sequence and conformational variations to occur in the V3 loop of the HIV-1 gp120. If the resulting changes in the gp120 V3 loop are accommodated by the flexible binding surface of CXCR4, thus promoting coreceptor switching from CCR5 to CXCR4, but not by that of an antibody, the virus can then evade neutralizing antibodies, especially after the host immunity is exhausted at the symptomatic phase of HIV-1 infection, while maintaining a high affinity for the coreceptor that is necessary for viral entry into the cell (Figure 4). These are intriguing and potentially important hypotheses and questions that should be addressed in the future studies.

**Development and Clinical Applications of Novel CXCR4-Targeted Molecules Designed Based on the Natural Chemokines.** CXCR4 inhibitors that have entered clinical trials so far were originated from chemical screening and medicinal chemistry efforts, as exemplified by the much studied AMD3100 and its derivatives. As a complementary approach, chemically synthesized and engineered molecules designed based on the natural chemokine ligands of CXCR4, such as agonist SDF-1$\alpha$ and antagonist vMIP-II, are also important therapeutic candidates for drug development and clinical applications. For instance, SMM-chemokines are a class of such molecules with enhanced biological properties compared to natural chemokines, and they were developed by using concepts and techniques of synthetic biology and protein medicinal chemistry. Being engineered from the natural

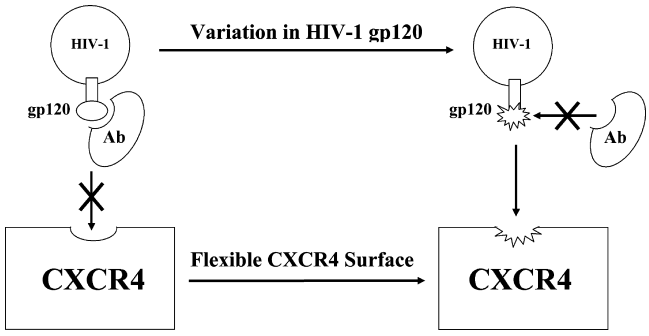



**Figure 4.** Hypothetical mechanism for how HIV-1 gp120 exploits the flexible ligand binding surface of CXCR4 to evade the recognition of a neutralizing antibody while retaining the ability to bind CXCR4 for cellular entry. The flexibility of the CXCR4−ligand interface may allow sequence and conformational variations of the V3 loop of the HIV-1 gp120. If the resulting changes in the gp120 V3 loop are accommodated by the flexible binding surface of CXCR4 but not by that of an antibody, the virus can then evade neutralizing antibodies while maintaining a high affinity for the coreceptor that is necessary for viral entry into the cell.

chemokines to target only the selective receptor and, perhaps in the future, a particular receptor-mediated disease pathway, these natural protein and peptide derived agents have been useful as not only tools to study the functions of the native ligands but also promising leads to be examined in the future translational research to test their clinical values. As the techniques of nanotechnology and synthetic biology (which have already been applied to generate **9** and DV1 as shown in Table 1) can be used to enhance the enzymatic stability and in vivo half-lives of these peptide and protein-based therapeutics, and they do not need to cross the cell membrane for their therapeutic targets (which are the two major road blocks for peptide and protein drugs), it can be anticipated that synthetic modified analogues of the natural chemokines of CXCR4 will have significant values in the clinical settings.

Finally, whether drug discovery research strategies and results from the above areas of future research topics on CXCR4 are applicable to other chemokine receptors or GPCRs in general will need to be investigated. The mechanisms underlying CXCR4 ligand binding, signaling, and dimerization/oligomerization, and the design and discovery of new drugs based on the understanding of these mechanisms and the high-resolution crystal structures of CXCR4 may offer new insights or ideas for drug discovery research that targets other chemokine receptors and GPCRs. If so, CXCR4 will then become a useful model system for obtaining a general mechanistic understanding of drug discovery strategies for GPCRs, which currently represent the largest class of therapeutic drug targets.

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**Won-Tak Choi** earned his M.D. and Ph.D. (in Biochemistry) from the University of Illinois at Urbana-Champaign in 2009. He worked with Professor Ziwei Huang on the biochemical characterization of chemokine receptor CXCR4 interactions with HIV-1, natural ligands, and de novo designed inhibitors. Following his graduation in 2009, Dr.

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**Srinivas Duggineni** received his Ph.D. in Organic Chemistry under the supervision of Professor Bijoy Kundu at the Central Drug Research Institute Lucknow, India, in 2005. Upon graduation, he joined Aurigene Drug Discovery Ltd. at Bangalore, India, from 2005 to 2007. Dr. Duggineni carried out postdoctoral research with Professor Ziwei Huang at the Sanford-Burnham Medical Research Institute, La Jolla, CA, from 2007 to 2009. He is currently continuing his postdoctoral research with Professor Ziwei Huang at the Department of Pharmacology, Cancer Research Institute, State University of New York at Syracuse, NY. His main research interests include synthesis of peptides, peptidomimetics, and small molecules for anticancer and anti-HIV therapeutics.

**Yan Xu** graduated with a concentration in Medicinal Chemistry at Jilin University, China, in 2009 and received a M.S. degree under the guidance of Professors Xiaohong Yang and Song Li. In 2010, she received financial support from China Scholarship Council and worked with Professor Ziwei Huang as an exchange Ph.D. student at the State University of New York at Syracuse, NY. Her main research interests include medicinal chemistry, peptide chemistry, and nanocarriers for cancer therapy.

**Ziwei Huang** received a Ph.D. in Chemistry in 1993 at the University of California at San Diego (UCSD) and then conducted postdoctoral research at the University of California at San Francisco. From 1995 to 2009, he held various faculty positions at the Sanford-Burnham Medical Research Institute, UCSD, CA, the University of Illinois at Urbana-Champaign, and Kimmel Cancer Center of Jefferson Medical College, PA. Since 2009, Dr. Huang is a Professor and Chairman of Department of Pharmacology, State University of New York (SUNY) at Syracuse, NY. In addition, he is the Director of SUNY Upstate Cancer Research Institute. His main research interests are the discovery and development of new synthetic probes and therapeutics targeting protein–protein interactions implicated in human diseases such as cancer, HIV infection, and neurodegeneration.

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## ■ ACKNOWLEDGMENTS

We apologize to our colleagues if we have missed or failed to cite their work and papers in this review because of the specific focus and space constraint of this review and the diverse research topics and rapid development of this field. We thank all former and current members of our laboratories and collaborators at other institutions who contributed to the work cited in this review. Our studies were supported by grants from the National Institutes of Health, the Carol M. Baldwin Breast Cancer Research Fund, the Connolly Endowment/Hendricks Fund, and the LUNGevity Foundation.

## ■ ABBREVIATIONS USED

CXCR4, CXC chemokine receptor 4; GPCR, G-protein-coupled receptor; HIV, human immunodeficiency virus; AIDS, acquired immune deficiency syndrome; SMM, synthetically and modularly modified; SDF-1$\alpha$, stromal cell-derived factor 1$\alpha$; vMIP-II, viral macrophage inflammatory protein II; MIP-1$\beta$, macrophage inflammatory protein 1$\beta$; RANTES, regulated on activation, normal T-cell expressed and secreted; TM, transmembrane; IC, intracellular loop; ECL, extracellular loop; PI3K, PI3 kinase; Akt, serine/threonine protein kinase; BAD, Bcl-2-associated death promoter; MEK, mitogen activated protein kinase kinase; JAK, Janus kinase; STAT, signal transducer and activator of transcription; cAMP, cyclic adenosine monophosphate; MAb, monoclonal antibody; BM, bone marrow; HSC, hematopoietic stem cell; HPC, hematopoietic progenitor cell; G-CSF, granulocyte colony-stimulating factor; PMN, polymorphonuclear neutrophil; NHL, non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; NSC, neural stem cell; CNS, central nervous system; hNSC, human neural stem cell; RA, rheumatoid arthritis; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; Th1, T helper 1; AHR, airway hyper-responsiveness; HAD, HIV-associated dementia; WHIM, warts, hypogammaglobulinemia, infections, and myelokathexis

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## ■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on December 2, 2011 with an error in the caption of Figure 4. The corrected version was reposted on December 6, 2011.