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Biology and clinical relevance of chemokines and chemokine receptors CXCR4 and CCR5 in human diseases

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Abstract

Chemokines and their receptors are implicated in a wide range of human diseases, including acquired immune deficiency syndrome (AIDS). The entry of human immunodeficiency virus type 1 (HIV-1) into a cell is initiated by the interaction of the virus's surface envelope proteins with two cell surface components of the target cell, namely CD4 and a chemokine co-receptor, usually CXCR4 or CCR5. Typical anti-HIV-1 agents include protease and reverse transcriptase inhibitors, but the targets of these agents tend to show rapid mutation rates. As such, strategies based on HIV-1 co-receptors have appeal because they target invariant host determinants. Chemokines and their receptors are also of general interest since they play important roles in numerous physiological and pathological processes in addition to AIDS. Therefore, intensive basic and translational research is ongoing for the dissection of their structure – function relationships in an effort to understand the molecular mechanism of chemokine – receptor interactions and signal transductions across cellular membranes. This paper reviews and discusses recent advances and the translation of new knowledge and discoveries into novel interventional strategies for clinical application.

Keywords

HIV; AIDS; CXCR4; CCR5; SDF-1α; vMIP-II; chemokines; chemokine receptors

Introduction

Chemokines are small soluble proteins of approximately 70 amino acid residues with a molecular weight of 8 – 10 kDa.[1] They act as potent chemoattractants of a large variety of mononuclear cell types to sites of inflammation or secondary lymphoid organs by interacting with chemokine receptors. Based on the positions of two conserved cysteine residues in their amino (N)-termini, chemokines can be divided into four subfamilies: CC, CXC, CX3C and C.[2,3] The two main subfamilies of chemokines are CXC and CC. CXC chemokines are primarily involved in the activation of neutrophils, whereas CC chemokines stimulate other leukocytes such as monocytes, lymphocytes and basophils. The highly conserved three-dimensional structures of all chemokines include a flexible N-terminus, a three-stranded antiparallel β-sheet and a C-terminal α-helix.[4] In the typical structure, the first two cysteine residues are situated close together, near the N-terminus, with the third

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cysteine residue residing in the center of the molecule, and the fourth cysteine residue located close to the C-terminal end.[5] 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 β-sheet with three β-strands and a C-terminal α-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. The structures of many chemokines have been determined by nuclear magnetic resonance (NMR) or X-ray crystallography, including those of stromal cell-derived factor (SDF)-1α,[6,7] viral macrophage inflammatory protein (vMIP)-II,[8,9] macrophage inflammatory protein (MIP)-1β,[10] and regulated on activation, normal T-cell expressed and secreted (RANTES).[11]

Chemokine receptors are a group of transmembrane (TM) proteins that belong to the superfamily of G-protein-coupled receptors (GPCRs).[2,12,13] They possess seven TM helices and transmit signals from extracellular ligands to intracellular biological pathways via heterotrimeric G-proteins. Chemokines and their receptors are implicated in a wide range of human acute and chronic inflammatory diseases (i.e. acute respiratory distress syndrome, allergic asthma, psoriasis and arthritis), neurological disorders, and cancer.[2–4,14] Chemokine receptors are also involved in the pathogenesis of human immunodeficiency virus type 1 (HIV-1) infection. HIV-1 enters cells through a fusion process in which the HIV-1 envelope glycoprotein gp120 binds to CD4, the main receptor for HIV-1 on the target cell surface.[15,16] However, it has long been known that CD4 alone is not sufficient for HIV-1 fusion and entry, and that additional receptors may be needed. In 1996, chemokine receptors CXCR4 and CCR5 were discovered to be the long-sought co-receptors for syncytium-inducing and non-syncytium-inducing HIV-1 strains, respectively.[17–20] The viral fusion process involves the initial binding of HIV-1 gp120 to its high-affinity receptor CD4, which results in conformational changes in gp120 and CD4.[21–23] The gp120 – CD4 complex then interacts with a chemokine co-receptor, such as CXCR4 or CCR5, to form a heterotrimeric complex of gp120 – CD4 – co-receptor.[24–26] During the asymptomatic stage of disease, macrophage (M)-tropic strains of HIV-1 involved in sexual transmission primarily use CCR5 as an entry co-receptor.[17–19] However, in 40–50% of HIV-1-infected individuals, T-cell (T)-tropic strains that predominantly use CXCR4 eventually replace M-tropic strains, leading to rapid disease progression.[27–29] Among many new directions in AIDS research opened by this discovery, an important area of investigation is the biochemical and biophysical characterization of the interactions of chemokine receptors with HIV-1 and natural as well as synthetic chemokine ligands.

Structure – fuction relationship of CXCR4 and its ligands

CXCR4 is a CXC chemokine receptor 4 which consists of 352 amino acid residues.[30] Like other GPCRs, CXCR4 consists of an amino (N)-terminus, three extracellular and intracellular loops, seven TM helices, and a carboxyl (C)-terminus. However, unlike other chemokine receptors which have a number of distinct ligands, CXCR4 has only one endogenous natural ligand known as an SDF-1α.[1] However, CXCR4 can also be recognized by an antagonistic ligand, vMIP-II, encoded by the Kaposi's sarcoma-associated herpes virus.[31] CXCR4 – SDF-1α interaction has essential physiological functions in immunomodulation, organogenesis, hematopoiesis and cerebellar neuron migration.[32–34] This is further demonstrated by knockout mice of CXCR4 and SDF-1α that die of hematopoietic, cardiac, vascular and cerebellar defects during embryogenesis.[32,33,35]

vMIP-II displays a broader spectrum of receptor activities than any mammalian chemokine, as it binds with high affinity to a number of both CXC and CC chemokine receptors, including CXCR4 and CCR5, and it inhibits cell entry of HIV-1 mediated by these receptors.[36,37] Synthetic peptides derived from the N-terminus of vMIP-II showed that the

N-terminus of vMIP-II is the major binding determinant for CXCR4[38] (Table 1). Only V1 peptide (1–21 residues) from the N-terminus of vMIP-II showed CXCR4 binding, and it selectively prevents CXCR4 signal transduction and co-receptor function in mediating the entry of T- and dual-tropic HIV-1 isolates.[38] An all-D-amino acid analog of V1 peptide, designated as DV1 peptide, displayed even higher binding affinity and antiviral activity than V1, demonstrating the remarkable stereochemical flexibility of the CXCR4 – peptide interface.[39]

Prior to the recent publication of high resolution crystal structures of CXCR4 by Wu *et al.*,[30] several groups have endeavored to characterize CXCR4 interactions with HIV-1, natural ligands and *de novo* designed inhibitors using molecular modeling, chimeras and site-specific mutagenesis. These studies demonstrated that the amino (N)-terminus and the second (ECL2) and third (ECL3) extra-cellular loops (ECLs) of CXCR4 are required for HIV-1 co-receptor activity.[40–50] They also indicated a requirement for multiple extracellular and TM domains of CXCR4 in chemokine interactions and receptor signaling.[41,42,46,50–55] In addition, a separation of binding and signaling functions was revealed by these chimeric and mutational studies, and it has been exploited to validate the accuracy of a two-site model that was initially developed for the C5a chemoattractant and its receptor. This model has the chemokine core domain being the ‘site one’ docking domain and the chemokine N-terminus being the ‘site two’ signaling trigger.[56] According to this model, the motif composed of amino acids 12–17 of the SDF-1α, 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-1α with the receptor groove formed by TM domains and/or ECLs, thereby triggering the receptor function.[6,56,57] The N-terminus of SDF-1α, being relatively flexible and unstructured in solution, has been confirmed as essential for CXCR4 recognition and signal transduction.[6,7,58]

Recently, the long-awaited crystal structure of CXCR4 was published by Wu *et al.*, who reported five independent crystal structures of CXCR4 bound to an antagonist small molecule IT1t and to a cyclic peptide CVX15 at 2.5 to 3.2 Å resolution.[30] These structures are now providing new clues about the interactions between CXCR4 and SDF-1α as well as with HIV-1 gp120. All structures have revealed consistent homodimers with an interface, including TM helices V and VI, which may be involved in regulating signaling. Moreover, the peptide and small molecule complexes of CXCR4 have identified the likely ‘site two’ of the chemokine-signaling trigger. The IT1t ligand was shown to occupy part of the binding pocket defined by side chains from helices I, II, III and VII, whereas CVX15 filled most of the binding-pocket volume by inducing major deviations in the base of the receptor N-terminus (residues 29–33), as well as a minor adjustment of extracellular tips of helices VI, VII and V. Compared with previous GPCR structures, the binding pocket of CXCR4 is larger, more open and located closer to the extracellular surface, and it includes acidic Asp^{187}, Glu^{288} and Asp^{97} that are important for SDF-1α binding. This suggests that Lys^{1}, the most critical residue in SDF-1α for receptor activation, could reach into the CXCR4 pocket and interact with one of these acidic residues. The importance of Glu^{288} for SDF-1α signaling was previously demonstrated by our laboratory.[50] Similarly, the basic character of gp120 V3 loop, which becomes exposed upon CD4 binding, could potentially penetrate the CXCR4 binding pocket, thereby interacting with one of these acidic residues. Taken together, the crystal structures of CXCR4 provide strong support for the two-site model, and they also suggest the possibility of a three-step interaction between CXCR4 and its ligand. The first step would be the electrostatic interaction of the body of the chemokine with the complementary surface of CXCR4. The second step would be the insertion of the N-terminal of chemokine into the cavity defined by the TM and some extra-cellular domains. The implied third step would be the folding of the N-terminus of CXCR4 across the top of the docked chemokine. As further details are resolved regarding the dynamic changes

involved in CXCR4 interactions with its natural ligand and HIV-1 gp120, new opportunities will surely emerge for drug discovery efforts that target specific functional states of the receptor.

Structure – fuction relationship of CCR5 and its ligands

CCR5 is a CC chemokine receptor 5 which consists of 352 amino acid residues. It has multiple natural chemokine ligands, including MIP-1α, MIP-1β, 'RANTES' protein and monocyte chemotactic protein (MCP)-2.[59] Among these ligands, RANTES and MIP-1α can bind to other CC chemokine receptors, while MIP-1β is known to be most specific for CCR5.[59] vMIP-II encoded by the Kaposi's sarcoma-associated herpes virus displays a broader spectrum of receptor activities, including CCR5.[31,36,37] Signal transduction through CCR5 is known to play important roles in both physiological and pathological processes, including inflammation and hematopoiesis.

As the major co-receptor for HIV-1 entry, CCR5 has undergone extensive studies using chimeric receptors,[51–54,60,61] site-directed mutagenesis,[62–67] NMR and molecular modeling.[68] These studies have shown the involvement of all extracellular domains of CCR5 in its HIV-1 co-receptor function, particularly the N-terminal domain and the second ECL (ECL2) of CCR5.[51–54,61] The ECL2 of CCR5 is responsible for the binding selectivity of the receptor, and it determines the range of chemokines to which it responds functionally.[69] The major interaction sites between MIP-1β and CCR5 include the amino (N)-terminus, N-loop (residues 13–19), 3_{10} turn (Arg18, Lys19 and Arg22), 20s region, 40s loop (Lys^{45}, Arg^{46} and Lys^{48}), and carboxyl (C)-terminus of MIP-1β,[70–73] and the N-terminus and ECL2 of CCR5.[51,68,69,74–76] Phe^{13} of MIP-1β is the most important residue for CCR5 binding.[77] In addition to these basic amino acids, Pro^{21} was found to be important in CCR5 binding, whereas Tyr^{15} is essential for the proper folding of MIP-1β.[70] Thus, the key residues in MIP-1β crucial for CCR5 binding include most of the basic amino acids and two hydrophobic groups, all arranged on one surface of the chemokine.[70]

As with other chemokines, the N-terminus of MIP-1β is of primary importance for signal transduction and cell activation, but it does not significantly contribute to receptor binding. According to the prevailing two-site model, the core moiety of CC chemokines first binds to the extracellular domains of CCR5 through electrostatic attractions, and the N-terminus subsequently binds to the second binding site spanning the TM helixes to activate the receptor. Several research groups have attempted to locate this second binding site important for receptor activation by alanine scanning mutagenesis of the TM domains.[66,78] These studies have revealed that the binding site for small molecule antagonists of CCR5, including TAK-779, AD101 and SCH-C (Table 2), are located near the extracellular surface of the receptor, within a cavity formed between TM helices I, II, III, V and VII. The important residues include Leu^{33}, Tyr^{37}, Asp^{76}, Thr^{82}, Trp^{86}, Tyr^{108}, Phe^{113}, Ile^{198} and Glu^{283}. Govaerts *et al.*[79] have provided further support by identifying the importance of Thr^{82} for receptor activation, but not for chemokine binding.

Docking of HIV-1 gp120 to CCR5 involves interactions between conserved residues in the V3 stem – C4 region of gp120 and the N-terminus of CCR5, as well as binding of the V3 crown to a second region of CCR5, which a number of studies suggest to be primarily ECL2.[51–54,61] Since TAK-779 does not disrupt interactions between gp120 and the N-terminus of CCR5,[66] it is certainly possible that the V3 crown interacts with the TM domain residues, although none of the TM domain mutants discussed above were significantly impaired with respect to viral entry. The binding of small molecule antagonists to the TM domains of CCR5 may induce a conformational change in the region of CCR5 (likely to be ECL2) that interact with the V3 crown. Thus, disruption of the binding site on CCR5 for the

V3 crown would inhibit the gp120 – CCR5 interaction and viral entry. Interesting, the Hartley group recently identified three analogues of the N-terminally modified chemokine PSC-RANTES, all of which exhibit *in vitro* potency against HIV-1 comparable to that of PSC-RANTES[80] (Table 2). Despite the subtle differences in sequence, volume and mass that distinguish these modified RANTES compounds, all three showed very distinct characteristics with respect to CCR5 binding affinity, G protein-linked signaling and receptor sequestration. The first, 6P4-RANTES, resembles PSC-RANTES in that it is a strong agonist that induces prolonged intracellular sequestration of CCR5. The second, 5P12-RANTES, has no detectable signaling activity and does not bring about receptor sequestration. The third, 5P14-RANTES, induces significant levels of CCR5 internalization without detectable signaling activity. As such, these compounds represent excellent molecular probes for unraveling the details of agonist versus antagonist activity associated with slightly different binding to the second binding site of CCR5. Thus, they can be useful in distinguishing sites important for binding, signaling and internalization.

Chemokine receptor inhibitors

In comparison to anti-HIV-1 agents that are directed against components of the rapidly mutating virus population, co-receptor-based therapeutic strategies have the appeal of targeting relatively invariant host determinants.[38,39,55,58,81–91] Furthermore, individuals with CCR5 mutations appear to be both healthy and highly resistant to HIV-1 infection, which demonstrates the feasibility of this approach for inhibiting viral infection.[3,92] Although previous studies have demonstrated the potential benefits of chemokine receptor inhibitors for combating AIDS and other diseases, the use of natural, non-specific chemokines in clinical applications is problematic due to the lack of selectivity and its potential side-effects. Consequently, the development of new inhibitors engineered with higher selectivity for targeted receptors and reduced toxicity is clearly desirable for their use in clinical applications and as specific probes in receptor biology, to study the role of particular ligands or receptors.

Several peptides and organic compounds, unrelated to natural chemokines that have antagonistic activity in CXCR4, were discovered much earlier through random screening[39,55,87–90] (Table 1). ALX40-4C (*N*-a-acetyl-nona-D-arginine amide acetate) and AMD3100 were the first CXCR4 antagonists to enter clinical trials.[55,93,94] ALX40-4C is a highly basic peptide designed as an arginine mimic of the HIV Tat protein, and it has been shown to prevent HIV-1 entry via CXCR4.[93,94] Moreover, T22,[83] an 18-amino acid synthetic polyphemusin, and its downsized analogs, T140 and TC14012 (14 amino acid residues), were shown to inhibit CXCR4-mediated HIV-1 entry in the nanomolar range.[95] Subsequent studies show that Arg^2, L-3-(2-naphthyl)alanine $(NaI)^3$, Tyr^5 and Arg^{14} constitute the critical pharmacophore of T140.[96] Screening of pentapeptide libraries containing these critical residues resulted in the identification of a small cyclic pentapeptide FC131 (cyclo(-NaI-Gly-D-Tyr-Arg-Arg-) that has similar anti-HIV activity to T140. Furthermore, CGP64222, R3G and NeoR were reported as Arg-mimic and cationic CXCR4 antagonists.[97] CGP64222, a basic peptide oligomer of nine residues, has been shown to inhibit the replication of a wide range of laboratory HIV strains through selective interaction with CXCR4. However, there is cause for concern regarding undesired side-effects of blocking the normal CXCR4 – SDF-1α function, as knockout mice lacking either CXCR4[33,35] or SDF-1α[32] died during embryogenesis with evidence of hematopoietic, cardiac, vascular and cerebellar defects.[98] Even if adverse effects are not observed during clinical trials, any CXCR4 antagonist has the potential to cause harm if it is used chronically as a highly active antiretroviral therapy.

An alternative route of designing specific CXCR4 inhibitors is to use natural chemokine ligands as design templates by mimicking specific regions of a chemokine ligand. This can be used to study the structure – function relationship of the native molecule and to develop novel agonists or antagonists of chemokine receptors.[38,39,58,84–87,90,91,99] This approach was first attempted on SDF-1α, the only known natural ligand of CXCR4.[58,84,85,91] Peptides derived from the N-terminus of SDF-1α were proven essential for CXCR4 recognition, signal transduction and antiviral activity. However, they were less potent than the native SDF-1α.[58,91] The attachment of positively charged residues to the N-terminal peptide sequence was found to enhance its ability to bind to CXCR4 and inhibit CXCR4-mediated T-tropic HIV-1 entry.[84] When combined with the observation that peptides and organic compounds, such as AMD3100,[82] T22,[83] and ALX40-4C,[55] have high positive charges and affinity for CXCR4, these studies indicate that electrostatic interaction may play a important role in CXCR4 recognition.[84]

CXCR4 can also be recognized by vMIP-II, which displays a broad spectrum of receptor activities.[36,37] By studying synthetic peptides derived from the N-terminus of vMIP-II, our laboratory demonstrated that the N-terminus of vMIP-II is the major determinant for CXCR4 recognition[38] (Table 1). Only V1 peptide (1–21 residues) showed CXCR4 binding, and it selectively prevented CXCR4 signal transduction and the co-receptor function in mediating the entry of T- and dual-tropic HIV-1 isolates. An all-D-amino acid analog of V1 peptide, designated as DV1 peptide, was also synthesized.[39] Despite dramatically different conformations of side-chain groups, DV1 displayed higher binding affinity toward CXCR4, and it showed a significant antiviral activity in inhibiting the replication of CXCR4-dependent HIV-1 strains. Unnatural D-peptides can be highly desirable and advantageous over natural L-peptides for therapeutic development, as they are highly stable and resistant to proteolytic degradation.

Understanding the potential adverse effects of the antagonistic inhibitors of CXCR4 in HIV-1 therapy, Sachpatzidis *et al.* identified RSVM and ASLW as novel allosteric agonists that are insensitive to the CXCR4 antagonists, AMD3100 and T140, or monoclonal antibodies, 12G5 and 44717.111. This was achieved by screening a semi-randomized 17-mer library in a yeast strain that was expressing a functional CXCR4 receptor.[90] In chemotaxis assays, RSVM behaves as a partial agonist, while ASLW behaves as a superagonist that displays a chemotactic index greater than the maximum observed in SDF-1α. Allosteric agonists may be therapeutically useful in combination with small molecule antagonists for anti-HIV therapy, since they could maintain essential receptor functions. The data also illustrate that other binding sites may exist for non-physiological agonists. Despite their early stage of development, allosteric agonists and other previously discussed potent CXCR4 peptide antagonists or agonists could serve as leads for the development of new therapeutic agents for HIV-1 infection and other diseases affecting the immune system.

Similarly, several small CCR5 antagonists have been reported to block HIV-1 infection[100–102] (Table 2). TAK-779, a non-peptide compound, binds to CCR5 primarily via polar interactions with Glu^{283} and Tyr^{37}, hydrophobic interactions with Phe^{10}, Phe^{112}, Phe^{113}, Ile^{198} and Trp^{248} deeply buried in the TM regions, and a face-to-face aromatic stacking contact with Tyr^{108}.[103,104] Moreover, two structurally related small molecules SCH-C (SCH-351125) and AD101 (SCH-350581) inhibit RANTES binding as well as HIV-1 entry and replication, and they have excellent oral bioavailability in rats, dogs, monkeys and humans.[78] In addition, Agrawal *et al.*[105] prepared peptide segments from the amino (N)-terminus and ECLs of CCR5 and evaluated their inhibitory effects on HIV-1 entry. In this study, peptides derived from ECL1 and ECL3 inhibited R5-tropic BaL and dual-tropic 89.6 HIV-1 strains, whereas peptides from ECL2 only inhibited the BaL strain.

Peptides from the N-terminus of CCR5 showed no effects on HIV-1 inhibition. The inhibitory effects of these CCR5-derived peptides were independent of co-receptors, illustrating that they target HIV envelopes rather than co-receptors.

Aminooxypentane (AOP)-RANTES, a chemically modified analog of RANTES, is a CCR5 antagonist that effectively inhibits CCR5-dependent HIV-1 strains[101] (Table 2). Additional efforts to increase the antiviral potency of RANTES derivatives, using unnatural amino acids and systematic SAR, led to the generation of more potent HIV-1 inhibitors. This was exemplified by PSC-RANTES (*N*-nonanoyl, des-Ser1[L-thioproline2, L-cyclohexylglycine3]-RANTES [2–68]), in which several unnatural, non-coded structures were incorporated into the N-terminal region of RANTES with a 50-fold increase in potency over AOP-RANTES.[106] More recently, Gaertner *et al.*[80] used a phage-display strategy to generate fully recombinant chemokine analogs of PSC-RANTES with potent anti-HIV activity, culminating in 5P12-RANTES. 5P12-RANTES is as an effective inhibitor of virus entry via CCR5 as its immediate precursors, but has two important advantages: first, it is a pure antagonist with no demonstrable signaling activity upon CCR5 binding, and second, it is possible to produce 5P12-RANTES by recombinant expression, rather than chemical synthesis, suggesting that production could occur at an ultralow cost.

Although several CCR5 antagonists have been evaluated in clinical trials, only Maraviroc (MCV) has been approved in 2007 by the US Food and Drug Administration (FDA) for treatment of HIV-infected patients experiencing virological failure due to resistance to other classes of antiretroviral drugs.[107] Maraviroc was subsequently approved for the treatment of antiretroviral naïve patients as well. Another CCR5 antagonist, Vicriviroc, is currently in advanced clinical development (phase III) but has yet to be approved by the FDA.[107] Two other products in development are antibodies to CCR5. PRO 140 is a humanized CCR5 monoclonal antibody that inhibits CCR5-tropic HIV-1 *in vitro*, and was recently shown to have potent antiviral activity after a single dose in a phase Ib monotherapy, dose-escalation trial.[108] HGS004 is a human immunoglobulin G4 monoclonal antibody against CCR5 that was also recently tested in a phase Ib trial that established its safety and *in vivo* activity against HIV-1.[109] Maraviroc and other CCR5 antagonists could potentially be used in a variety of other clinical situations, such as the prevention of HIV transmission, intensification of HIV treatment and prevention of transplant rejection.[107] As such, the future roles for CCR5 antagonists in both the prevention and treatment of HIV infection are likely to expand.

Synthetically and modularly modified-chemokines: a new class of unnatural synthetic molecules as leads for therapeutic development

We have been working toward the development of a systematic strategy based on chemokine structures to synthesize a new family of unnatural chemokines that, unlike natural chemokines, have higher receptor binding selectivity. In this regard, we have reported our progress in developing such a strategy by employing SMM (synthetically and modularly modified)-chemokines as a potential general method for the *de novo* design of novel chemokine receptor-selective ligands.[72] Proof of this concept was shown by applying this strategy to transform vMIP-II, a very non-selective chemokine, into new analogs with significantly enhanced selectivity and potency for CXCR4 or CCR5, two principal co-receptors for HIV-1 entry, through modifying 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. However, RCP168 and RCP188 drastically decreased or even completely abolished cross-binding activities for other receptors.

Furthermore, RCP168 is more effective in inhibiting HIV-1 infection than SDF-1α, and its anti-HIV activity is comparable to that of T-20.

In addition to high receptor selectivity, another important biological property of such *de novo*-designed ligands is signaling activity. RCP168, a vMIP-II analog with its N-terminal (1–10) residues replaced with D-amino acids, did not trigger either Ca^{2+} signaling or receptor internalization,[72] which is distinct from the natural ligand of CXCR4, SDF-1α. More interestingly, RCP168 did not interfere with the Ca^{2+} signaling induced by SDF-1α at its effective CXCR4 binding concentration and only showed its effect at concentrations over 20 times higher than usual. Moreover, RCP168 significantly inhibits HIV-1 entry, in contrast to its much weaker activity in interfering with SDF-1α signaling. Thus, these disparate inhibitory activity profiles of RCP168 in differentiating HIV-1 co-receptor function versus the normal function of CXCR4 may prove to be advantageous in clinical applications, as RCP168 may not induce unwanted Ca^{2+} signaling or interfere with SDF-1α signaling important for the normal physiological functions at the concentrations used for inhibiting HIV-1 infection. The mechanistic basis for the disparate activities of RCP168 was demonstrated by the mutational mapping analysis of the binding sites of RCP168 and other D-amino acid-containing SMM-chemokines on CXCR4. This analysis revealed that RCP168 binding sites on CXCR4 overlap significantly with HIV-1 but differ from SDF-1α.[110]

The high-resolution crystal structure of RCP168, when compared with previous reports of vMIP-II,[8,9] revealed that the enhanced selectivity of RCP168 was associated with the structural changes at the N-terminus. This was due to the D-amino acid modification and surprisingly the 30s loop as a result of a conformational change propagation from the distal N-terminus through a disulfide bridge.[111] Based on this structural insight and with the aim of generating higher CXCR4 selectivity, new analogs containing modifications at the 30s loop were designed by replacing the 30s loop of RCP168 with the corresponding region of SDF-1α. Indeed, this analog, RCP303, exhibited more CXCR4 selective binding profiles than the parent molecule, as the 30s loop substitution led to a substantial reduction in binding to both CCR5 and CCR2. This finding further confirmed the role of the 30s loop in affecting receptor binding selectivity. In light of this finding, one may rationalize a structural basis for the conformational change cascade in chemokine – receptor interactions, which may include: (1) the initial binding of the N-terminus of a chemokine to the receptor; (2) the resulting conformational changes in the N-terminus (including the N-loop) and subsequently the 30s loop as facilitated by the disulfide bridge; and finally (3) the triggered recognition between the 30s loop and the receptor, leading to multipoint (at least including the N-terminus and the 30s loop) contact between the chemokine and its receptor.

To understand the signaling mechanism of CXCR4 or CCR5 in neuronal apoptosis associated with HIV-associated dementia (HAD), we also applied SMM-chemokine analogs as chemical probes of the mechanism(s) whereby these SMM-chemokines prevented or promoted neuronal apoptosis.[112] Because of the profound activities of chemokine receptors in HAD, developing selective and potent inhibitors of chemokine receptors, while understanding the physiological or pathological processes of HAD, are crucial when devising novel strategies for clinical interventions. In this study, we demonstrated that unlike natural agonist SDF-1α, which causes neuronal death via a p38 MAPK-dependent pathway,[113] antagonistic CXCR4-selective SMM-chemokines can effectively prevent $gp120_{IIIB}$-induced neuronal apoptosis. Furthermore, compared with natural CCR5 agonist ligands that are known to promote neuronal survival by activating Akt,[114] our data on unnatural CCR5 antagonists indicated that inhibition of CCR5 is neurotoxic via the p38 MAPK pathway, demonstrating that distinct CCR5-mediated signaling pathways can be activated by different chemokine ligands, which results in different cell fates. Taken

together, using novel synthetic SMM-chemokines as mechanistic probes and/or potential inhibitors, we have obtained new insights into the distinct signaling pathways of neuronal apoptosis associated with HAD, which are activated by different chemokine receptor ligands that are either agonists or antagonists. This study also demonstrated a strategy for using chemically engineered inhibitors of chemokine receptors to study the signaling mechanism and intervention strategy of neuronal cell death and survival. A similar strategy may find its application in the study of other neurodegenerative diseases, as chemokine pathways to neuronal protection or damage may, at least in part, be common to other central nervous system (CNS) disorders including stroke, spinal cord injury and Alzheimer's disease.

More recently, we tested the functionality of novel 'dual-moiety' CXCR4 chemokine agonists in human neural stem cells (hNSCs) that express CXCR4 (unpublished data). Recent advances in regenerative medicine unveiled the importance of chemotaxis during the engagement of neural stem cells toward the areas of neurodegeneration. Exposure of hNSCs to SDF-1α and subsequent induction of CXCR4-mediated signaling triggers a series of intracellular processes, which lead to survival, proliferation and, most importantly, migration of neural stem cells for injury repair. A panel of 'dual-moiety' chemokine analogs were designed by linking a highly potent and selective CXCR4 binding moiety, DV1 (D-[1–21]-vMIP-II), to a critical CXCR4-activating moiety, SD1 ([1–8]-SDF-1α), with various spacer sequences in between. Binding assays showed that 'dual-moiety' chemokine analogs possess potent CXCR4-selective binding affinity. More importantly, most of these 'dual-moiety' chemokine analogs significantly enhanced the calcium influx in CXCR4-expressing cells compared with the effect of SD1. Moreover, the prototypic analog, SDV1a, also chemoattracted CXCR4-expressing cells. Further investigations of SDV1a using hNSCs demonstrated its high capacity to activate CXCR4-downstream signaling events *in vitro* as well as cause extensive migration inside the transplanted healthy mouse brain. The effectiveness of mobilizing hNSCs with *de novo* designed agonists may lead to new translational therapeutics for the clinical repair of CNS injuries and other neurodegenerative conditions.

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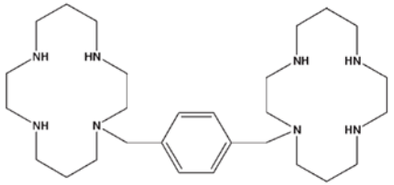
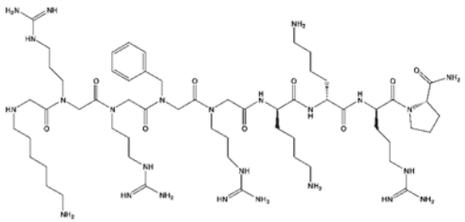
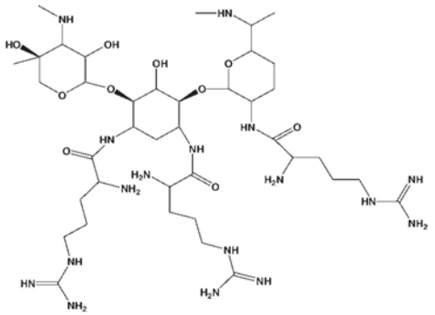
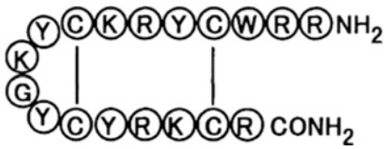
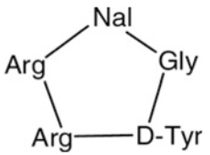
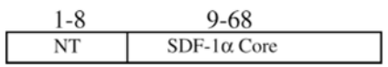
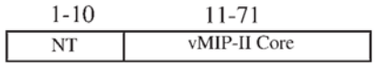
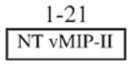
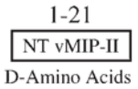
Table 1

List of CXCR4 inhibitors, their chemical structures, sequences and modifications

Inhibitors	Modification diagrams or chemical structures	Amino acid sequences*	References
ALX40-4C		AcNH-D-Arg-(D-Arg)$_8$-COOH	55,93,94
AMD3100			82
CGP64222			97
R3G			97

Inhibitors	Modification diagrams or chemical structures	Amino acid sequences*	References
T22		RRWCYRKCYKGYCYRKCR	83
T140	R-R-Na1-C-Y-R-K-D(K)- P-Y-R-Cit-C-R	R-R-Na1-C-Y-R-K-D(K)-P-Y-R-Cit-C-R	95,96
TC14012	R-R-Na1-C-Y-Cit-K-D(Cit)- P-Y-Cit-C-R	R-R-Na1-C-Y-Cit-K-D(Cit)-P-Y-R-Cit-C-R	95
FC131			96
SDF-1*α*		KPVSLSYRCPCRFFESHVARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLKWIQEYLEKALNK	1,6,7,58,84,85,91
vMIP-II		LGASWHRPDKCCLGYQKRPLPQVLLSSWYPTSQLCSKPGVIFLTKRGRQVCADKSKDWVKKLMQQLPVTAR	8,9,31
V1		LGASWHRPDKCCLGYQKRPLP	38
DV1		*LGASWHRPDKCCLGYQKRPLP*	39

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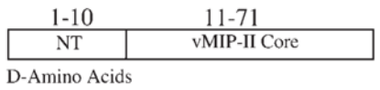
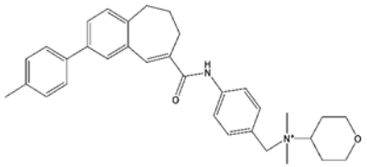
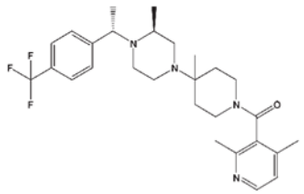
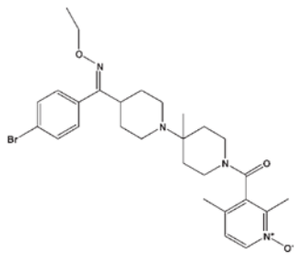
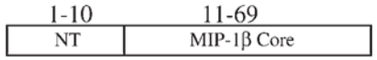
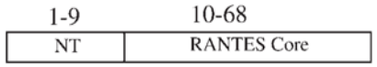
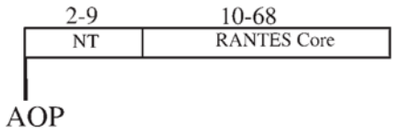
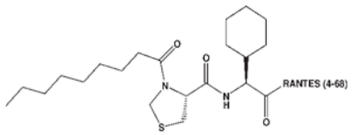
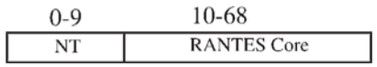
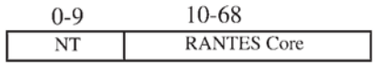
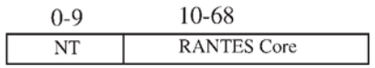
Inhibitors	Modification diagrams or chemical structures	Amino acid sequences*	References
RCP168		*LGASWHRPDK*CCLGYQKRPLPQVLLSSWYPTSQLCSKPGVIFLTKRGRQVCADKSKDWVKKLMQQLPVTAR	72

*D-Amino acids are shown in italic

Table 2

List of CCR5 inhibitors, their chemical structures, sequences and modifications

Inhibitors	Modification diagrams or chemical structures	Amino acid sequences	References
TAK-779			66,103,104
AD101			78
SCH-C			78
MIP-1β		APMGSDPPTACCFSYTARKLPRNFVVDYYETSSLCSQPAVVFGTKRSKQVCADPSESWVQEYVYDLELN	10,59
RANTES		SPYSSDTTPCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKKWVREYINSLEMS	11

Inhibitors	Modification diagrams or chemical structures	Amino acid sequences	References
AOP-RANTES		AOP-PYSSDTTPCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKKWVREYINSLEMS	101
PSC-RANTES		PSC-SSDTTPCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKKWVREYINSLEMS	106
5P12-RANTES		QGPPLMATQSCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKKWVREYINSLEMS	80
5P14-RANTES		QGPPLMSLQVCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKKWVREYINSLEMS	80
6P4-RANTES		QGPPGDIVLACCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKKWVREYINSLEMS	80

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