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Agonists for the Chemokine Receptor CXCR4

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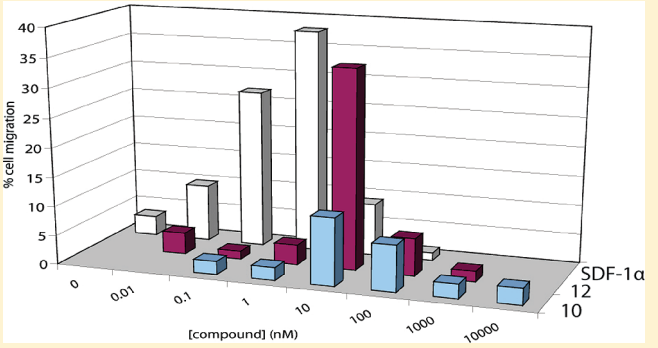
Supporting Information

ABSTRACT: The development of agonists for the chemokine receptor CXCR4 could provide promising therapeutic candidates. On the basis of previously forwarded two site model of chemokine−receptor interactions, we hypothesized that linking the agonistic N-terminus of SDF-1 to the T140 backbone would yield new high-affinity agonists of CXCR4. We developed chimeras with the agonistic SDF-1 N-terminus grafted to a T140 side chain and tested their binding affinity and chemotactic agonist activity. While chimeras with the peptide grafted onto position 12 of T140 remained high-affinity antagonists, those bearing the peptide on position 14 were in part agonists. One chimera was a full CXCR4 agonist with 25 nM affinity, and several chimeras showed low nanomolar affinities with partial agonist activity. Our results confirmed that we have developed high-affinity agonists of CXCR4.



KEYWORDS: CXCR4, agonist, CXCL12, T140, chemotaxis

The chemokine receptor CXCR4 is a prominent member of the rhodopsin-like G-protein-coupled receptor (GPCR) family. It is also the first peptidergic GPCR whose structure has been determined by X-ray crystallography.[1] CXCR4 recognizes and is activated by the chemokine SDF-1, also called CXCL12. CXCR4 is an important target for synthetic ligand development, and many peptide and nonpeptide ligands have been developed.[2−9] However, for CXCR4, all of these ligands are antagonists, or inverse agonists, like the cyclopeptide T140 and its analogues.[10] T140 is a potent CXCR4 inverse agonist derived from the horseshoe crab peptide polyphemusin. Besides N-terminal peptide fragments of SDF-1α having low affinities[11] or cropped versions of SDF-1α,[12,13] no synthetic high-affinity agonists are available.

The CXCR4/SDF-1 axis is a main player in hematopoietic stem cell (HSC) homing to bone marrow[14] and also directs metastatic dissemination of epithelial cancers to this tissue.[15] In both cases, SDF-1 provides directional cues for migration of motile cells into the bone marrow niche, as well as for their retention there. Consequently, blockade of the CXCR4/SDF-1 axis by synthetic CXCR4 antagonists has become a major strategy to prevent metastatic dissemination.[16] However, one drawback of the long-term use of CXCR4 antagonists that became already apparent in initial clinical trial assessing the antiretroviral activity of AMD3100 (a small molecule CXCR4 antagonist) is the washout of HSC from their bone marrow niches.[17] As a consequence, CXCR4/SDF-1 short-term inhibition is now used for the mobilization of HSC to the periphery to gain easier access to HSC grafts.[18] Finally, mobilization of metastasized cancer cells from bone marrow niches during chemotherapy is believed to remove these cells from their protective microenvironment, an approach currently under clinical evaluation.[19]

Recent data suggest that systemic application of CXCR4 agonists, rather than antagonists, might represent a viable alternative to CXCR4/SDF-1 inhibition.[20] In line with the rationale that CXCR4 agonism is beneficial in the cancer setting, cancer cells have been shown to silence SDF-1 expression, and forced re-expression of SDF-1 reduced metastasis dissemination.[21,22] The mechanistic basis for this might be either blurring of SDF-1 gradients required to provide directional information or inducing CXCR4 downregulation from the cell surface by receptor internalization.[12]

Here, we set out to design potent synthetic CXCR4 agonists. Our strategy was based on photolabeling experiments with T140 photoanalogs and the resulting in silico docking studies.[23] That work showed several possible binding modes, in some of which the side chains of residues 12 and 14 of T140 were directed to the transmembrane bundle of CXCR4. We therefore hypothesized that the graft of low-affinity CXCR4 agonist peptides derived from the N-terminal sequence of SDF-1 on the high-affinity scaffold T140 would confer agonist properties to the combined high-affinity chimeric molecules. We here show that depending on the T140 residues chosen to graft the SDF-1 N-terminal peptides, the resulting peptides were indeed highly potent

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Table 1. Sequences and Affinities of T140-SDF-1α Chimeras Expressed as IC_{50}

No	Compound	Sequence	IC50 (nM)	
	SDF-1α	H-KPVSLSYRCPCRFFESHVARANVKHLKILNTPNC-ALQIVARLKNNNRQVCIDPKLKWIQEYLEKALNK-OH	0.08± 0.07	N=11
	T140	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH (S-S)	1.56±0.71	N=4
1	T140-Lys[12]-ε[SDF(1-7)]	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Lys-Cys-Arg-OH (S-S); H-Lys-Pro-Val-Ser-Leu-Ser-Tyr	2.22±1.75	N=3
2	T140-Lys[12]-ε[SDF(1-8)]	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Lys-Cys-Arg-OH (S-S); H-Lys-Pro-Val-Ser-Leu-Ser-Tyr-Arg	1.36±0,80	N=3
3	T140-Lys[14]-ε[SDF(1-6)]	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Lys-OH (S-S); H-Lys-Pro-Val-Ser-Leu-Ser	62.02±12.18	N=3
4	T140-Lys[14]-ε[SDF(1-7)]	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Lys-OH (S-S); H-Lys-Pro-Val-Ser-Leu-Ser-Tyr	170.0±57.41	N=3
5	T140-Lys[14]-ε[SDF(1-8)]	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Lys-OH (S-S); H-Lys-Pro-Val-Ser-Leu-Ser-Tyr-Arg	21.67±7.20	N=3
6	T140-Lys[14]-ε[SDF-Ser[9](1-9)]	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Lys-OH (S-S); H-Lys-Pro-Val-Ser-Leu-Ser-Tyr-Arg-Ser	21.23±4.00	N=3
7	T140-Arg[12], Lys[14]-ε[SDF(1-6)]	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Arg-Cys-Lys-OH (S-S); H-Lys-Pro-Val-Ser-Leu-Ser	28.33±10.9	N=3
8	T140-Arg[12], Lys[14]-ε[SDF(1-7)]	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Arg-Cys-Lys-OH (S-S); H-Lys-Pro-Val-Ser-Leu-Ser-Tyr	38.61±10.01	N=3
9	T140-Arg[12], Lys[14]-ε[SDF(1-8)]	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Arg-Cys-Lys-OH (S-S); H-Lys-Pro-Val-Ser-Leu-Ser-Tyr-Arg	16.51±4.08	N=3
10	T140-Arg[12], Lys[14]-ε[SDF-Ser[9](1-9)]	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Arg-Cys-Lys-OH (S-S); H-Lys-Pro-Val-Ser-Leu-Ser-Tyr-Arg-Ser	5.67±0.24	N=3
11	T140-Arg[12], Lys[14]-ε[SDF-Ser[9](1-10)]	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Arg-Cys-Lys-OH (S-S); H-Lys-Pro-Val-Ser-Leu-Ser-Tyr-Arg-Ser-Pro	9.57±4.76	N=3
12	T140-Arg[12],Lys[14]-ε[SDF-Ser[9],Ala[10](1-10)]	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Arg-Cys-Lys-OH (S-S); H-Lys-Pro-Val-Ser-Leu-Ser-Tyr-Arg-Ser-Ala	24.92±5.20	N=3

CXCR4 agonists that efficiently induce CXCR4-dependent chemotaxis.

Two series of T140-SDF-1α chimeras were synthesized (Table 1). The first series has the N-terminal portion of SDF-1α (chain length 7 or 8 residues) coupled to position 12 of T140 (T140(Lys[12]-ε[SDF(1−7)]) (**1**) and T140(Lys[12]-ε[SDF(1−8)]) (**2**)). The second series has the N-terminal of SDF-1α (chain length 6−10 residues) coupled to position 14 of T140 (T140(Lys[14]-ε[SDF(1−6)]) (**3**), T140(Lys[14]-ε[SDF(1−7)]) (**4**), T140(Lys[14]-ε[SDF(1−8)]) (**5**), and T140(Lys[14]-ε[SDF(1−8, Ser[9])] (**6**). The coupling acceptor residue on position 12 (Cit) or 14 (Arg) was replaced by a lysine. A similar series bearing the peptide graft on position 14, but with an additional citrulline to arginine substitution on position 12 to compensate for the loss of charge brought about by the modification on position 14, was also synthesized (T140(Arg[12], Lys[14]-ε[SDF(1−6)]) (**7**), T140(Arg[12], Lys[14]-ε[SDF(1−7)]) (**8**), T140(Arg[12], Lys[14]-ε[SDF(1−8)]) (**9**), T140(Arg[12], Lys[14]-ε[SDF(1−8, Ser[9])] (**10**), T140(Arg[12],

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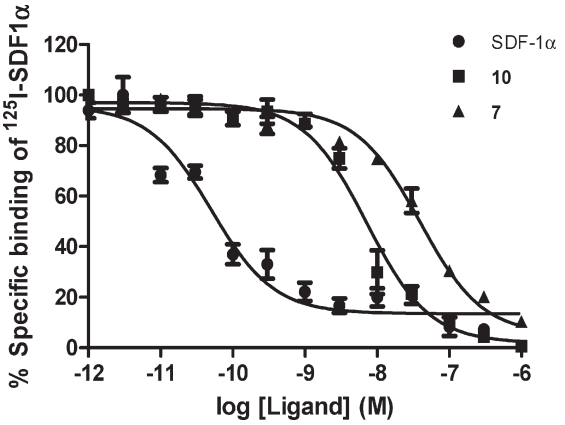

Figure 1. Competition binding assay with ^{125}I-SDF-1α of analogues 7 and **10** containing varying SDF-1α chain lengths on HEK293 cells stably expressing human CXCR4 were carried out as described in the Experimental Procedures. IC_{50} values were determined using GraphPad Prism 5 for Windows. These results are representative of at least three separate experiments.

Lys^{14}-ε[SDF(1−8, Ser^{9}, Pro^{10})] (**11**), and T140(Arg^{12}, Lys^{14}-ε[SDF(1−8, Ser^{9}, Ala^{10})] (**12**)). For all compounds with a SDF-1α side chain ranging from 9 to 10 amino acids (**6** and **10**−**12**), the cysteine on position 9 of SDF-1α was substituted by an isosteric amino acid, serine. Finally, a variation of **11** was synthesized with an alanine replacing the proline at position 10 of SDF-1 (**12**) to add flexibility to the peptide.

To determine the affinity of the T140-SDF-1α chimeras, we performed radioligand competition binding assays using ^{125}I-SDF-1α as a tracer on HEK293 cells stably expressing human CXCR4. Homologous binding using SDF-1α was assessed using a one-site binding model and exhibited an affinity of 0.08 ± 0.07 nM, which corresponds to the high-affinity site of SDF-1α[24] (Figure 1). Compounds **1** and **2**, both with the SDF-1α chain situated on position 12 of T140, showed affinities of 1.36 ± 0.8 and 2.22 ± 1.75 nM, respectively.

We next evaluated the chimeras with their SDF-1α chain on position 14 and citrulline at position 12 (like in T140). The affinities for these compounds beginning with the shortest SDF-1α side chain were 62.02 ± 12.18 (**3**), 170.0 ± 57.41 nM (**4**), 21.67 ± 7.2 nM (**5**), and 21.23 ± 4.00 nM (**6**) for the longest derivative.

For the second series of chimeras with the SDF-1α chain on position 14 and the additional citruline to arginine substitution at position 12, compound **7**, with the shortest SDF-1α side chain of the series, showed an affinity of 28.33 ± 10.9 nM. Compounds **8** and **9** showed affinities of 38.61 ± 10.01 and 16.51 ± 4.08 nM, respectively. Thus, arginine on position 12 seemed to provide superior affinity than citrulline: for instance, **10** (with Arg12) (5.67 ± 0.24 nM) versus **5** (with Cit12) (21.23 ± 4.00 nM). Analogue **11**, with the longest SDF-1 peptide of this series, presented an affinity of 9.57 ± 4.76 nM, thus an almost 3-fold increase as compared to **12**, at 24.92 ± 5.20 nM. Analogue **12** is identical to **11** except for a Pro to Ala substitution at position 10 of the grafted SDF-1 peptide. This modification of the original SDF-1 N-terminal sequence, besides a necessary Cys to Ser switch at position 9, seems to be responsible for the observed loss of affinity. The restoration of the charge profile of T140 by introducing Arg on position 12 instead of isosteric citrulline to compensate for the loss of a positive charge at position 14 (acylated Lys instead of Arg) appears to provide a favorable interaction with CXCR4. The length of the grafted peptide seems to have an influence on affinity as well, with 8−10 amino acids being optimal.

To assess the functional characteristics of the compounds, chemotaxis assays were performed using REH cells. As expected, SDF-1α induced migration following a classical bell-shaped dose−response from as low as 0.01 nM, with a peak at 1 nM followed by a rapid decrease at higher chemokine concentrations (Figure 2). Compounds **1** and **2** induced no migration of REH cells but acted as antagonists toward SDF-1-induced chemotaxis (Supporting Information, Figure S1). Also, some of the T140-SDF-1α chimeras with the side chain on residue 14 of T140, **3**, **6**, **7**, and **9** did not induce significant chemotactic activity in comparison to the control (migration medium only). Peptide **5**, with citrulline preserved in position 12 of T140, appears to be a low potency agonist with a peak at 100 nM. Compounds **8** and **10** acted as agonists with reduced efficacy, inducing weaker migration than SDF-1α, with peaks at 10 nM, only one log above the natural chemokine agonist SDF-1. Compound **11** is a low efficacy agonist with maximum induced migration at 10−100 nM. Compounds **12** and **4** showed similar efficacy as SDF-1, with peaks at 10 nM and 1 μM, respectively, analogue **4** thus being of very low potency. While the compound with a proline, **11**, showed better affinity, the proline to alanine exchange at position 10 (compound **12**) of the chain, designed to provide additional flexibility to the grafted peptide, seemed thus to improve efficacy in chemotaxis. There is no correlation between efficacy and affinity of the compounds **4**−**12**.

We have successfully synthesized CXCR4 agonists by combining an inverse agonist (T140) scaffold with the N-terminal peptide of SDF-1, a low-affinity agonist. Two analogue series, with this N-terminal peptide of SDF-1 grafted as a side chain at two positions of T140, namely, positions 12 and 14, were prepared with a peptide graft of varying chain length. The pharmacological properties of these analogue series were then evaluated. From the structure−affinity relationship, it appears that positioning of the N-terminal chain of SDF-1α on the twelfth amino acid of T140 conserves high affinity for CXCR4, but the resulting chimeras remain antagonists. However, attachment of SDF-1α N-terminal peptides to the side chain of position 14 of T140 appeared to confer agonist properties to the analogues. Full chemotactic efficacy and improved affinities were obtained using position 14 of T140 for the peptide graft and simultaneously substituting Cit to Arg at position 12 of the T140 template. Analogue **4**, with a 7 residue peptide graft, is a full agonist of chemotaxis, but because of its low affinity (IC_{50} = 170.0 nM), it is not very potent (peak at 1 μM). Analogue **12**, with a 10 residue peptide graft, is also a full agonist with much better potency (chemotaxis peak at 10 nM). The Pro-Ala exchange in position 10 of the grafted SDF-1α peptide in compound **12** lowered affinity but induced full agonist properties when compared to its counterpart **11** with the native SDF-1α (Ser^{9}) 1−10 sequence, suggesting increased peptide flexibility being required for better efficacy. Future structure−function studies will be needed to clarify this and to further improve those chimeric chemokine agonists. A model rationalizing the different results obtained as a function of the graft acceptor position of T140 on the CXCR4 is shown in Figure S2 in the Supporting Information.

In conclusion, we have synthesized two full CXCR4 agonists with chemotactic potencies only one log unit less that the endogenous CXCR4 ligand, SDF-1. We also created several

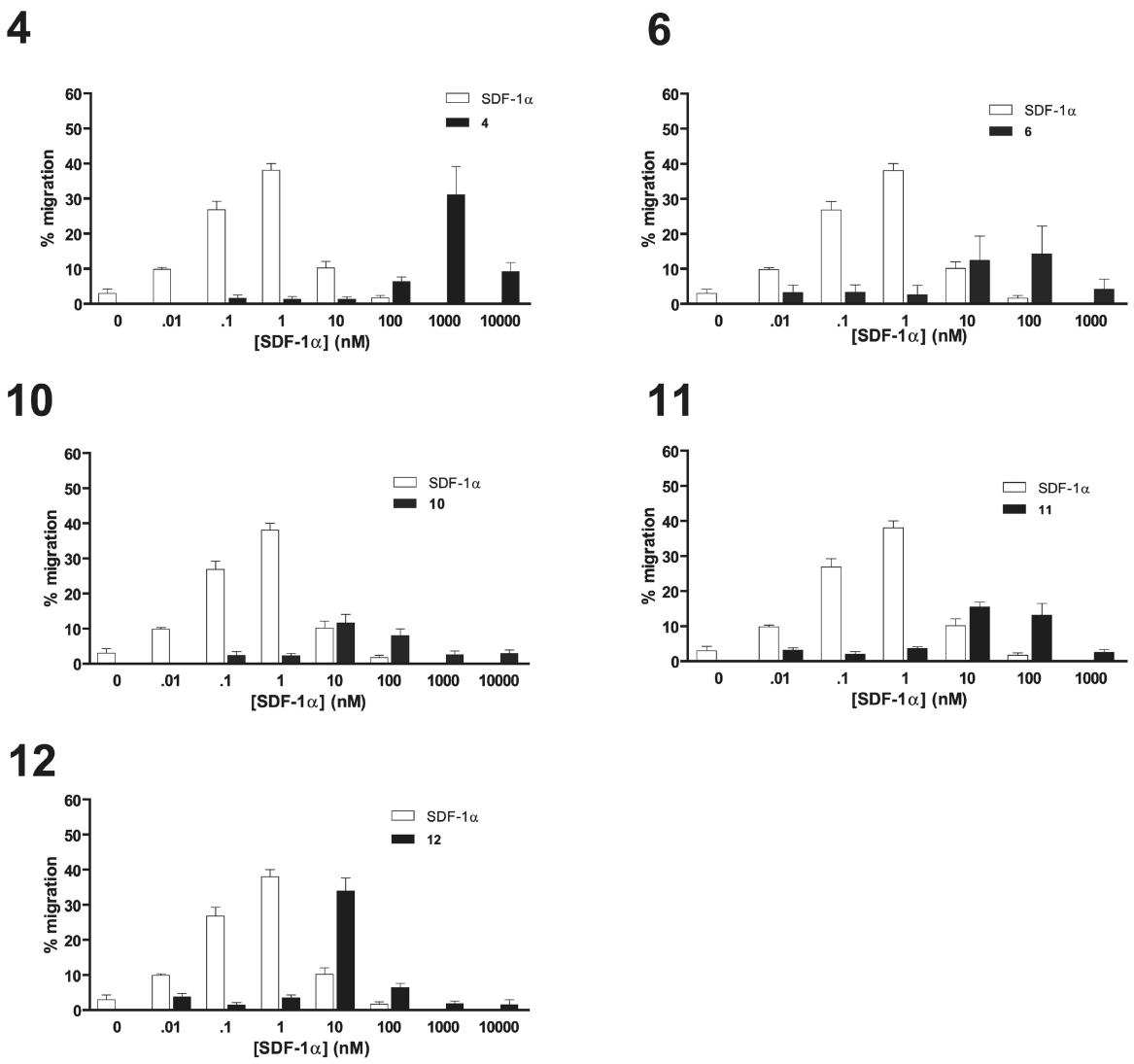

Figure 2. Compounds **4**, **6**, and **10**−**12**: Transwell migration assay where chemotaxis is expressed as % of REH cells initially seeded onto the Neuroprobe ChemoTx plate that did migrate to the bottom chamber. Experiments were carried out in triplicate, as described in the Experimental Procedures. Data were plotted using GraphPad Prism 5 for Windows. These results are mean values of at least three independent experiments.

partial agonists. Our work was based on the premises that the inverse agonist cyclopeptide T140 will provide binding affinity, while the grafted N-terminus chain will provide agonist properties, in line with the two site model suggested for SDF-1-CXCR4 interaction.[25] These compounds are promising lead compounds for the future design of CXCR4 agonists.

EXPERIMENTAL PROCEDURES

Polyethylenimine and puromycin were from Sigma (Oakville, ON, Canada), ^{125}I-SDF-1α (2200 Ci/mmol) were from PerkinElmer Life and Analytical Sciences (Woodbridge, ON, Canada). DMEM (Dulbecco's modified Eagle's medium), FBS (fetal bovine serum), and penicillin/streptomycin were obtained from Gibco Life Technologies (Gaithersburg, MD). Human SDF-1α was from PeproTech (Rocky Hills, NJ).

T140 synthesis was done as previously described.[23] Peptides synthesis with arginine in position 14 was made on automatic synthesizer using Fmoc strategies, starting with FmocArg(Pbf)NovasynTGA subst (0.22 meq/g) from Novabiochem. The synthesis of T140 was followed by deprotection of lysine's side chain to continue synthesis with SDF-1 analogues. The peptides with lysine in position 14 were made manually using Fmoc strategies on Wang resin. Peptides were cleaved with 95% TFA adding EDT and TLS as scavengers. The crude peptides were then dissolved and agitated in 2 M $(NH_4)_2CO_3$, pH 6.5, for 4 h at room temperature for cyclization. Purifications were made on C_{18} column using a 10−35% gradient of acetonitrile with 0.05% TFA. The pure peptides were characterized on mass spectra (MALDI-ToF). Lyophilized peptides were stored at −20 °C and subsequently dissolved in distilled water prior to use.

HEK293 cells were grown in DMEM containing 10% (v/v) FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C. HEK293 stably expressing human CXCR4 were grown using puromycin (3 μg/mL) as a selection agent. Confluent cells (95%) in 100 mm diameter Petri dishes were used for binding assays. REH cells were grown in RPMI 1640 medium 10% (v/v) FBS, supplemented with 2 mM L-glutamine in 50 mL cell culture flasks.

Cell membrane preparation and binding assays were performed as described previously[17] with minor modifications. Briefly, HEK293 cells stably expressing human CXCR4 were washed once with PBS and subjected to one freeze−thaw cycle. Broken cells were then gently scraped in resuspension buffer (50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, and

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5 mM $MgCl_2$), centrifuged at 3500 g for 15 min at 4 °C, and resuspended in binding buffer (50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 140 mM NaCl, and 0.1% BSA). For competition binding assays, broken cells (1 μg of protein) were incubated for 1 h at room temperature in binding buffer with 0.03 nM [^{125}I]-SDF-1α as a tracer and increasing concentrations of competitor in a final volume of 0.5 mL. Bound radioactivity was separated from free ligand by filtration, and receptor-bound radioactivity was quantified by γ-radiation counting. Radioligand binding assays were collected in triplicate and are presented as means ± SEMs. Binding curves were fitted using a one site model, and IC_{50} values were determined using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA) using nonlinear regression.

REH (B-ALL, acute lymphoblastic B-cell leukemia) cells were washed using RPMI medium, and 25000 cells per well were seeded in 96-well migration assay plates with 5 μm pores (Neuroprobe ChemoTx system) containing RPMI medium with 0.2% BSA. Dilutions of the various peptides in RPMI medium (0.2% BSA) were added to the bottom chamber in a final volume of 29 μL. When assessing antagonist activity, 1 nM SDF-1α was placed in the bottom chamber, and antagonist at identical concentrations (100 nM) was placed in both the upper and the lower chamber. Cells were allowed to migrate for 3 h at 37 °C, 5% CO_2. After incubation, the porous membrane was removed, and the migrated cells located in each bottom chamber were subsequently counted using a BIORAD T10 automated cell counter. Migration experiments were repeated three times in triplicate, and data were plotted using GraphPad Prism version 5.00.

■ ASSOCIATED CONTENT

Supporting Information. Migration assay results for the antagonistic compounds **1** and **2** and a binding model of the chimeras to CXCR4. Mass spectrometry information for the peptides **1**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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