

Pharmacological Properties of Peptides Derived from Stromal Cell-Derived Factor 1: Study on Human Polymorphonuclear Cells

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ABSTRACT

Small compounds capable of blocking the stromal cell-derived factor 1 (SDF-1) receptor CXCR4 may be potentially useful as anti-inflammatory, antiallergic, immunomodulatory, and anti-human immunodeficiency virus (HIV) agents. SDF-1-derived peptides have proven to target CXCR4 efficiently despite a 100-fold lower affinity (or more) than SDF-1. Here we studied the binding and antiviral properties of a series of substituted SDF-1-derived N-terminal peptides and tested their functional effects on human polymorphonuclear cells, because these cells are very reactive to chemokines and chemoattractants. All peptides bound to CXCR4 and inhibited HIV entry in a functional assay on CD4⁺ HeLa cells. A 10-residue substituted dimer, derived from the 5–14 sequence of SDF-1, displayed the high-

est affinity for CXCR4 (K_i value of 290 nM, a reduction of only 15-fold compared with SDF-1) and was also the best competitor for HIV entry (IC_{50} value of 130 nM). Whereas most peptides displayed CXCR4-independent functional effects on human polymorphonuclear cells, including the modulation of calcium fluxes and the activation of superoxide anion production at high concentration (10 μ M), the peptide dimer was devoid of these nonspecific effects at antiviral concentrations. Overall, this study shows that appropriate modifications of SDF-1-derived N-terminal peptides may ameliorate their binding and viral blocking properties without generating significant unspecific side effects.

Stromal cell-derived factor 1 (SDF-1), a member of the CXC chemokine family, is a potent chemoattractant for hematopoietic cells, including bone marrow progenitors (Aiuti et al., 1997), lymphocytes (Bleul et al., 1996b), monocytes, and polymorphonuclear cells (Bleul et al., 1996a). SDF-1 also stimulates proliferation of B-cell progenitors in vitro (Nagasawa et al., 1994), and SDF-1 deficient mice displayed profound defects in B-cell lymphopoiesis and myelopoiesis (Nagasawa et al., 1996). SDF-1 is thus likely to attract hematopoietic cells in appropriate microenvironments in which they differentiate or proliferate in response to local stimuli. SDF-1 is the ligand for CXCR4, a G protein-coupled receptor that is expressed not only in hematopoietic cells but also in a large variety of tissues, such as brain, microglia (Lavi et al., 1997),

and endothelia (Gupta et al., 1998). Accordingly, CXCR4-deficient mice exhibited cardiac defects, abnormal cerebellar development, and anatomical changes of gastrointestinal tract vascularization (Tachibana et al., 1998; Zou et al., 1998). CXCR4 was found to function as a coreceptor for the entry of T-tropic strains of human immunodeficiency virus (HIV) in CD4⁺ T lymphocytes (Feng et al., 1996) and SDF-1 specifically blocks HIV infection through CXCR4 (Bleul et al., 1996a; Oberlin et al., 1996).

Small compounds capable of blocking CXCR4 may be potentially useful as anti-inflammatory, antiallergic, immunomodulatory, and antiviral agents. Different classes of compounds have been identified so far that antagonize HIV binding to CXCR4 and/or CXCR4 signaling by interacting with the receptor. These include heterocyclic compounds called bicyclams (Schols et al., 1997); T22, an 18-amino-acid residue peptide derived from polyphemusin II (Murakami et al., 1997); and ALX-40-4C, a highly cationic oligopeptide containing nine arginines (Doranz et al., 1997) and SDF-1-

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ABBREVIATIONS: SDF-1, stromal cell-derived factor 1; HIV, human immunodeficiency virus; PMN, neutrophil polymorphonuclear cells; HBSS, phenol red-free Hanks' balanced salt solution containing sodium hydrogen carbonate and calcium and magnesium salts; IL-8, interleukin-8; Gro α , growth-related oncogene- α chemokine; AM, acetoxymethylester; PTX, pertussis toxin; C5a, complement fraction C5-a; fMLP, formyl-Met-Leu-Phe peptide.

derived peptides (Heveker et al., 1998; Loetscher et al., 1998; Luo et al., 1999). Whereas SDF-1-derived peptides have proven to efficiently block HIV infection and/or to antagonize CXCR4 in functional assays, the peptides studied so far displayed a relative low affinity for the receptor.

In the present study, we have investigated whether amino acid substitutions may increase binding and antiviral properties of SDF-1 derived peptides. In addition, to characterize the functional properties and potential side effects of these compounds in a sensitive human cellular model, pharmacological studies were conducted on human polymorphonuclear cells (PMN). Among the tested peptides, we identified a substituted 10-amino-acid residue dimer that displayed a higher binding affinity for CXCR4 than did other peptides reported so far (15-fold reduction compared with SDF-1) and a comparable increase in antiviral activity. In contrast with other peptides, this dimer did not show major side effects on human PMN.

Materials and Methods

SDF-1 Derived Peptides. Peptides were prepared on a multiple peptide synthesizer (Abimed, Langenfeld, Germany) according to the standard 9-fluorenylmethoxycarbonyl protection protocols. These peptides were purified to greater than 95% and characterized by matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry. Peptides containing one cysteine residue were oxidized/dimerized as described previously (Volkmer-Engert et al., 1998). Dimeric peptides were purified and separated from reduced species by preparative high-performance liquid chromatography. For dose-response experiments, peptide stock solutions (2 mM) were prepared in water and diluted into complete Dulbecco's modified Eagle's medium to the final concentration as indicated.

Competition Binding Assays. Binding studies were carried on CEM T-cells, which were found to give reliable saturable SDF-1 binding in previous studies (Crump et al., 1997). Aliquots of 2×10^6 CEM cells were incubated for 4 h at 4°C (a temperature known to inhibit endocytosis) in 0.5 ml of RPMI medium containing 20 mM HEPES and 1% bovine serum albumin in the presence of 0.2 nM 125 I-SDF-1 (PerkinElmer Life Sciences) and various concentrations of competitors. Bound and free 125 I-SDF-1 were separated by centrifugation through a 12% sucrose cushion in the same buffer. Non-specific binding was measured in the presence of a 500 nM unlabeled SDF-1. Data were analyzed and affinity constants calculated using the Prism software (ver. 2; GraphPad, San Diego, CA). IC_{50} values were calculated from the inhibition curve with the use of the equation $y = ns + (B_o - ns) / 1 + 10^{x - \text{Log } IC_{50}}$, where B_o corresponds to maximal total binding in the absence of competitor and ns to non specific binding. K_i values were calculated from IC_{50} values using the equation of Cheng and Prusoff (1973).

Antiviral Activity of SDF-1 Derived Peptides in Vitro. HIV-1 infectivity was scored by using the CD4⁺ HeLa P4.2 cell line (Clavel and Charneau, 1994), which is stably transfected with a *lacZ* reporter gene, inducible by the viral protein Tat. Viral stocks were from chronically infected CEM cells with infectious titers in the range of 10^5 to 10^6 U/ml. The cells were infected in microtiter plates as described previously (Heveker et al., 1998) in the absence or presence of various concentrations of SDF-derived peptides. After 24-h infection, cells were lysed in 50 μ l of buffer containing 1% Nonidet P-40; finally, 50 μ l was added containing 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 50 mM β -mercaptoethanol, 80 mM sodium phosphate, pH 7.4, 10 mM MgCl₂, and 6 mM chlorophenol red β -galactopyranoside monosodium salt. The absorbance was measured at 575 nm.

PMN Collection. Venous blood samples were collected from healthy donors onto heparin (10 U/ml; Choay, Gentilly, France) and mixed with an equal volume of 2% dextran T 500 (Pharmacia, Bois

d'Arcy, France) for 45 min at room temperature. Cell suspension was carefully layered over a Lymphoprep gradient (density 1.077 g/ml; Flobio, Courbevoie, France) and centrifuged for 20 min at 1500 rpm at room temperature. The gradient fraction containing PMN was collected. After hypotonic lysis of contaminating erythrocytes, purified PMN were rinsed twice in phenol red-free Hanks' balanced salt solution, containing 4 mM NaHCO₃, 1.25 mM CaCl₂ and 0.75 mM MgSO₄ (HBSS; Sigma, St. Louis, MO), and resuspended in HBSS at a final concentration of 5×10^6 PMN/ml for chemotaxis assays or 1×10^7 PMN/ml for studies on superoxide anion production.

Calcium Mobilization Studies. C5-a complement fraction (C5-a) and formyl-Met-Leu-Phe peptide (fMLP) were from Sigma, whereas interleukin-8 (IL-8), the growth-related oncogene alpha chemokine (Gro α), and SDF-1 were purchased from R&D Systems (Oxon, UK). Changes in cytosolic free calcium concentration were measured in PMN loaded with 1 μ M Fura 2-acetoxymethylester (AM) in phenol red-, calcium-, and magnesium-free Hanks' balanced salt solution, containing sodium hydrogen carbonate and 20 mM HEPES at 37°C for 1 h (Pozzan et al., 1983; Nasmith and Grinstein, 1987). Cells were washed and resuspended in a 20 mM HEPES-buffered HBSS. In some studies, pertussis toxin (PTX, 1 μ g/ml; Sigma) or the anti-CXCR4 12G5 antibody (6 μ g/ml) were preincubated with cells at 37°C for 3 h and 30 min, respectively, before the addition of peptides. Fura 2-AM fluorescence assays were performed with aliquots of 5×10^6 PMN in 2 ml of 20 mM HEPES-buffered HBSS, using a fluorometer equipped with a thermally controlled cuvette holder and a magnetic stirrer (Jobin Yvon 3D, Longjumeau, France). Excitation and emission wavelengths for Fura 2-AM were set at 340 and 510 nm, respectively. After each series of stimulation with peptides or chemoattractants, the maximal fluorescence (F_{max}) was measured by adding 0.1% Triton X-100 (final concentration) and minimal background fluorescence (F_{min}) was determined immediately after by adding 25 mM EGTA (final concentration). The actual free Ca²⁺ concentration was calculated with the following equation: $[Ca^{2+}]_i$, nM = $224 (F - F_{\text{min}}) / (F_{\text{max}} - F)$, where F is the measured fluorescence, 224 nM the dissociation constant for Fura 2-AM and F_{max} and F_{min} are as defined above. Tracings were reproduced and scanned using an Agfa Snapscan scanner (Agfa-Gevaert S.A., Rueil Malmaison, France). Tracings correspond to experiments performed on cells from one donor and are representative of experiments on at least three different donors.

Chemotaxis Assays. A modified version (Keller et al., 1979) of the original Boyden chamber technique (Boyden, 1962) was used for chemotaxis studies. Briefly, 0.1 ml of cell suspension in HBSS, supplemented with 1% bovine serum albumin, was added to the upper compartment of the chamber and 0.2 ml of the same buffer containing appropriate chemotactic compounds was added to the lower compartment. A cellulose filter with pores of 3 μ m in diameter (Millipore, Bedford, MA) was placed between the two compartments. The chambers were incubated for 90 min at 37°C. Migration was stopped with ethanol and filters were stained with hemalum. Cell migration was determined by means of the "leading front technique" (Zigmond and Hirsch, 1973); five high-power fields were analyzed for each filter.

Superoxide Anion Generation. Superoxide anion generation was measured by the reduction of ferricytochrome C (horse heart type III) as described previously (Johnston et al., 1975). PMN (10^6 cells/ml) and 75 μ l of ferricytochrome C (5 mg/ml) were incubated with or without peptides for 10 min at 37°C. The final volume of the reaction mixture was adjusted to 500 μ l with 10 mM phosphate-buffered saline, pH 7.4. Incubation was stopped by placing the tubes in an ice-water bath. Cells were centrifuged at 1200 rpm for 10 min. The absorbance of the supernatants was read at 550 nm in a spectrophotometer (DU 40; Beckman Coulter, Fullerton, CA) and the results were expressed in nanomoles of released O⁻ per minute per 10^6 cells.

Fig. 2. Receptor binding of SDF-1 peptides. Competition for specific binding of ^{125}I -SDF-1 to CEM by indicated peptides or unlabeled SDF-1. Bars indicate S.D. of two to four experiments in triplicate. ■, SDF-1; △, S2; ▼, L5H; ◇, 1-13; □, S1D; ◆, S1.

was shown to behave like a CXCR4 agonist, capable of promoting intracellular Ca^{2+} rise (Heveker et al., 1998). However, in the same study, the "S" peptide did not induce any Ca^{2+} signal up to 50 μM , contrasting with its significant effect observed here on PMN, at a concentration of 100 nM. In addition, "S1" and "S2" peptides, which displayed close antiviral properties and/or binding affinities for CXCR4, elicited very different Ca^{2+} signals. A potential explanation for these discrepancies could be that some SDF-1-derived peptides may signal through another receptor in human PMN. To test this hypothesis, cells were preincubated with saturating concentrations of the 12G5 anti-CXCR4 antibody before the addition of 1 μM peptide or 10 nM SDF-1 (Table 2). The antibody blocked the SDF-1-promoted rise of intracellular Ca^{2+} in PMN but had no effect on Ca^{2+} fluxes induced by the "S" lead and derived peptides. These data indicate that the Ca^{2+} signal promoted in PMN by these peptides was mediated by a different receptor than CXCR4. The observation that preincubation with pertussis toxin completely blocked the intracellular Ca^{2+} rise elicited by "S", "S1", and "S1D" peptides is consistent with the involvement of another receptor coupled to heterotrimeric G proteins of the $\text{G}_i\text{-G}_o$ group.

None of the peptides could significantly modify Ca^{2+} responses promoted by fMLP (Table 2), whereas sequential treatments with SDF-1 and peptides (10 μM), or vice

versa, produced different effects. A first stimulation with 1 nM SDF-1 caused homologous desensitization of the CXCR4-dependent Ca^{2+} signaling pathway in PMN; desensitization resulted in a major inhibition of the Ca^{2+} response elicited by a subsequent stimulation of PMN with 10 nM SDF-1 (Fig. 4). When PMN were first stimulated with SDF-1 and then submitted to a second stimulus by "S", "S1", and "S1D" peptides, at a concentration of 10 μM , desensitization was not observed (Fig. 4). In the reverse experiments, 10 μM "S", "S1", and "S2" peptides had no effect on the subsequent stimulation by 1 nM SDF-1 (Table 2 and Fig. 4). These data are consistent with the hypothesis above that the Ca^{2+} flux elicited by "S", "S1", and "S2" peptides involves a CXCR4-independent pathway. The "1-13" and "S1D" peptides caused inhibitions of $\approx 50\%$ and almost 100% inhibition, respectively, of the Ca^{2+} flux promoted by a following stimulation of PMN with SDF-1 (Table 2 and Fig. 4). In the case of the "1-13" peptide, the inhibition of the subsequent SDF-1 signal is attributable to CXCR4 desensitization because the phenomenon was observed even when the peptide was washed off the cells before PMN stimulation with SDF-1. In contrast, the inhibition caused by preincubation with the "S1D" peptide was observed only when the peptide was present at saturating concentration in the reaction medium, indicating that "S1D" is a CXCR4 antagonist.

Functional Effects of Peptides on Polymorphonuclear cells. Chemokines are known to govern important physiological functions of PMN, such as chemotaxis, epithelial adhesion, superoxide production, and granule release (Bokoch, 1995).

In agreement with previous reports indicating that SDF-1 is chemotactic for most hematopoietic cells, SDF-1 induced a bell-shaped chemotactic response of PMN, maximal at 10 nM, that was substantially inhibited by the 12G5 anti-CXCR4 antibody (Fig. 5A). All peptides, including those with low intrinsic activity in Ca^{2+} assays, induced similar chemotactic bell-shaped responses (not shown). The maximal chemotactic activity of peptides was 50 to 100% of that observed for SDF-1 and was obtained for peptide concentrations between 10 and 100 nM (Fig. 5B). The 12G5 antibody, at a concentration causing a 70% inhibition of maximal SDF-1-promoted chemotaxis (Fig. 5A), failed to block chemotaxis induced by peptides. "S1" and "S1D" peptides were also tested in chemotaxis inhibition assays against fMLP, C5a, and IL-8. In these experiments, peptides were incubated with cells in the upper part of the Boyden chamber at a concentration of 1 μM , whereas chemoattractants or IL-8 were added to the lower part of the chamber. None of the tested peptides inhibited the chemotactic response promoted by fMLP, C5a, or IL-8, indicating that the peptides did not interact with fMLP, C5a, or IL-8 receptors (data not shown).

Superoxide production by polymorphonuclear cells is physiologically involved in the inflammatory response to pathogens (Babior, 1978). Inappropriate production of superoxide, on the other hand, was shown to be harmful and involved in a number of pathological conditions (Halliwell and Gutteridge, 1990). Because SDF-1-derived peptides were capable of activating intracellular Ca^{2+} release and/or chemotaxis in PMN, we tested whether peptides could also elicit $\text{O}_2^{\cdot -}$ production (Fig. 6). The positive control fMLP promoted a robust $\text{O}_2^{\cdot -}$ production at a concentration of 100 nM whereas the $\text{O}_2^{\cdot -}$ produced by polymorphonuclear cells in the presence

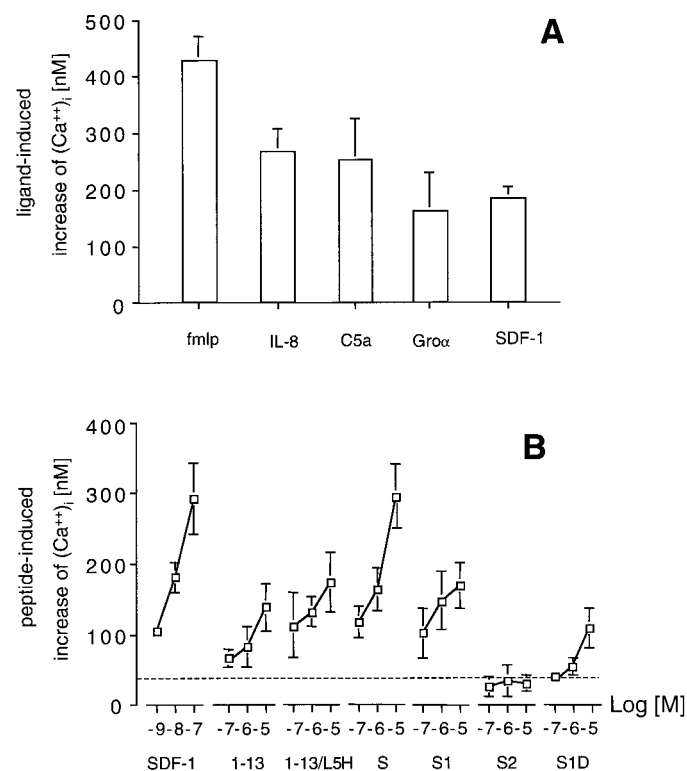


Fig. 3. Calcium signaling of chemoattractants and SDF-1-derived peptides. Polymorphonuclear cells were loaded with Fura-2, stimulated with indicated chemoattractants or SDF-1-derived peptides, and the resulting $[\text{Ca}^{2+}]_i$ -dependent fluorescence changes were recorded. A, comparison of signals promoted by 10 nM concentrations of each chemoattractant. B, increase of $[\text{Ca}^{2+}]_i$ promoted by various concentrations of SDF-1-derived peptides. Dashed line corresponds to the significance limit over background. Data correspond to experiments performed in triplicate on cells from one donor and are representative of experiments on at least three different donors. Bars represent S.D.

of SDF-1 was not different from that measured in the presence of the vehicle alone. Peptides "1-13", "1-13/L5H", "S", and "S1" stimulated superoxide production at a concentration of 10 μ M; in contrast, peptides "S2" and "S1D" did not induce any significant $O_2^{\cdot -}$ production when incubated with PMN at the same concentration (Fig. 6A). We tested the effect of the anti-CXCR-4 antibody on the $O_2^{\cdot -}$ production elicited by two of the most potent peptides and by fMLP. As expected, no inhibition could be observed (Fig. 6B).

Discussion

SDF-1 acts as a chemokine on virtually all human leukocytes and their precursors. In PMN, which represent highly susceptible targets of multiple attractants, the signals induced by chemokines, such as IL-8, or by chemoattractants, such as fMLP and C5a, usually encompass elevation of intracellular Ca^{2+} , chemotaxis, and superoxide anion production. These functions are mediated by PTX-sensitive heterotrimeric proteins of the G_i group (Bokoch, 1995).

We showed that the CXCR4 ligand, SDF-1, although promoting robust chemotaxis and PTX-sensitive intracellular Ca^{2+} rise, does not induce superoxide anion production in PMNs. The molecular mechanism underlying this phenomenon, which suggests that SDF-1 is not involved in acute PMN response but rather in control of myelopoiesis and redistribution of mature PMN within tissues (Nagasawa et al., 1996), remains unclear. It was reported that different signaling duration, which is regulated by receptor phosphorylation, may trigger different cellular responses. For example, the IL-8 receptor CXCR2 does not activate phospholipase D in RBL-2H3 cells, whereas a truncated mutant of this receptor, which is more resistant to endocytosis and desensitization, activates the enzyme (Richardson et al., 1998). CXCR4 was reported to undergo rapid ligand-induced endocytosis (Tarasova et al., 1998) and to be rapidly desensitized by both G-protein coupled receptor kinases and protein kinase C (Haribabu et al., 1997; Signoret et al., 1997); it thus seems conceivable that the short signaling time of CXCR4 in PMN may account for its inability to mediate superoxide anion

production despite the fact that this receptor is coupled to G_i proteins. An alternative explanation is that CXCR4 may activate additional signaling pathways and/or couple to a specific set of scaffolding proteins, which would prevent superoxide anion production. Interestingly, it was recently shown that CXCR4 may activate the Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathway (Vila-Coro et al., 1999), a finding not reported so far for receptors that activate superoxide anion production.

We described previously small peptides derived from the amino terminal sequence of SDF-1 that are ligands of CXCR4 and inhibit HIV-1 entry via the CXCR4 coreceptor into target cells; some of these peptides also induced intracellular Ca^{2+} signals mediated by CXCR4 (Heveker et al., 1998). In the present study, we extended our observations by studying in detail the pharmacological properties of several novel peptides and peptide analogs that bind to CXCR4, using human polymorphonuclear granulocytes as a model. These peptides were initially selected among many others because of a functional assay measuring the inhibition of cell infection by T-tropic strains of HIV-1 that require CXCR4 as coreceptor. Subsequent inhibition binding studies using ^{125}I -SDF-1 as radiolabeled ligand showed that peptide binding affinities for CXCR4 were proportional to peptide potencies in inhibiting viral infection. Binding results extended previous findings indicating that the presence of both cysteine residues from the characteristic N-terminal chemokine C-X-C motif is not strictly required for peptide binding to CXCR4 (Loetscher et al., 1998). A purified peptide dimer containing a thioester bond at the level of the second cysteine residue of the CXC motif, a substitution of the cysteine residue at position 9, and a substitution of the two phenylalanine residues at positions 13 and 14 by D-phenylalanines displayed highly increased binding affinity for CXCR4 and antiviral activity. The reason for this increase in affinity remains unclear. This thioester bond itself might be part of the peptide binding domain, because both cysteine residues of the C-X-C-motif are thought to be engaged in disulfide bridges within the SDF-1 molecule (although not directly with each other). Alterna-

TABLE 2

Ca^{2+} signaling promoted by SDF-1-derived peptides

Ca^{2+} signals promoted by 10 μ M concentrations of each peptide or 100 nM SDF-1. PMN were preincubated for 30 min at 37°C with 6 μ g/ml of the 12G5 anti-CXCR4 antibody before the addition of 1 μ M peptide or 10 nM SDF-1; residual activity corresponds to the ratio between the Ca^{2+} signal measured in the presence of the antibody and that measured with control cells stimulated by the same concentration of peptide or SDF-1. PMN were preincubated for 3 h at 37°C with 1 μ g/ml PTX before the addition of 1 μ M peptide or 10 nM SDF-1; residual activity corresponds to the ratio between the Ca^{2+} signal measured in cells preincubated with the toxin and that measured with control cells stimulated by the same concentration of peptide or SDF-1. PMN were preactivated or not with 10 μ M concentrations of each indicated peptide or 1 nM SDF-1 and then stimulated with SDF-1 or fMLP (10 nM); residual activity corresponds to the ratio between the Ca^{2+} signal measured in non-preactivated PMN and that measured in preactivated cells. fMLP caused a homologous desensitization of Ca^{2+} signaling larger than 50%.

Peptide	Peptide-Promoted Ca^{2+} Fluxes Maximal [Ca^{2+}] nM	Inhibition of Peptide Effect by Anti- CXCR4 mAb (residual activity)	Inhibition of Peptide Effect by PTX (residual activity)	Effect of Peptide Preincubation on Agonist- Promoted Ca^{2+} Fluxes (residual activity)	
				SDF-1 ^a	fMLP ^a
			% of control	% of control	
1-13	105 \pm 24	N.D.	N.D.	55 [†]	95
1-13 (L5H)	148 \pm 38	N.D.	N.D.	N.D.	100
5-14 (S)	292 \pm 31	85 \pm 10	0**	100	95
S1	143 \pm 28	93.5 \pm 5.8	0**	100	100
S2	29.5 \pm 11	109 \pm 9	N.D.	100	100
S1D	108 \pm 28	90 \pm 10	0**	0	100
SDF-1	292 \pm 50	22 \pm 4**	0**	5	95

^a Data of one experiment representative of two independent experiments giving similar results. [†], cells preactivated with the 1-13 peptide were washed with buffer before addition of SDF-1.

N.D., not determined.

** $p < 0.01$.

tively, the higher affinity of peptide dimers may be caused by increased avidity. Interestingly, recent reports indicated that the CXCR4 receptor forms dimers at the cell surface, and that receptor dimerization is a prerequisite for receptor signaling (Vila-Coro et al., 1999); it cannot be excluded that a dimeric peptide can bridge the ligand binding sites of two adjacent CXCR4. Such a scenario was reported recently for the interleukin-5 receptor: a disulfide-cross-linked peptide dimer that forms spontaneously in solution was shown to bind simultaneously to two receptors (England et al., 2000). Finally, both explanations may be true.

On examining the signaling effects of SDF-1-derived peptides on PMN, we found that all tested peptides, except the S2 peptide, which lacks both cysteine residues of the CXC motif, promoted a significant Ca^{2+} response at concentrations of 1 μM and above. This result is in apparent contradiction with previous findings that the first two residues, the lysine at position 1 and the proline at position 2 on both SDF-1 and derived peptides, are indispensable for the induction of CXCR4-mediated Ca^{2+} fluxes (Crump et al., 1997). In fact, in the case of peptides lacking the first two amino acid residues of the SDF-1 sequence, peptide-induced Ca^{2+} fluxes were not attributable to CXCR4 activation but probably to other serpentine G_i -coupled receptors, as shown by experi-

ments conducted in the presence of anti-CXCR4 antibodies or pertussis toxin. The other receptor(s) targeted by peptides of the "S" series in addition to CXCR4 could not be identified in this study; however, experiments conducted with inhibiting antibodies and sequential stimulation of PMN with appropriate agonists excluded that CXCR2 or fMLP receptor mediated the CXCR4-independent calcium signaling effects. Moreover, sequence alignment analysis did not show any evident homology between the 5 to 14 N-terminal sequence of SDF-1 and that of other known chemokines that could have

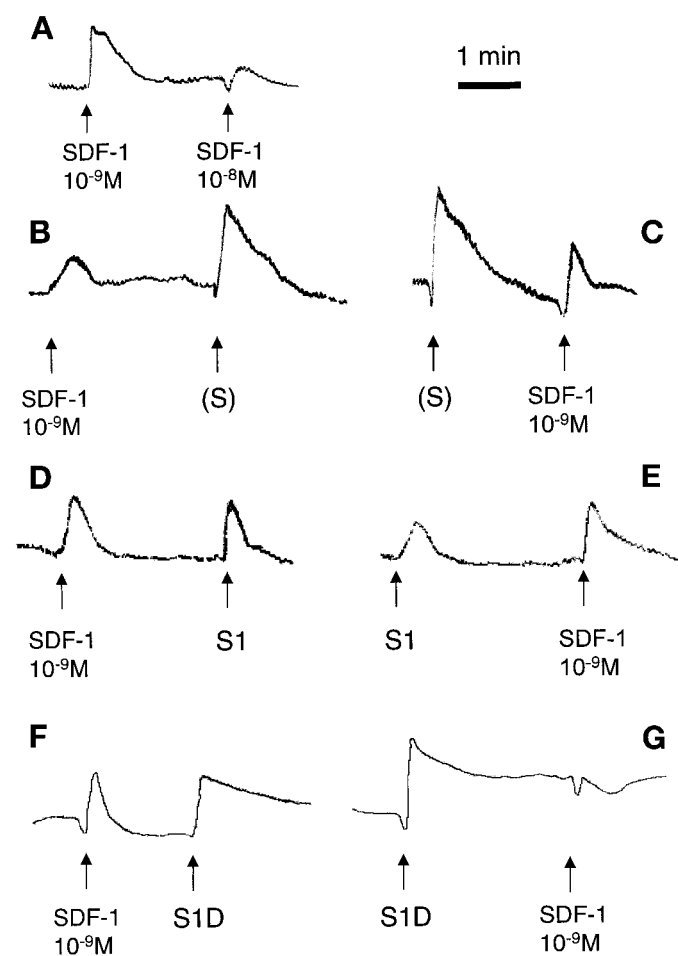


Fig. 4. Calcium influx in PMN induced by SDF-1 and SDF-1-derived peptides. PMN were sequentially treated with SDF-1 and indicated peptides (10 μM) or vice versa. Data shown were recorded on cells from a same donor and are representative of experiments performed on three different donors. See explanation in the text.

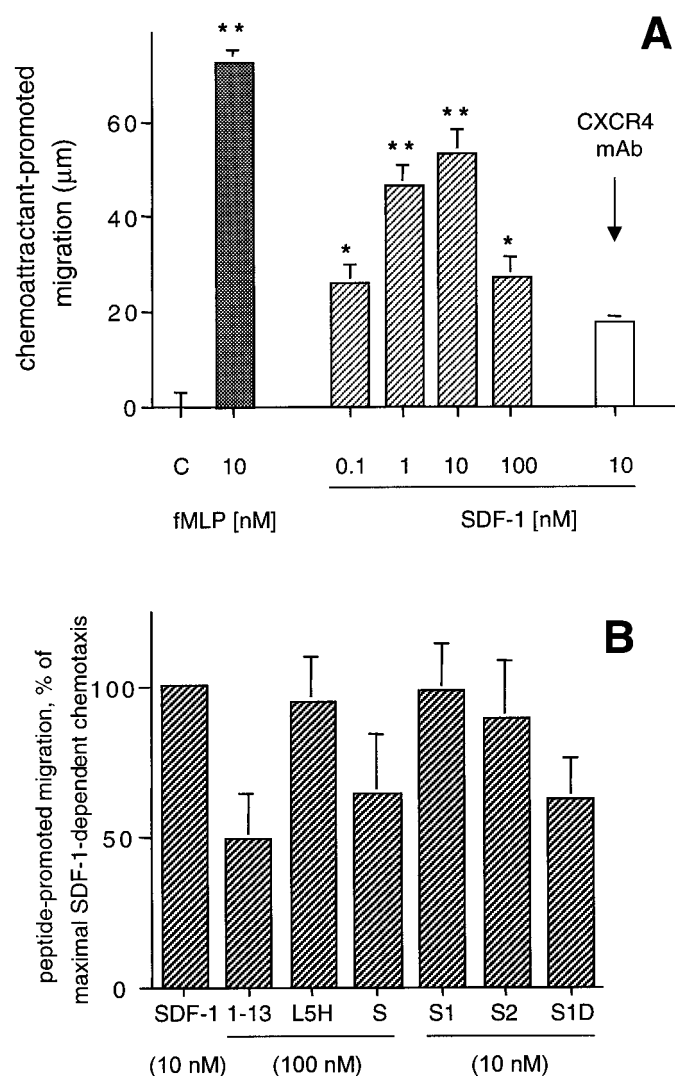


Fig. 5. PMN chemotaxis promoted by SDF-1 and SDF-1-derived peptides. Cells were added to the upper compartment of Boyden chambers and indicated chemoattractant compounds or SDF-1-derived peptides were added to the lower compartment. Cell migration was determined by measuring the distance of the leading front. Specific migration was calculated by subtracting the distance of the leading front measured in control experiments (no chemoattractant in the lower chamber). A, chemotaxis promoted by SDF-1 is compared with that elicited by fMLP. The effect of the 12G5 anti-CXCR4 antibody was determined in experiments performed with 10 nM SDF-1. * $p < 0.05$; ** $p < 0.01$. B, comparison of the maximal chemotactic effect of SDF-1-derived peptides and SDF-1. For peptides, the concentrations used in the assays ranged from 0.1 nM to 10 μM and typical bell-shaped chemotactic responses were observed (data not shown). Maximal chemotaxis was observed at a concentration of 100 nM for "1-13", "1-13/L5H", and "S" peptides and at a concentration of 10 nM for "S1", "S2", and "S1D" peptides. Data are triplicates of experiments on cells from a single donor and are representative of three or four different experiments. Bars represent S.D.

been indicative for a potential second target for the peptides. Importantly, the antiviral and antagonist effects of the dimeric "S1D" peptide on SDF-1 binding to CXCR4 and on HIV infection were observed for peptide concentrations that were 1 order of magnitude lower than those promoting CXCR4-independent Ca^{2+} signals in PMN, indicating that the "S1D" peptide is selective for CXCR4. Whereas all peptides displayed at least some chemotactic effect on PMN, none of them inhibited chemotaxis induced by SDF-1 or strong chemoattractants such as fMLP, C5a, or IL-8. At high concentrations (10 μM), all peptides except "S2" and the "S1D" dimeric peptide also promoted a significant superoxide anion production in PMN. These effects were clearly not attributable to the activation of CXCR4 but may be explained, as in the case of Ca^{2+} signaling, by the activation of another unidentified receptor.

Thus far, the pharmacological properties of SDF-1-derived peptides have mostly been studied in cell lines. In the case of the 1-13/L5H peptide, for example, no signaling activity could be observed in HeLa cells at concentrations up to 100 μM (Heveker et al., 1998), in contrast to what we have observed on PMN. PMN have proven here to be a sensitive

model for detecting side effects of SDF-1–derived synthetic peptides. These effects are not simply anecdotal because undesired activation of PMN oxidative burst was shown to be particularly harmful in many pathological conditions (Halliwell and Gutteridge, 1990). The development of immunomodulating or antiviral chemokine-derived peptides that target chemokine receptors is a rapidly evolving area of investigation. Our results indicate that tests on PMN should be included in the early screening procedures of the pharmacological properties of these compounds.

In conclusion, using a combinatorial peptide chemistry approach and a fast screening procedure based on antiviral properties, we have identified an SDF-1 chemokine-derived dimeric peptide with markedly ameliorated properties in terms of binding affinity for CXCR4 and anti-HIV activity. This novel CXCR4 antagonist, which displays only moderate nonspecific effects on human PMN at pharmacological concentrations, constitutes a new lead for the generation of low-molecular-weight CXCR4 blockers.

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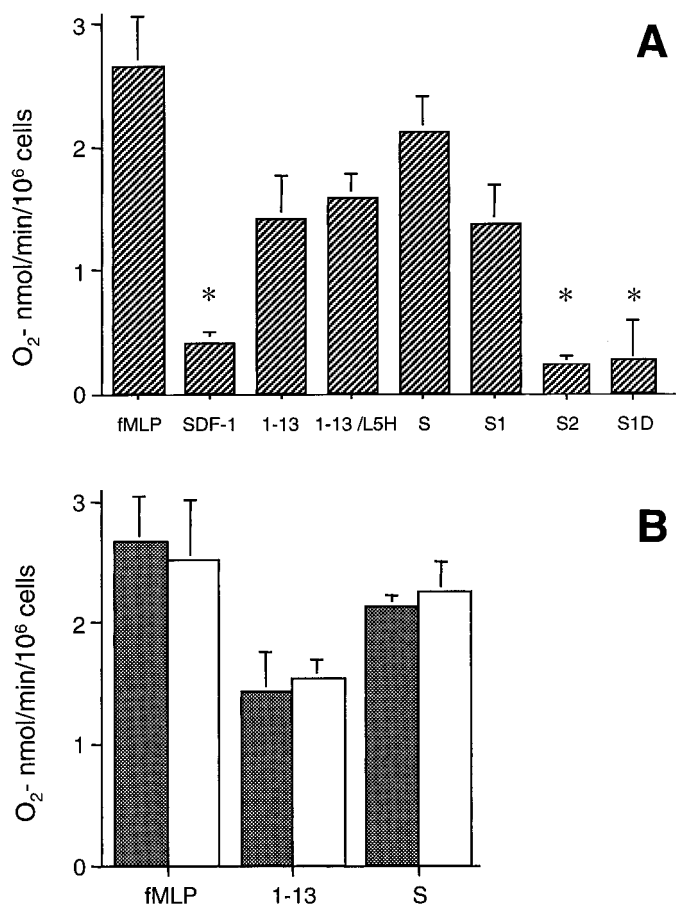


Fig. 6. Superoxide anion production promoted by chemoattractant compounds and SDF-1–derived peptides in PMN. Cells were incubated with or without SDF-1 or peptides for 10 min at 37°C. Superoxide anion generation was measured by the reduction of ferricytochrome C as described in Materials and Methods. A, fMLP was used at 100 nM whereas SDF-1 and peptides were at a concentration of 10 μM . Asterisks indicate the absence of significant difference with control experiments conducted with vehicle alone. B, some of the experiments were carried out in the presence of a saturating concentration of the 12G5 anti-CXCR4 antibody. ■, control; □, +CXCR4 monoclonal antibody.

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