Identification of oligopeptide sequences which inhibit migration induced by a wide range of chemokines

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We have identified an amino acid sequence, termed peptide 3, corresponding to amino acids 51–62 of the mature human monocyte chemoattractant protein-1 (MCP-1), which inhibits human mononuclear-cell and THP-1-cell migration induced by a wide range of chemokines. For example, peptide 3 inhibited MCP-1-induced THP-1 migration in a transwell assay with an ED₅₀ of approx. 8 µM. Peptide 3 binds directly to THP-1 cells with an association constant of approx. 10 µM, and is therefore likely to be a direct receptor antagonist for CC and CXC chemokine receptors. By performing a structure–function analysis of this peptide, we have identified a sequence variant that shows an approx. 3–4-fold greater potency as an inhibitor of chemokine-induced migration [Leu₄Ile₁₁ peptide 3 (1–12)].

Furthermore, unlike peptide 3, which binds to the Duffy antigen receptor for chemokines on human erythrocytes with a similar affinity to the specific chemokine receptors on THP-1 cells, the Leu₄Ile₁₁ peptide 3 (1–12) sequence variant shows at least 20-fold greater selectivity for the specific receptors. Derivatives of Leu₄Ile₁₁ peptide 3 (1–12) are therefore the best candidates among the molecules we have investigated for use as a chemokine inhibitor *in vivo*.

Key words: Duffy antigen receptor binding, interleukin-8, monocyte chemoattractant protein-1, stromal cell derived factor-1α, THP-1 cells.

INTRODUCTION

Chemokines are a family of low-molecular-mass (8–12 kDa) structurally related proteins that exhibit a variety of pro-inflammatory activities [1,2]. Recently these proteins have been found to play a significant role in many disease processes, such as rheumatoid arthritis [3], transplant rejection [4], the progression of HIV infection [5,6] and atherosclerosis [7,8]. There are two major subfamilies of chemokines; ‘CC’ [for example, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α)] and ‘CXC’ [for example, interleukin-8 (IL-8) and stromal cell-derived factor-1α (SDF-1α)], where designation is based on whether the first two cysteines in a conserved motif are adjacent, or are separated by an intervening residue respectively [1,2]. Generally, CC chemokines are chemoattractants for monocytes [9,10], whereas CXC chemokines are potent chemoattractants and activators of neutrophils or lymphocytes but not monocytes [11]. This distinction *in vitro* has led to the proposition that particular infiltrates of leucocytes may be attributed to the synthesis and release of particular chemokines *in vivo*. For example, studies have shown that intradermal injection of MCP-1 produces a predominantly monocytic infiltrate in rat or mouse skin [12,13], whereas IL-8 injected into rabbits produces predominantly neutrophil accumulation at the injection site [14].

One problem in investigating the roles of chemokines and their possible role in the pathogenesis of disease is the fact that chemokine receptors each bind several different chemokines, and each chemokine binds several different receptors [15]. As a result, chemokine receptors are often termed ‘promiscuous’ [16]. They are all members of the seven-transmembrane-spanning G-protein-coupled receptor family, and have been divided into two

groups: CXC receptors (1–4) and CC receptors (1–8), since chemokine-ligand promiscuity does not cross CC to CXC boundaries [1,2]. One notable exception is the Duffy antigen receptor for chemokines (DARC) [1], another seven-transmembrane receptor that binds a wide variety of chemokines, of both the CC and CXC subfamilies (as well as acting as a binding protein for the malarial parasite *Plasmodium vivax*). DARC is expressed on erythrocytes and post-capillary endothelial cells [17,18], and it has been suggested to function as either a chemokine reservoir, or as a method of presenting chemokines to circulating leucocytes [1,2].

Agents that modulate the activity of chemokines may be useful for the prevention and treatment of a wide range of inflammatory disease processes. Studies have shown that the deletion of amino acid residues 2–8 of MCP-1 (MCP-1Δ2–8) results in a polypeptide lacking chemoattractant activity [19,20] in transwell migration assays. Furthermore, MCP-1Δ2–8 inhibits cell migration induced by the chemokine MCP-1. The mechanism of inhibition of MCP-1 by MCP-1Δ2–8 is unclear. One possibility is that MCP-1Δ2–8 forms heterodimers with the wild-type MCP-1, preventing the wild-type MCP-1 from eliciting a biological response [19]. Neutralizing antibodies to MCP-1 have also been shown to inhibit the inflammatory effects of MCP-1 protein both *in vitro* and in animal models *in vivo*, for example in the rat model of glomerulonephritis [21,22]. N-terminal modifications to another CC chemokine, RANTES (regulated on activation, normal T-cell expressed and presumably secreted), have been shown to render it inactive as a chemoattractant in a mononuclear-cell migration assay and also able to act as a competitive inhibitor of RANTES signalling [23,24]. Furthermore, Kledal and colleagues [25] found that Kaposi’s sarcoma-associated herpes virus encodes a chemokine vMIP-II, which bound with a high affinity to both

Abbreviations used: DARC, Duffy antigen receptor for chemokines; fMLP, *n*-formyl-Met-Leu-Phe; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RANTES, regulated on activation, normal T-cell expressed and presumably secreted; SDF-1α, stromal cell-derived factor-1α; TGF-β1, transforming growth factor-β1.

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CC and CXC chemokine receptors, and was able to inhibit the migration of freshly isolated human monocytes induced by CC chemokines [25]. However, none of these agents described is likely to be a useful inhibitor of chemokine activity *in vivo* since they are all relatively large polypeptides and are likely to have undesirable pharmacokinetic properties.

As a first step towards designing small-molecule inhibitors of MCP-1 activity we have synthesized peptides (up to 15 amino acids in length) corresponding to regions of identity between mouse and human MCP-1 amino acid sequences. Since both mouse and human MCP-1 bind to, and positively signal through, the human chemokine receptors, structural determinants necessary for binding may be conserved between these two proteins. The effects of these synthetic MCP-1-derived peptides have been examined *in vitro* using a transwell migration assay for both human mononuclear cells and the human myelomonocytic cell line THP-1. Once inhibitors have been identified *in vitro* they can be tested for anti-inflammatory activity *in vivo* and may ultimately be useful therapeutically for controlling the inflammatory response during many disease processes.

MATERIALS AND METHODS

Chemoattractant proteins

The following recombinant proteins (all purchased from R&D Systems, Abingdon, Oxon., U.K.) were used in the transwell migration assay at a concentration that caused a maximal mononuclear-cell and THP-1-cell migratory response to the chemoattractant in question: human MCP-1 and human MIP-1 α at 50 ng/ml; human IL-8 at 100 ng/ml; and human SDF-1 α , amino acids 22–89, at 75 ng/ml. Human transforming growth factor- β 1 (TGF- β 1, 100 ng/ml) and *n*-formyl-Met-Leu-Phe (fMLP, 100 nM; Sigma) were used as non-chemokine chemoattractant controls in the transwell migration assays.

Synthesis of oligopeptides

Peptides (6–15 amino acids in length) were synthesized by standard solid-phase chemistry and purified to greater than 95% purity using a LiChrosphere 100RP-18 column and eluting with 0.1% orthophosphoric acid in 90% acetonitrile (Affiniti Research Products, Exeter, Devon, U.K.). The purity of each peptide was confirmed by HPLC and mass-spectroscopy techniques.

Preparation of human mononuclear cells

Fresh venous blood was taken from the ante-cubital fossa region using a 19 gauge butterfly needle. Donors were males and females aged 25–30. For every 10 ml of fresh blood collected 0.75 ml of heparin sulphate was added, and the mixture left at room temperature for 5 min. This anti-coagulated blood (30 ml) was carefully layered over 15 ml of Lymphoprep (Nycomed Pharma, Oslo, Norway), and centrifuged at 778 *g* for 15 min using no brake in accordance with the instructions from Nycomed. The purified mononuclear cells were removed carefully with a glass pipette, washed with Dulbecco's A PBS (pH 7.3; Oxoid, Basingstoke, Hants, U.K.) and centrifuged at 778 *g* for 5 min. This was repeated twice before counting the mononuclear cells and reconstituting the cell pellet to 1×10^7 cells/ml in Geys balanced salt solution (Sigma) + 1 mg/ml BSA (BSA fraction V powder, fatty acid free; Sigma). The mononuclear cells were used within 1–2 h in a transwell migration assay.

Flow cytometry of human mononuclear cells

The proportion of monocytes in the mononuclear-cell preparation was determined by flow cytometry using a Beckton Dickinson FACSsort equipped with an argon-ion laser at 488 nm and 200 mW light output. Briefly, the percentage of monocytes in each sample was determined using a mouse monoclonal anti-CD14 R-phycoerythrin-conjugated antibody (mouse IgG2b, clone FMC 17; Harlan Seralab, Loughborough, Leicestershire, U.K.). The percentages of B- and T-lymphocytes were determined using anti-CD20 (mouse IgG1, clone B-Ly1; Dako, Copenhagen, Denmark) and anti-CD3 (mouse IgG1, clone UCHT-1; Harlan Seralab) FITC-conjugated mouse monoclonal antibodies respectively. Each antibody (10 μ l) was incubated together with 1×10^6 cells for 30 min in the dark at 4 °C. The appropriately conjugated antibody isotypes (Harlan Seralab) were used as controls. Using the standard Becton Dickinson FITC and phycoerythrin filter sets, 10000 events per sample were collected. Percentages of positively stained cells were estimated using the Lysys II software program (Becton Dickinson).

Transwell migration assay using human mononuclear cells

Transwell migration assays were a modification of the protocol described by Denholm and Stankus [26], and were performed in 96-well disposable chemotaxis chambers fitted with an 8- μ m polycarbonate filter (PVP free, ChemoTX, Neuroprobe, Cabin John, MA, U.S.A.). Briefly, 29 μ l of medium plus or minus chemoattractant was added to the lower compartment of each well. The framed filter was aligned with the holes in the corner of the filter frame and placed over the wells. Mononuclear cells (2.5×10^5) in 25 μ l of Gey's balanced salt solution + 1 mg/ml BSA were added to the upper compartment. Peptides to be tested were dissolved in Milli Q water, serially diluted in the Gey's balanced salt solution + 1 mg/ml BSA as necessary, and were incubated with the mononuclear cells in the upper compartment of the migration chamber. The chamber was then incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 1.5 h.

After incubation, any cells remaining in the upper compartment were removed gently from the top of the filter with a pipette, and 20 μ l of 20 mM EDTA in Dulbecco's A PBS was added into each upper well, and incubated for 20 min at 4 °C. The filter was flushed carefully with media using a gentle flow, and removed. The number of cells that had migrated was determined by counting the cells in each well using a haemocytometer and determining the means $\pm$ S.E.M. for triplicate wells.

Transwell migration assay using a human myelomonocytic cell line; THP-1 cells

THP-1 cells (European Collection of Cell Cultures, Salisbury, Wiltshire, U.K.) were maintained at a density of between 4×10^5 and 1×10^6 cells per ml in RPMI 1640 supplemented with 10% fetal calf serum and 20 μ M 2-mercaptoethanol. Transwell migration assays were performed in the same way as with the mononuclear cells except the 96-well disposable chemotaxis chamber was fitted with a 5- μ m polycarbonate filter (PVP free, ChemoTX, Neuroprobe), and 5×10^4 THP-1 cells in 25 μ l of RPMI 1640 culture media were added to the upper compartment. In most cases, the serially diluted peptides to be tested were added to the upper compartment of the chemotaxis chamber but, in some cases, the peptide was added with the chemoattractant in the lower compartment. When using the THP-1 cells the chamber was incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 4 h.

After incubation, any cells remaining in the upper compartment were removed and incubated with 20 mM EDTA as above. The number of cells that had migrated was determined using the vital stain MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma]. A standard curve consisting of a 2-fold dilution series of THP-1 cells (top standard, 50000 cells in 29 μ l) was constructed. Migrated cells and cells in the wells for the standard curve were stained by addition of 3 μ l of MTT stock solution (5 mg/ml in RPMI 1640 without Phenol Red; Sigma) and incubated at 37 °C for 4 h. The media were carefully aspirated from each well, and the converted Blue Formazan dye was solubilized in 20 μ l of DMSO. Absorbance of converted dye was measured at a wavelength of 595 nm using an ELISA plate reader. The number of cells migrated in each well was determined by interpolation of the standard curve.

Measurement of 125 I-MCP-1 binding to human erythrocytes

To prepare human erythrocytes, 9 ml of freshly drawn blood were transferred to a tube containing 1 ml of 3.8% sodium citrate, and left at room temperature for 15 min. This anticoagulated blood (5 ml) was carefully layered over 3.5 ml of Polymorphprep (Nycomed Pharma), and centrifuged at 500 g for 35 min in accordance with the manufacturer's instructions. The erythrocytes were carefully removed and reconstituted to the original volume (5 ml) with binding medium (PBS+1 mg/ml fatty acid-free BSA, pH 7.4), and centrifuged at 900 g for 10 min. This was repeated four times, prior to counting the cells and adjusting the volume to give 1×10^8 erythrocytes per well in a 'V'-bottomed microtitre plate (Corning Costar, High Wycombe, Bucks, U.K.).

The cells were sedimented by spinning for 5 min at 670 g and resuspended in 25 μ l of binding medium containing 0.5 nM 125 I-MCP-1 (specific activity 2000 Ci/mmol; Amersham Pharmacia Biotech) in the presence or absence of non-labelled MCP-1 or peptides. Each condition was assayed in triplicate. The cells were incubated at 37 °C for 30 min. Binding medium (50 μ l) containing 20% sucrose was added to each well, and centrifuged at 670 g for 5 min. The supernatant was removed immediately, and the radioactivity in the cell pellet was reconstituted in 50 μ l of binding medium and quantified in a gamma counter. Non-specific binding was determined in the presence of a 500-fold excess of unlabelled ligand.

RESULTS

The amino acid sequences of human MCP-1 and the mouse homologue JE/MCP-1 were aligned (Figure 1). The two sequences shared an overall homology of 35.1% (Figure 1). We noted three distinct regions of homology (90% in each case) between the human and murine MCP-1 amino acid sequences; the first was a 13-mer sequence (amino acids 1–13 in mature MCP-1), termed peptide 1; the second was a 15-mer sequence (amino acids 28–42, peptide 2); and the third was a 12-mer sequence (amino acids 51–62, peptide 3). Each peptide was synthesized using standard solid-phase chemistry to $\geq 95\%$ purity as determined by HPLC and mass-spectroscopy techniques.

The effect of peptides on human mononuclear-cell chemotaxis

Each peptide was tested in the upper compartment of a transwell migration assay with the human mononuclear cells for the ability to inhibit chemokine-induced migration. The mononuclear population consisted of approx. 8% monocytes and 80% lymphocytes as determined by standard flow-cytometry techniques (results

not shown). Throughout these experiments we have selected MCP-1 and MIP-1 α as representative CC chemokines, and IL-8 and SDF-1 α as representatives of the CXC chemokine family. In addition, TGF- β 1 was selected as a migration-inducing agent unrelated to the chemokine family. Peptide 1 had no effect on the migration of human mononuclear cells in response to any of the CC or CXC chemokines tested (ED_{50} 100 μ M, < 10% inhibition at 100 μ M; Figure 2). Peptide 2 was a weak inhibitor of chemokine-induced migration (ED_{50} 100 μ M, 19% inhibition for MCP-1 at 100 μ M, $P = 0.09$; Figure 2). In contrast, peptide 3 was a highly effective inhibitor in response to all chemokine-induced mononuclear-cell migration (Figure 2). The ED_{50} was between 8 and 14 μ M when MCP-1 was used to induce migration. At concentrations of 100 μ M, peptide 3 abolished mononuclear-cell migration induced by all of the CC and CXC chemokines tested (Figure 2). In marked contrast, TGF- β 1, a non-chemokine migration-inducing agent, was not significantly inhibited by any of the three peptides, even at 100 μ M (inhibition < 10%, $P > 0.05$; Figure 2). This indicates the peptides did not affect human mononuclear-cell viability for the duration of the experiment.

The effect of MCP-1 peptides using a human myelomonocytic cell line; THP-1 cells

It was impractical to characterize the structure–function relationship of peptide 3 using human mononuclear cells because of the large amounts of blood required. We therefore used the human myelomonocytic THP-1 cell line in a transwell migration assay with different families of chemokines in the lower compartment to induce THP-1-cell migration. Each peptide was incubated in the upper compartment with the THP-1 cells at 100 μ M final concentration. Furthermore, each assay was performed in triplicate, and the means of six separate assays are documented in Table 1. For each chemokine tested (MCP-1, MIP-1 α , SDF-1 α and IL-8) peptide 1 had no significant effect on THP-1-cell migration (inhibition < 14%, $P > 0.05$; Table 1). Similarly, peptide 2 had no effect on the THP-1 migration induced by the CXC chemokines SDF-1 α and IL-8 (inhibition < 13%, $P > 0.05$; Table 1), but had a small statistically significant effect on the CC chemokines MCP-1 and MIP-1 α (19% inhibition, $P < 0.05$; Table 1). As observed for human mononuclear cells, peptide 3, but not peptides 1 and 2, abolished THP-1 migration in response to all the chemokines tested (inhibition > 90% with all chemokines, $P < 0.0001$; Table 1). In marked contrast, fMLP and TGF- β 1, two non-chemokine chemotactic agents for THP-1 cells, were not significantly inhibited with any of the three peptides, even at 100 μ M (inhibition < 10%, $P > 0.05$; Table 1). Taken together, these results demonstrate that peptide 3 specifically inhibits THP-1 migration induced by the chemokines tested, but has no effect on the migration induced by non-chemokine chemoattractants.

In order to investigate if there were any differences in the ED_{50} values for peptide 3 as an inhibitor of CC versus CXC chemokines, the ED_{50} values were determined for peptide 3 using THP-1 migration induced by each of the chemokines listed above (Figure 3). Each assay was performed in triplicate at six different concentrations of peptide 3 (1, 5, 10, 25, 50 and 100 μ M final concentrations of peptide in the upper compartment of the transwell THP-1 migration assay) in three separate experiments. Peptide 3 had an ED_{50} against MCP-1 and MIP-1 α of 8 and 7.5 μ M respectively (Figure 3), and against SDF-1 α and IL-8 of 13.5 and 10 μ M respectively (Figure 3). Although peptide 3 showed weak selectivity for CC chemokines over CXC chemokines (1.8-fold, $P < 0.01$), nevertheless, at 100 μ M, peptide 3

	1	11	21	31	41
Mouse MCP-1:	AQPDVNA [*] NAPL [*]	TCCYSFTSKM [*]	IPMSRLESYK [*]	RITSSRC [*] PK [*] E [*]	AVVFVTKLKR [*]
Human MCP-1:	AQPDAINAPV	TCCYNFTNRK	ISVQRLAS [*] YR [*]	RITSSKCPKE	AVIFKTIVAK
	⇐ Peptide 1 ⇒		⇐ Peptide 2 ⇒		
	51	61	71	81	91
Mouse MCP-1:	EVCADPKKEW [*]	VQTYIKNLDR [*]	NQMRSEPTTL [*]	FKTASALRSS [*]	APLNVKLTRK [*]
Human MCP-1:	EICADPKQKW	VQDSMDHLDK	Q-----	-----	-----
	⇐ Peptide 3 ⇒				
	101	111	121		
Mouse MCP-1:	SEANASTTFS	TTTSSTSVGV	TSVTVN [*]		
Human MCP-1:	-----	-----	TQTPKT		

Figure 1 Alignment of the human and mouse MCP-1 amino acid sequences

The sequences were extracted from the original publications [28,29] and aligned using the CLUSTALW algorithm with default parameters. Amino acids conserved between the two sequences are marked *. Peptides 1, 2 and 3 (12–15 amino acids in length), synthesized by solid-phase chemistry, are shown in bold. Peptide 1 is amino acids 1–13, peptide 2 is amino acids 28–42 and peptide 3 is amino acids 51–62 of mature human MCP-1.

inhibited > 99 % of the migration induced by all of the chemokines tested.

As one strategy to investigate the mechanism of action by which the peptides function as inhibitors, the peptides were added with the chemokines in the lower compartment in the transwell THP-1 migration assay, in contrast to all the other experiments where the peptides were incubated with the cells in the upper compartment. Under these conditions, peptide 1 inhibited MCP-1-induced migration to a greater extent than when incubated with the cells [60 % inhibition, $P < 0.05$, compared with 10 % inhibition at 100 μ M final concentration (not significant); results not shown]. This result is consistent with published reports which state that the N-terminus of MCP-1 is involved in dimerization thought to be necessary for MCP-1 signalling [19]. In contrast, peptide 3 was much less effective when incubated with the chemokine in the lower compartment than with the cells in the upper compartment (17 % inhibition at 100 μ M compared with > 99 % inhibition when incubated in the upper compartment with the cells; results not shown). This suggests that peptide 3 inhibits MCP-1-induced migration by interacting with the cells rather than by binding to the MCP-1 ligand. In order to confirm this proposition, the binding affinity of N-terminally biotinylated peptide 3 for THP-1 cells was determined. This derivative bound to the surface of THP-1 cells with an association constant of approx. 10 μ M. Taken together, these results suggest that peptide 3 inhibits THP-1 migration by acting as a direct receptor antagonist to the cells rather than binding directly to the MCP-1 ligand. Furthermore, because the peptide inhibits THP-1 migration induced by both CC and CXC chemokines, peptide 3 is presumably an antagonist to both CC and CXC receptors on the cell surface.

The effect of peptide-3 analogues on THP-1-induced migration

To investigate what structural characteristics of full-length pep-

tide 3 [designated peptide 3 (1–12)] were necessary for inhibition, we synthesized analogues of peptide 3 at greater than 95 % purity. In all cases, migration induced by fMLP and TGF- β 1, two non-chemokine chemotactic agents for THP-1 cells, was not significantly inhibited with any of the derivatives of peptide 3, even at 100 μ M (inhibition < 10 %, $P > 0.05$; Table 2). Peptide 3 (3–12) had very similar properties to peptide 3 (1–12) (Table 2), which suggests that the glutamate (E) and isoleucine (I) residues at positions 1 and 2 respectively of the peptide-3 sequence were unimportant for receptor binding, and that the C-terminal portion of peptide 3 was probably more important for inhibiting chemokine-induced THP-1 migration. To investigate this further we also synthesized two halves of the 12-mer peptide 3 (1–12). Peptide 3 (7–12) inhibited THP-1 migration induced by CC and CXC chemokines similarly to peptide 3 (1–12), and showed no selectivity for any of the chemokines tested, with an ED₅₀ of $\leq$ 8 μ M in each case (Table 2). In contrast, peptide 3 (1–6) was much less potent at inhibiting the CC chemokines compared with peptide 3 (1–12), but was only slightly less efficient at inhibiting CXC chemokines compared with peptide 3 (1–12) (ED₅₀ of 16 and 19 μ M for IL-8 and SDF-1 α respectively, Table 2). This demonstrates that the C-terminal portion of peptide 3 is able to inhibit chemokine-induced THP-1 migration to the same extent as the full-length peptide 3.

We aligned the chemokine sequences in the peptide-3 region (Figure 4). There are five conserved amino acids present in almost all the CC and CXC chemokines (denoted by asterisks, Figure 4). We therefore proposed that the nature of the amino acid in the non-conserved positions may play a role in the selectivity of receptor binding of the different chemokines. As a result, we synthesized a number of analogues of peptide 3 (1–12), substituting amino acids from various chemokines into the MCP-1-derived sequence.

In CC chemokines, position 4 of peptide 3 (1–12) is usually an alanine (A), whereas in CXC chemokines this is commonly a

Figure 2 Effects of peptides 1, 2 and 3 on human mononuclear-cell migration

A transwell migration assay was performed using freshly prepared human mononuclear cells. Migration was induced by: (A) 50 ng/ml MCP-1; (B) 50 ng/ml MIP-1 α ; (C) 75 ng/ml SDF-1 α ; and (D) 100 ng/ml IL-8. In addition, 100 ng/ml TGF- β 1 (E) was used as a non-chemokine chemoattractant. Each condition was assayed in triplicate wells for each of four individuals and the results shown are the means $\pm$ S.E.M. from the four individuals. * P < 0.05 compared with chemoattractant alone (Student's unpaired t test). Pep, peptide.

Table 1 Percentage inhibition of THP-1 cells at 100 μ M final concentration of each peptide

Migration of THP-1 cells in transwell assays induced by MCP-1, MIP-1 α , SDF-1 α , IL-8, fMLP and TGF- β 1 were performed in triplicate in the absence or presence of 100 μ M peptide 1, 2 or 3. The percentage inhibition of migration is shown. * P < 0.05 for the presence of peptide compared with the control (Student's unpaired t test).

Peptide	MCP-1	MIP-1 α	SDF-1 α	IL-8	fMLP	TGF- β 1
Peptide 1	10	13	14	9	1	5
Peptide 2	19*	19*	13	10	3	10
Peptide 3	100*	93*	99*	100*	1	6

leucine (L). Leu₄ peptide 3 (1–12) was 3–4-fold more potent as an inhibitor of CXC chemokine-induced migration compared with peptide 3 (1–12) (Table 2), but inhibited CC chemokine-induced migration with similar potency to the original peptide 3 (1–12) sequence (ED₅₀ of approx. 7–8 μ M, Table 2). Therefore, substituting a leucine at position 4 in the MCP-1 peptide 3 (1–12) sequence compared with an alanine results in a somewhat selective inhibition of THP-1 migration induced by CXC chemokines.

In the MIP-1 α -sequence peptide-3 region, position 7 is a serine (S), whereas in most other CC and CXC chemokines it is a lysine (K) residue (Figure 4). Hence, Ser₇ peptide 3 (1–12) was compared with peptide 3 (1–12). Surprisingly, Ser₇ peptide 3 (1–12) showed

Figure 3 ED₅₀ for peptide-3 inhibition of THP-1 migration induced by CC or CXC chemokines

THP-1 migration in a transwell assay was induced with (top left) MCP-1, (top right) MIP-1α, (bottom left) SDF-1α and (bottom right) IL-8 at the same concentrations shown in Figure 2. The average number of THP-1 cells migrated with control (RPMI 1640 + 10% fetal calf serum) in the lower compartment of the transwell migration plate was 5000 ± 200 cells. When chemoattractant was placed in the lower compartment there was a significant increase in the number of THP-1 cells migrated (typical values were: MCP-1, 12280 ± 240; MIP-1α, 11560 ± 270; SDF-1α, 14600 ± 1100; and IL-8, 7800 ± 320 cells). Each point is the mean ± S.E.M. of three separate experiments, with each condition in each experiment assayed in triplicate wells.

Table 2 ED₅₀ values of peptide 3 and its derivatives with CC and CXC chemokines

The ED₅₀ for inhibition of THP-1 migration in a transwell assay induced by either 50 ng/ml MCP-1, 50 ng/ml MIP-1α, 75 ng/ml SDF-1α, 100 ng/ml IL-8 or 10 ng/ml TGF-β1 were determined for a number of variants of the peptide-3 sequence, exactly as shown for peptide 3 in Figure 3. The ED₅₀ values shown are the means ± S.E.M. of three separate experiments. All the variants inhibited migration in response to all four chemokines by more than 90% at 100 μM. None of the variants showed statistically significant inhibition of migration in response to fMLP or TGF-β1 at 100 μM (the highest concentration tested). Residues in bold indicate positions where amino acids have been substituted in the original peptide-3 sequence.

Peptide		ED ₅₀ (μM)					
		MCP-1	MIP-1α	SDF-1α	IL-8	fMLP	TGF-β1
Peptide 3 (1–12)	(EICADPKQKWVQ)	8 ± 2	8 ± 3	14 ± 2	10 ± 1	> 100	> 100
Peptide 3 (1–6)	(EICADP)	25 ± 5	20 ± 3	19 ± 4	16 ± 2	> 100	> 100
Peptide 3 (7–12)	(KQKWVQ)	7 ± 1	5 ± 3	6 ± 1	5 ± 2	> 100	> 100
Peptide 3 (3–12)	(CADPKQKWVQ)	8 ± 1	7 ± 1	9 ± 2	9 ± 4	> 100	> 100
Leu ₄ peptide 3 (1–12)	(EICLDPKQKWVQ)	8 ± 2	7 ± 2	3 ± 1	3 ± 1	> 100	> 100
Ser ₇ peptide 3 (1–12)	(EICAD P SQKWVQ)	7 ± 1	6 ± 2	4 ± 3	3 ± 2	> 100	> 100
Ile ₁₁ peptide 3 (1–12)	(EICADPKQKW I Q)	6 ± 3	4 ± 1	7 ± 2	2 ± 1	> 100	> 100
Leu ₄ Ile ₁₁ peptide 3 (1–12)	(EICLDPKQKW I Q)	2 ± 0	2 ± 1	4 ± 1	4 ± 2	> 100	> 100

some CXC specificity (approx. 3-fold) but the effect on MCP-1- and MIP-1α-induced THP-1 migration was unaltered (Table 2). Therefore, despite the substitution of a serine at position 7, as found in some CC chemokines, Ser₇ peptide 3 (1–12) was selectively more potent as an inhibitor of CXC chemokines rather than CC chemokines.

Many chemokines have valine (V) at position 11; however, SDF-1α, a CXC chemokine, has an isoleucine (I) at this position. Ile₁₁ peptide 3 (1–12) again showed some CXC chemokine selectivity, although it was not as marked as Leu₄ peptide 3 (1–12). Surprisingly, Ile₁₁ peptide 3 (1–12) showed the greatest selectivity for IL-8 (which has a valine at this position) rather

	1	12	
MCP-1	ET CAD PKQKW Q	Peptide 3 (1–12)	Ref:(28)
	* * * * *		
MIP-1 α	QVCAD P SEEW Q		Ref:(30)
SDF-1 α	QVCID P KLKW Q		Ref:(31)
IL-8	EL CLD PKENW Q		Ref:(32)
CC Chemokines:			
MCP-1	ETCADPKQKWQ		
MCP-2	EVCADPKERWVR		
MCP-3	ETCADPTQKWQ		
MCP-4	ETCADPKEKWQ		
MIP-1 α	QVCADPSEEWQ		
MIP-1 β	QVCADPSESWQ		
EOTAXIN	DICADPKKKWQ		
LARC	SVCANPKQTWVK		
PARC	QICADPNKKWQ		
RANTES	QVCANPEKKWVR		
CXC Chemokines:			
IL-8	ELCLDPKENWQ		
SDF-1 α	QVCIDPKLKWQ		
IP-10	KRCLNPESKAIK		
MIG	QTCLNPDSADVK		

Figure 4 Alignment of peptide 3 (1–12) derived from the human MCP-1 sequence with the cognate region of the representative CC and CXC chemokines used in this study

Conserved amino acids are shown in bold and marked with an asterisk. Cognate sequences from other CC and CXC chemokines are also shown. LARC, liver- and activation-regulated chemokine; PARC, pulmonary- and activation-regulated chemokine; IP-10, human interferon- γ -inducible protein-10; MIG, monokine induced by interferon- γ .

than SDF-1 α . We also analysed an analogue of peptide 3 that had both the Leu₄ and Ile₁₁ substitutions (Table 2). Leu₄Ile₁₁ peptide 3 (1–12) did not show any significant selectivity but was approx. 4-fold more potent as an inhibitor of all the chemokines tested (Table 2), compared with peptide 3 (1–12).

Binding characteristics of peptides 1, 2 and 3 for the DARC receptor on erythrocytes

Based on the results described above, peptide 3 (1–12) may be an antagonist of multiple chemokine receptors, as it inhibits THP-1 migration induced by a range of CC and CXC chemokines. It is therefore possible that peptide 3 (1–12) also binds to DARC receptors expressed on erythrocytes, which would limit any use of peptide 3 (1–12) as a chemokine inhibitor *in vivo* since erythrocytes would severely limit peptide 3 (1–12) bioavailability in the circulation. Therefore, we investigated the DARC-binding characteristics of peptides 1, 2 and 3 to human erythrocytes. We used inhibition of ¹²⁵I-MCP-1 binding to erythrocytes as an indirect measure of peptide binding to DARC (see the Materials and methods section). Consistent with other published results, Scatchard analysis of ¹²⁵I-MCP-1 binding to the DARC receptor on human erythrocytes was linear and characteristic of a single class of binding sites, with a K_d of approx. 5 nM (Figure 5A) [16]. Peptide 1 was a weak inhibitor of MCP-1 binding to DARC (ED_{50} = 90 μ M; Figure 5). In marked contrast, peptide 2, which had little or no effect on THP-1 migration, showed high-affinity binding to DARC (ED_{50} = 350 nM; Figure 5). Peptide 3 (1–12) also binds to DARC, although it binds to DARC on erythrocytes with only a similar affinity to that with which it binds to THP-1 cells (6–8 μ M; Figure 5). The analogues of peptide 3 inhibited MCP-1 binding to the DARC to a lesser extent than peptide 3 (1–12). For example, peptide 3 (7–12) had an ED_{50} of 15 μ M and Ser₇ peptide 3 (1–12) an ED_{50} of 25 μ M (results not shown).

Leu₄Ile₁₁ peptide 3 (1–12) was a weak inhibitor of MCP-1 binding to DARC (ED_{50} = 90 μ M), similarly to peptide 1 (Figure 5). As a result of the weak binding of Leu₄Ile₁₁ peptide 3 (1–12) to DARC and its potent inhibition of THP-1 migration induced by both CC and CXC chemokines, Leu₄Ile₁₁ peptide 3 (1–12) is the best candidate among the peptides tested here for use as a chemokine inhibitor *in vivo*.

DISCUSSION

We have identified an oligopeptide sequence derived from the human MCP-1 sequence, designated peptide 3 (1–12), which is an inhibitor of both human mononuclear-cell and THP-1-cell migration induced by CC or CXC chemokines *in vitro*. Peptide 3 (1–12) shows little selectivity as an inhibitor of migration induced by either family of chemokines, with typical ED_{50} values of approx. 8 μ M against CC chemokines and approx. 10–14 μ M against CXC chemokines (Figure 3). Peptide 3 (1–12) and its derivatives had no effect on THP-1 migration induced by non-chemokines such as TGF- β 1 or fMLP, even at 100 μ M (Tables 1 and 2). The effects of chemokine-induced migration cannot therefore be due to the effects of the peptide on cell viability or any other non-specific mechanism inhibiting migration. Taken together, these results demonstrate that peptide 3 (1–12) is a specific inhibitor of migration induced by chemokines, but inhibits irrespective of the nature of the chemokine used.

Consistent with previous reports suggesting that MCP-1 functions as a dimer [19], peptide 1, an N-terminal 13-mer, was a more efficient inhibitor of MCP-1-induced migration when incubated with the chemokine in the lower compartment than when incubated with the cells in the upper compartment. In marked contrast, peptide 3 (1–12) was much less effective when incubated with the chemokine than with the cells. Since a biotinylated peptide 3 (3–12) bound to the surface of THP-1 cells

Figure 5 Binding of peptides 1, 2 and 3 to DARC on human erythrocytes

(A) Binding data for 125 I-MCP-1 binding to freshly prepared human erythrocytes transformed by Scatchard analysis, as described in the Materials and methods section. 125 I-MCP-1 binding to human erythrocytes was linear and characteristic of a single class of binding sites with a K_d of approx. 5 nM, consistent with the published characteristics of the DARC receptor [16]. Inhibition of specific binding of 0.5 nM 125 I-MCP-1 to human erythrocytes by (B) peptide 1, (C) peptide 2 (note the change in the x-axis scale), (D) peptide 3 and (E) Leu₄Ile₁₁ peptide 3 (1-12) are shown.

with an association constant of approx. 10 μ M, it is likely that peptide 3 (1-12) inhibits THP-1 migration by acting as a receptor antagonist on the cells rather than binding to the MCP-1 ligand.

By analysing the structure-function relationship of various oligopeptide sequence variants of peptide 3 (1-12) we have demonstrated that the C-terminal 6-mer is as good an inhibitor

of THP-1 migration induced by either subfamily of chemokines as the original peptide-3 sequence (Table 2). Many of the oligopeptide sequences we investigated had very similar properties to the original peptide-3 sequence (Table 2). However, one sequence variant of peptide 3, Leu₄Ile₁₁ peptide 3 (1–12), showed a 3–4-fold greater potency for CC and CXC chemokines than the peptide 3 (1–12) sequence (Table 2).

Before predicting whether any chemokine inhibitor might have any anti-inflammatory properties *in vivo*, it is important to take into consideration the binding characteristics of the inhibitor to the DARC receptor on erythrocytes. It is well established that many chemokines have a high affinity for the DARC receptor [15,16,27]. Therefore, any chemokine inhibitor that may have significant affinity for the DARC receptor will be an unsuitable candidate for use *in vivo*. We therefore investigated DARC-receptor-binding characteristics of the peptides we identified using an iodinated MCP-1-binding assay. We inferred that the original peptide-3 sequence had a very similar affinity for the DARC receptor on erythrocytes as for binding to THP-1 cells (expressing specific chemokine receptors but not DARC). Therefore, it would be unlikely that this peptide could be used to inhibit chemokine signalling *in vivo* as its bioavailability in plasma would be expected to be low, as there is a huge excess of erythrocytes which would act as a sink, binding all the available peptide molecules. However, several of the peptide-3-related sequences that we investigated showed a lower affinity for the DARC receptor while retaining similar affinities for the specific chemokine receptors on THP-1 cells.

Leu₄Ile₁₁ peptide 3 (1–12) bound relatively weakly to DARC receptors on erythrocytes ($ED_{50} = 90 \mu\text{M}$; Figure 5), whereas its ED_{50} for inhibiting THP-1 migration induced by chemokines was 2–4 μM (Table 2). Leu₄Ile₁₁ peptide 3 (1–12) is therefore likely to be the best candidate, of the peptides we investigated, for use as a chemokine inhibitor *in vivo*. However, any anti-inflammatory properties of Leu₄Ile₁₁ peptide 3 (1–12) will need to be investigated in animal models of inflammatory diseases, for example in the rat glomerulonephritis model, where it has been demonstrated clearly that the inflammatory response is chemokine-dependent, since neutralizing antibodies to MCP-1 reduced the inflammation by 60% compared with control animals [21,22].

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REFERENCES

- Rollins, B. J. (1997) *Blood* **90**, 909–928
- Luster, A. D. (1998) *N. Eng. J. Med.* **338**, 436–445
- Koch, A. E., Kunkel, S. L., Harlow, L. A., Johnson, B., Evanoff, H. L., Haines, G. K., Burdick, M. D., Pope, R. M. and Strieter, R. M. (1992) *J. Clin. Invest.* **90**, 772–779
- Kondo, T., Watarai, Y., Novick, A. C., Toma, H. and Fairchild, R. L. (1997) *Transplantation* **63**, 732–742
- Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clarklewis, I., Sodroski, J. and Springer, T. A. (1996) *Nature (London)* **382**, 829–833
- Cocchi, F., Devico, A. L., Garzinodemo, A., Arya, S. K., Gallo, R. C. and Lusso, P. (1995) *Science* **270**, 1811–1815
- Nelken, N. A., Coughlin, S. R., Gordon, D. and Wilcox, J. N. (1991) *J. Clin. Invest.* **88**, 1121–1127
- Ylaherttuala, S., Lipton, B. A., Rosenfeld, M. E., Sarkioja, T., Yoshimura, T., Leonard, E. J., Witztum, J. L. and Steinberg, D. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5252–5256
- Uguccioni, M., Dapuzzo, M., Loetscher, M., Dewald, B. and Baggiolini, M. (1995) *Eur. J. Immunol.* **25**, 64–68
- Valente, A. J., Graves, D. T., Viallevalentin, C. E., Delgado, R. and Schwartz, C. J. (1988) *Biochemistry* **27**, 4162–4168
- Larsen, C. G., Anderson, A. O., Appella, E., Oppenheim, J. J. and Matsushima, K. (1989) *Science* **243**, 1464–1466
- Conti, P., Pang, X. Z., Boucher, W., Letourneau, R., Reale, M., Barbacane, R. C., Thibault, J. and Theoharides, T. C. (1997) *Blood* **89**, 4120–4127
- Conti, P., Pang, X. Z., Boucher, W., Letourneau, R., Reale, M., Barbacane, R. C., Thibault, J. and Theoharides, T. C. (1997) *Int. Immunol.* **9**, 1563–1570
- Foster, S. J., Aked, D. M., Schroder, J. M. and Christophers, E. (1989) *Immunology* **67**, 181–183
- Ahuja, S. K., Gao, J. L. and Murphy, P. M. (1994) *Immunol. Today* **15**, 281–287
- Neote, K., Darbonne, W., Ogez, J., Horuk, R. and Schall, T. J. (1993) *J. Biol. Chem.* **268**, 12247–12249
- Hadley, T. J., Lu, Z. H., Wasniewska, K., Martin, A. W., Peiper, S. C., Hesselgesser, J. and Horuk, R. (1994) *J. Clin. Invest.* **94**, 985–991
- Peiper, S. C., Wang, Z. X., Neote, K., Martin, A. W., Showell, H. J., Conklyn, M. J., Ogborne, K., Hadley, T. J., Lu, Z. H., Hesselgesser, J. and Horuk, R. (1995) *J. Exp. Med.* **181**, 1311–1317
- Zhang, Y. J. and Rollins, B. J. (1995) *Mol. Cell. Biol.* **15**, 4851–4855
- Gong, J. H. and Clarklewis, I. (1995) *J. Exp. Med.* **181**, 631–640
- Brown, Z., Robson, R. L. and Westwick, J. (1996) *J. Leukocyte Biol.* **59**, 75–80
- Wada, T., Yokoyama, H., Furuichi, K., Kobayashi, K. I., Harada, K., Naruto, M., Su, S. B., Akiyama, M., Mukaida, N. and Matsushima, K. (1996) *FASEB J.* **10**, 1418–1425
- Pattison, J., Nelson, P. J., Huie, P., Vonleutichau, I., Farshid, G., Sibley, R. K. and Krensky, A. M. (1994) *Lancet* **343**, 209–211
- Elsner, J., Petering, H., Hochstetter, R., Kimmig, D., Wells, T. N. C., Kapp, A. and Proudfoot, A. E. J. (1997) *Eur. J. Immunol.* **27**, 2892–2898
- Kledal, T. N., Rosenkilde, M. M., Coulin, F., Simmons, G., Johnsen, A. H., Alouani, S., Power, C. A., Lutichau, H. R., Gerstoft, J., Clapham, P. R. et al. (1997) *Science* **277**, 1656–1659
- Denholm, E. M. and Stankus, G. P. (1995) *Cytokine* **7**, 436–440
- Horuk, R., Colby, T. J., Darbonne, W. C., Schall, T. J. and Neote, K. (1993) *Biochemistry* **32**, 5733–5738
- Rollins, B. J., Stier, P., Ernst, T. and Wong, G. G. (1989) *Mol. Cell. Biol.* **9**, 4687–4695
- Rollins, B. J., Morrison, E. D. and Stiles, C. D. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3738–3742
- Obaru, K., Fukada, M., Maeda, S. and Shimada, K. J. (1986) *Biochemistry* **99**, 885–894
- Shirozu, M., Nakano, T., Inazawa, J., Tashiro, K., Tada, H., Shinohara, T. and Honjo, T. (1995) *Genomics* **28**, 495–500
- Yoshimura, T., Matsushima, K., Tanaka, S., Robinson, E. A., Appella, E., Oppenheim, J. J. and Leonard, E. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 9233–9237

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