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(54) Title: PEPTIDE ANTAGONIST OF MULTIPLE CHEMOKINE RECEPTORS AND USES THEREOF

(57) Abstract: The present invention provides a peptide, with the structure of Formula (I), having antagonist activity toward multiple chemokine receptors: NH_2-F P M F K R G R - X wherein X is: C; K; a covalent linker; C* --- C* R G R K F M P F - NH_2, wherein C* --- C* represents a disulphide bridge; K - R G R K F M P F - NH_2, wherein K - R represents an amide bond between the ε-amino group of K and the carboxyl group of R; or a covalent linker additionally bound to a second NH_2 - F P M F K R G R at its C-terminal R, and wherein NH_2 represents the N-terminus. The present invention also teaches methods for the production and testing of the peptide antagonist, in addition to methods of chemically modifying the peptide of Formula (I) to enhance bioavailability and/or activity. Also provided is a therapeutic method comprising administration of the peptide antagonist to a patient in need of a reduction of chemokine receptor(s) function in the treatment of inflammatory diseases or disorders or for the prevention or reduction of HIV infection.

PEPTIDE ANTAGONIST OF MULTIPLE CHEMOKINE RECEPTORS AND USES THEREOF

FIELD OF THE INVENTION

The present invention pertains to the field of peptide antagonists of chemokine receptors and therapeutic uses thereof.

BACKGROUND

Peptide antagonists for chemokine receptors are useful in the treatment of diseases and disorders in which chemokine receptors play a role. For example, chemokine receptors, such as CCR-1, CCR-2, CCR-2A, CCR-2B, CCR-3, CCR-4, CCR-5, CXCR-3, CXCR-4, have been implicated as being important mediators of inflammatory and immunoregulatory disorders and diseases, including asthma and allergic diseases, as well as autoimmune pathologies such as rheumatoid arthritis and atherosclerosis. Accordingly, agents which modulate chemokine receptors would be useful in the treatment and prevention of such conditions.

Further, it has recently been recognised that for efficient entry into target cells, human immunodeficiency viruses require the chemokine receptors CCR-5 and CXCR-4, as well as the primary receptor CD4 (Levy, N. Engl. J. Med., 335(20), 1528-1530 (Nov. 14, 1996). Accordingly, an agent which could block chemokine receptors in humans who possess normal chemokine receptors should prevent infection in healthy individuals and slow or halt viral progression in infected patients (see Science, 275, 1261-1264 (1997)).

Chemokines are chemotactic cytokines that are released by a wide variety of cells to attract macrophages, T cells, eosinophils, basophils and neutrophils to sites of inflammation (reviewed in Schall, Cytokine, 3, 165-183 (1991) and Murphy, Rev. Immun., 12, 593-633 (1994)). There are two classes of chemokines, C-X-C ((α) and C-C (β), depending on whether the first two cysteines are separated by a single amino acid (C-X-C) or are adjacent (C-C). The α-chemokines, such as interleukin-8 (IL-8), neutrophil-activating protein-2 (NAP-2) and melanoma growth stimulatory activity protein (MGSA) are chemotactic primarily for neutrophils, whereas β-chemokines, such as RANTES, MIP-1α, MIP-1β, monocyte chemotactic protein-1 (MCP-1), MCP-2, MCP-3 and eotaxin are chemotactic for macrophages, T-cells, eosinophils and basophils (Deng, et al., Nature, 381, 661-666 (1996)).

The chemokines bind specific cell-surface receptors belonging to the family of G-protein-coupled seven-transmembrane-domain proteins (reviewed in Horuk, Trends Pharm. Sci., 15, 159-165 (1994)) which are termed "chemokine receptors." On binding their cognate ligands, chemokine receptors transduce an intracellular signal though the associated trimeric G protein, resulting in a rapid increase in intracellular calcium concentration. There are at least seven human chemokine receptors that bind or respond to β-chemokines with the following characteristic pattern: CCR-1 (or "CKR-1" or "CC-CKR-1") [MIP-1α, MIP-1β, MCP-3, RANTES] (Ben-Barruch, et al., J. Biol. Chem., 270, 22123-22128 (1995); Beote, et al, Cell, 72, 415-425 (1993)); CCR-2A and CCR-2B (or "CKR-2A"/"CKR-2A" or "CC-CKR-2A"/"CC-CKR-2A") [MCP-1, MCP-3, MCP-4]; CCR-3 (or "CKR-3" or "CC-CKR-3") [eotaxin, RANTES, MCP-3] (Combadiere, et al., J. Biol. Chem., 270, 16491-16494 (1995); CCR-4 (or "CKR-4" or "CC-CKR-4") [MIP-1α, RANTES, MCP-1] (Power, et al., J. Biol. Chem., 270, 19495-19500 (1995)); CCR-5 (or "CKR-5" or "CC-CKR-5") [MIP-1α, RANTES, MIP-1.beta.] (Sanson, et al., Biochemistry, 35, 3362-3367 (1996)); and the Duffy blood-group antigen [RANTES, MCP-1] (Chaudhun, et al., J. Biol. Chem., 269, 7835-7838 (1994)). The β-chemokines include eotaxin, MIP ("macrophage inflammatory protein"), MCP ("monocyte chemoattractant protein") and RANTES ("regulation-upon-activation, normal T expressed and secreted").

An antagonist that is capable of affecting multiple chemokine receptors would be expected to act as a broad spectrum therapeutic in the treatment and prevention of conditions including, but not limited to, inflammatory diseases and disorders, cancer and HIV infection.

Peptide antagonists of receptors are well known in the art. For example International patent application Nos. WO 96/40772 and WO 98/09642 pertain to small peptides and peptide dimers which act as agonists and antagonists of various receptor activities. These applications make use of fragments of receptor ligands which are able to modulate single receptor activities. International patent application No. WO 99/47158 expands on these findings and provides small peptide antagonists for a single receptor, CXCR4, derived from the N-terminal portion of SDF-1. The therapeutic use of these peptide antagonists is limited by the fact that they are specific for single receptor targets and, therefore, will modulate only one component involved in the disease or disorder. Therefore, a need remains for peptide antagonists with the ability to modulate the activity of multiple chemokine receptors.

This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No

admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention. Publications referred to throughout the specification are hereby incorporated by reference in their entireties in this application.

SUMMARY OF THE INVENTION

One object of the present invention is to provide a peptide antagonist of multiple chemokine receptors and uses thereof. This peptide antagonist modulates the activity of multiple chemokine receptors. In accordance with one aspect of the present invention there is provided a peptide, with the structure of Formula I, having antagonist activity toward multiple chemokine receptors:

$$NH_2 — F\ P\ M\ F\ K\ R\ G\ R — X \qquad (I)$$

wherein X is: C; K; a covalent linker; C* --- C* R G R K F M P F — NH_2 wherein C*--- C* represents a disulphide bridge; K — R G R K F M P F — NH_2 wherein K — R represents an amide bond between the ε-amino group of K and the carboxyl group of R; or a covalent linker additionally bound to a second NH_2 — F P M F K R G R at its C-terminal R.

In accordance with another aspect of the present invention there is provided a composition comprising the peptide of Formula I.

In accordance with another aspect of the present invention there is provided a use of the peptide of Formula I in the treatment and prevention inflammatory and immunoregulatory disorders and diseases in mammals, including humans.

In accordance with another aspect of the present invention there is provided a use of the peptide of Formula I in the treatment of mammals, including humans, to prevent or reduce HIV-1 infection.

BRIEF DESCRIPTION OF THE FIGURES AND TABLES

Table 1 lists the inhibition of chemokine binding on receptors by various chemokine N-terminal peptides. The results are presented as IC_{50} values measured in μM. * IC_{50} is the concentration of the ligands that inhibit each chemokine's maximal binding on the

receptor by 50 % (the concentration of the chemokine used was 4 nM); ** no binding; *** SDF-1 dimer is an agonist, whereas SDF, P2G dimer is an antagonist for CXCR4.

Table 2 shows the inhibition of chemokine binding on receptors and chemotaxis by the MCRA dimer peptide. * IC_{50} is the concentration of the MCRA dimer that inhibits the chemokine receptor binding or activity by 50 % (the concentration of the chemokine used was 4 nM for binding and 10 nM for chemotaxis); ** not detectable; *** and **** the IC_{50} of inhibition of chemotaxis by MCRA dimer was determined when MIP-1α was used at 2 nM, or MCP-1 was used at 5 nM.

Table 3 presents the level of cell infiltration (scores) into the lung following treatment with a control peptide or the MCRA dimer, measured at 24, 48 and 52 hours after treatment. The scores are presented in graphical form in figure 16.

Table 4 presents the level of cell infiltration (scores) into the bronchoalveolar lavage fluid (BAL) following treatment with a control peptide, MCRA(1-8)$_2$K, an MCP-1 antagonist and MCRA(1-8)$_2$K plus the MCP-1 antagonist. The scores are presented in graphical form in figure 18.

Table 5 presents level of cell infiltration (scores) into the lung following treatment with a control peptide, MCRA(1-8)$_2$K, an MCP-1 antagonist and MCRA(1-8)$_2$K plus the MCP-1 antagonist. The scores are presented in graphical form in figure 19.

Figure 1 demonstrates that the MCRA monomer and dimer (at a concentration of up to 1 μM) had no significant activity on their parent receptor (CXCR3), except at extremely high concentrations (e.g. 10 μM). The activity of 10 μM MCRA dimer was found to be equal to that of 1 nM native MCRA. Thus, the activity of MCRA peptides is around 1×10^4 fold lower than that of the native MCRA ligand.

Figure 2 demonstrates that the MCRA dimer has no activity on certain indicated chemokine receptors (no calcium induction) but has desensitised the activity of Eotaxin, SDF-1, and RANTES, very weak activity for MCP-1, and no inhibition for IL-8.

Figure 3 indicates the inhibition of Eotaxin binding to CCR3 by the MCRA monomer and dimer.

Figure 4a indicates that the MCRA(1-8)$_2$K binds to CCR3 receptor with slightly higher affinity than the MCRA dimer. Figure 4b shows that the MCRA(1-9)K1 dimer bound to

CCR3 receptor with the same potency as MCRA dimer. The MCRA(1-9)K1 dimer was prepared by replacement of the N-terminal phenylalanine of MCRA(1-8)$_2$K with lysine .

Figure 5 demonstrates the inhibition of Eotaxin chemotaxis of CCR3 cells with the MCRA dimer.

Figure 6 indicates the inhibition of RANTES binding to CCR5 by the MCRA monomer and dimer.

Figure 7 demonstrates that the MCRA(1-8)2K binds to CCR5 receptor with a slightly lower affinity than that of the MCRA dimer.

Figure 8 indicates the inhibition of RANTES chemotaxis of CCR5 cells with the MCRA dimer.

Figure 9 indicates the inhibition of SDF-1 binding to CXCR4 by the MCRA monomer and dimer.

Figure 10a demonstrates that the MCRA(1-8)$_2$K has the same potency as the MCRA dimer with respect to binding to the CXCR4 receptor. Figure 10b shows that MCRA(1-9)K1 dimer has the same potency as MCRA dimer with respect to binding to the CXCR4 receptor.

Figure 11 shows the inhibition of SDF-1 chemotaxis on CXCR4 cells with the MCRA dimer.

Figure 12 shows the inhibition of MIP-1α binding to CCR1 by the MCRA monomer and dimer.

Figure 13 shows the inhibition of MIP-1α chemotaxis of CCR1 cells with the MCRA dimer.

Figure 14 shows the inhibition of MCP-1 binding to CCR2 by the MCRA monomer and dimer.

Figure 15 indicates the inhibition of MCP-1 chemotaxis of CCR2 cells with the MCRA dimer.

Figure 16 demonstrates the low binding on CXCR3 receptor by MCRA monomer and dimer.

Figure 17 demonstrates that the MCRA dimer treatment systemically inhibits the cell infiltration into the lung in an asthmatic mouse model using C57 B/6 mice induced with chicken ovalbumin to develop asthma characterised by infiltration into the lung.

Figure 18 shows the number of cells found in the BAL fluid of asthmatic C57 B/6 mice following treatment with a control peptide, MCRA(1-8)$_2$K, an MCP-1 antagonist and MCRA(1-8)$_2$K plus the MCP-1 antagonist. This figure demonstrates the effect of MCRA(1-8)$_2$K on cell infiltration into the lungs of asthmatic mice.

Figure 19 shows the score of cell infiltrates found in the BAL fluid of asthmatic C57 B/6 mice following treatment with a control peptide, MCRA(1-8)$_2$K, an MCP-1 antagonist and MCRA(1-8)$_2$K plus the MCP-1 antagonist. This figure demonstrates the effect of MCRA(1-8)$_2$K on cell infiltration into the lungs of asthmatic mice.

DETAILED DESCRIPTION OF THE INVENTION

In response to the need for broad spectrum chemokine receptor antagonists, the present invention provides a new peptide that exhibits antagonist activity toward various chemokine receptors.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in spectroscopy, drug discovery, cell culture, molecular genetics, diagnostics, amino acid and nucleic acid chemistry described below are those well known and commonly employed in the art. Standard techniques are typically used for signal detection, recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection). Furthermore, the standard single letter code for reference to amino acids is used throughout the disclosure.

The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which are provided throughout this document. Standard

techniques are used for chemical syntheses, chemical analyses, and biological assays. As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

"Protein" refers to a whole protein, or fragment thereof, such as a protein domain or a binding site for a second messenger, co-factor, ion, etc. It can be a peptide or an amino acid sequence that functions as a signal for another protein in the system, such as a proteolytic cleavage site.

"Nucleic acid" refers to DNA and RNA and can be either double stranded or single stranded. The invention also includes nucleic acid sequences which are complementary to the claimed nucleic acid sequences.

"Isolated polynucleotide" or "isolated nucleic acid" refers a polynucleotide of genomic, cDNA, or synthetic origin or some combination thereof, which by virtue of its origin the "isolated polynucleotide" (1) is not associated with the cell in which the "isolated polynucleotide" is found in nature, or (2) is operably linked to a polynucleotide which it is not linked to in nature.

"Isolated protein" refers a protein of natural or synthetic origin or some combination thereof, which by virtue of its origin the "isolated protein" (1) is not associated with proteins found it is normally found with in nature, or (2) is isolated from the cell in which it normally occurs or (3) is isolated free of other proteins from the same cellular source, e.g. free of human proteins, or (4) is expressed by a cell from a different species, or (5) does not occur in nature. "Isolated naturally occurring protein" refers to a protein which by virtue of its origin the "isolated naturally occurring protein" (1) is not associated with proteins that it is normally found with in nature, or (2) is isolated from the cell in which it normally occurs or (3) is isolated free of other proteins from the same cellular source, e.g. free of human proteins.

"Polypeptide" as used herein as a generic term to refer to native protein, fragments, or analogs of a polypeptide sequence. Hence, native protein, fragments, and analogs are species of the polypeptide genus.

The term "multiple chemokine receptor antagonist peptide" or "MCRA peptide", as used herein, refers to a peptide of Formula I, or a fragment, derivative or analogue thereof.

"Naturally-occurring" as used herein, as applied to an object, refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that

is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

"Polynucleotide" refers to a polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA or RNA.

"Corresponds to" refers to a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

The term "modifying group" refers to groups that are not naturally coupled to MRCA peptides in their native form. Accordingly, the term "modifying group" is not intended to include hydrogen.

The term "covalent linker" refers to a covalent bridge forming moiety and includes, but is not limited to ornithine, L-amino-N-butyric acid, and benzene.

Other chemistry terms herein are used according to conventional usage in the art, as exemplified by The McGraw-Hill Dictionary of Chemical Terms (ed. Parker, S., 1985), McGraw-Hill, San Francisco, incorporated herein by reference).

Characterization of the Multiple Chemokine Receptor Antagonist Peptide

The present invention provides a peptide, and variants thereof, having antagonist activity toward multiple chemokine receptors, referred to as the Multiple Chemokine Receptor Antagonist (MCRA) peptide, with the structure of Formula I:

$$NH_2 — F\ P\ M\ F\ K\ R\ G\ R — X \qquad (I)$$

wherein X is: C; K; a covalent linker; C* --- C* R G R K F M P F — NH_2, wherein C*--- C* represents a disulphide bridge; K — R G R K F M P F — NH_2, wherein K — R represents an amide bond between the ε-amino group of K and the carboxyl group of R;

or a covalent linker additionally bound to a second NH_2 — F P M F K R G R at its C-terminal R. When X is C*---C* R G R K F M P F — NH_2, the peptide is referred to as the MCRA dimer. When X is K — R G R K F M P F — NH_2, the peptide may be referred to as MCRA(1-8)$_2$K. It should be understood that NH_2 is used to indicate the location of the N-terminus of the MRCA peptide, or portion thereof, and the free amino group may or may not be positively charged.

In one embodiment of the present invention the MCRA peptide is a peptide having an amino acid sequence that is F P M F K R G R C or F P M F K R G R K.

In one embodiment of the present invention the MCRA dimer has the structure of Formula II:

$$\begin{array}{l} NH_2\text{—F P M F K R G R C*} \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \vdots \\ NH_2\text{—F P M F K R G R C*} \end{array} \qquad \text{(II)}$$

wherein C* --- C* represents a disulphide bridge between the sulphur atoms of the cysteine residues.

In another embodiment of the present invention the MCRA dimer has the structure of Formula III:

$$\begin{array}{l} NH_2\text{—F P M F K R G R K} \\ \qquad\qquad\qquad\qquad\qquad\quad \vert \\ \quad NH_2\text{—F P M F K R G R} \end{array} \qquad \text{(III)}$$

wherein K — R represents an amide bond between the ε-amino group of K and the carboxyl group of R.

The peptides of the present invention exhibit antagonist activities towards multiple chemokine receptors, i.e. two or greater. In certain instances the MCRA peptide of the present invention demonstrates a chemokine receptor antagonist activity profile comprising antagonistic activity against multiple chemokine receptors with varying potency.

In one aspect of the present invention the MCRA peptide is a multiple antagonist for CCR3, CCR5, CXCR4, CCR1, and CCR2. The receptor binding potencies of the MCRA dimer on the aforementioned receptors have been determined and are shown in Table 2.

In one embodiment of the present invention the MCRA peptides are used to inhibit migration of various types of leukocytes (including monocyte, Th2 type T lymphocyte and eosinophils). This is possible because the MCRA peptide exhibits multiple potent antagonist activities toward various chemokine receptors which mediate these migrations.

Preparation of Multiple Chemokine Receptor Antagonist Peptide

The MCRA peptide can be produced using standard synthetic techniques known in the art, such as those described in Bodansky, M. Principles of Peptide Synthesis, Springer Verlag, Berlin (1993) and Grant, G.A. (ed.) Synthetic Peptides: A User's guide, W.H. Freeman and Company, New York (1992). Automated Peptide Synthesizers are commercially available, (e.g., Advanced Chemtech Model 396; Milligen/Biosearch. Additionally, one or more modulating groups can be attached to the MCRA peptide by standard methods, for example using methods for reaction through an amino group (e.g., the alpha-amino group at the amino terminus of a peptide), a carboxyl group (e.g., at the carboxy terminus of a peptide), a hydroxyl group, (e.g., on a tyrosine, serine, or threonine residue) or other suitable reactive group on an amino acid side chain (e.g., see Greene, T.W. and Wuts P.G.M. Protective groups in Organic Synthesis, John Wiley and Sons, Inc., New York (1991)).

In another aspect of the present invention, the MCRA peptide can be prepared according to standard recombinant DNA techniques using a nucleic acid molecule encoding the peptide. A nucleotide sequence encoding the peptide can be determined using the genetic code and an oligonucleotide molecule having this nucleotide sequence can be synthesized by standard DNA synthesis methods (e.g., using an automated DNA synthesizer). Alternatively, a DNA molecule encoding a peptide can be derived from the natural precursor protein gene (i.e. the chemokine for CXCR3) or cDNA (e.g., using the polymerase chain reaction (PCR) and/or restriction enzyme digestion) according to standard molecular biology techniques.

The invention also provides an isolated nucleic acid comprising a nucleotide sequence encoding the MCRA peptide of the present invention. In some embodiments, the peptide may comprise an amino acid sequence having at least one amino acid deletion compared to the MCRA peptide. In alternative embodiments, the isolated nucleic acid encodes a peptide wherein one or more amino acids are deleted from the N-terminus, C-terminus,

and/or an internal site of the MCRA peptide. In yet further embodiments, the isolated nucleic acid encodes a peptide fragment having one or more amino acids deleted compared to native MCRA peptide.

To facilitate expression of a peptide compound in a host cell by standard recombinant DNA techniques, the isolated nucleic acid encoding the peptide may be incorporated into a recombinant expression vector. Accordingly, the invention also provides recombinant expression vectors comprising the nucleic acid molecules of the invention. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a plasmid, which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors".

In recombinant expression vectors of the present invention, the nucleotide sequence encoding a peptide may be operatively linked to one or more regulatory sequences, selected on the basis of host cells to be used for expression. Regulatory sequences include those that direct constitutive expression of a nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences) and those that direct expression in a manner that can be regulated (e.g., only in the presence of an inducing agent). It will be appreciated by those skilled in the art that the design of the expression vector may depend on such factors as the choice of host cell to be transformed, the level of expression of peptide compound desired, etc. The expression vectors of the invention can be introduced into host cells thereby to produce peptide compounds encoded by nucleic acids as described herein.

The recombinant expression vectors of the present invention can be designed for expression of peptide compounds in prokaryotic or eukaryotic cells. For example, peptide compounds can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goddel; Gene Expression Technology: Methods in Enzymology 185 Academic Press, San Diego, Calif. (1990). Alternatively, the recombinant expression

vector may be transcribed and translated *in vitro*, for example using the T7 promoter regulatory sequences and T7 polymerase. Example of vectors for expression in yeast *S. cerivisae* include pYepSec1 (Baldari et al. (1987) EMBO J. 6:229-234), pMFa (Kurjan and Herskowitz, (1982) Cell 30:933-943), pJRY88 (Schultz et al. (1987) Gene 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, Calif.). Baculovirus vectors available for expression of proteins or peptides in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith et al. (1983) Virology 170:31-39). Examples of mammalian expression vectors include pCDM8 (Seed, B., (1987) Nature 329:840) and pMT2PC (Kaufman et al. (1987), EMBO J. 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40.

In addition to the regulatory sequences discussed above, the recombinant expression vector may contain additional nucleotide sequences. For example, the recombinant expression vector may encode a selectable marker gene to identify host cells that have incorporated the vector. Such selectable, marker genes are well known in the art. Moreover, to facilitate secretion of the peptide compound from a host cell, in particular mammalian host cells, the recombinant expression vector preferably encodes a signal sequence operatively linked to sequences encoding the MCRA peptide of the present invention. The signal sequence acts to carry the MCRA peptide into the secretory pathway of the cell and is then cleaved, allowing for release of the mature peptide compound (i.e., the MCRA peptide without the signal sequence) from the host cell. Use of a signal sequence to facilitate secretion of proteins or peptides from mammalian host cells is well known in the art.

The MCRA peptide can be produced as a fusion protein. For example, the expression vector pUR278 (Ruther et al. (1983) EMBO J. 2, 1791), can be used to create lacZ fusion proteins. The pGEX vectors can be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can be easily purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

Alternatively, any fusion protein can be readily purified by utilising an antibody specific for the fusion protein being expressed. For example, a system described in Janknecht et al. (1981) Proc. Natl. Acad. Sci. *USA* 88, 8972, allows for the ready purification of non-denatured fusion proteins expressed in human cell lines. In this system, the gene of

interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+} nitriloacetic acid-agarose columns, and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

In addition, a host cell may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in a specific, desired fashion. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product can be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, choroid plexus cell lines.

Accordingly, the invention also provides host cells containing the recombinant expression vectors of the invention. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It us understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term used herein. A host cell may be any prokaryotic or eukaryotic cell. The MCRA peptide can be produced in a prokaryotic host (e.g., *E. coli* or *B. subtilis*) or in a eukaryotic host (e.g., *Saccharomyces* or *Pichia*; mammalian cells, e.g., COS, NIH 3T3, CHO, BHK, 293, or HeLa cells; or insect cells). In a preferred embodiment, the peptide compound is expressed in mammalian cells *in vivo* in a mammalian subject to treat the subject through gene therapy (discussed further below). Preferably, the peptide compound encoded by the recombinant expression vector is secreted from the host cell upon being expressed in the host cell.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced in to the host cells along with the gene of interest. Preferred

selectable markers include those that confer resistance of drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker may be introduce in to a host cell on the same vector that as that encoding the peptide compound or may be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

Proteins and polypeptides can also be produced by plant cells. For plant cells viral expression vectors (e.g., cauliflower mosaic virus and tobacco mosaic virus) and plasmid expression vectors (e.g., Ti plasmid) are suitable. Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, Md.; also, see, e.g., Ausubel et al. (1994) Current Protocols in Molecular Biology, John Wiley & Sons, New York). The methods of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel *et al.* (1994) Current Protocols in Molecular Biology, John Wiley & Sons, New York; expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (Pouwels *et al.*, 1985, Supp. 1987).

Modification of MCRA Peptide

In one embodiment of the present invention the MCRA peptide is modified for such purposes as enhancing therapeutic or prophylactic efficacy, stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*), or post-translational modifications (e.g., to alter the phosphorylation pattern of protein). Such modified peptides, when designed to retain at least one activity of MCRA peptide, are considered functional equivalents of the polypeptides described in more detail herein. Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition.

In another embodiment of the present invention there is provided conservative variants of the MCRA peptide of Formula I that also exhibit antagonist activity toward multiple chemokine receptors. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (i.e. isosteric and/or isoelectric mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are can be divided into four families:

(1) Acidic = aspartate, glutamate;
(2) Basic = lysine, arginine, histidine;
(3) Nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and
(4) Uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine.

In similar fashion, the amino acid repertoire can be grouped as
(1) Acidic = aspartate, glutamate;
(2) Basic = lysine, arginine, histidine;
(3) Aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl;
(4) Aromatic = phenylalanine, tyrosine, tryptophan;
(5) Amide = asparagine, glutamine; and
(6) Sulphur-containing = cysteine and methionine. (See, for example, Biochemistry, 2d ed., by L. Stryer, (Ed.) W H Freeman and Co. (1981)).

In an other aspect of this invention, the residue can be deleted, added or replaced by any other residue as long as the resulting analogues retain antagonistic activities similar to MCRA. The derived peptides have substantial sequence similarity to MCRA, such as 40% sequence identity, 60% sequence identity, or 80% sequence identity to MCRA sequence, or a fragment thereof.

One embodiment of the present invention provides an analogue in which the N-terminal phenylalanine of MCRA is replaced with lysine (this analogue is referred to as MCRA(1-9)K1 in figure 4b and 10b). This analogue exhibits similar antagonistic activities to the MCRA dimer.

Whether a change in the amino acid sequence of a peptide results in a functional homologue (e.g. functional in the sense that the resulting polypeptide mimics the wild-type form) can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type peptide, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

Generally, those skilled in the art will recognise that peptides as described herein may be modified by a variety of chemical techniques to produce compounds having essentially the same activity as the unmodified peptide, and optionally having other desirable properties. For example the C-terminal may be modified by pegylation. For example,

carboxylic acid groups of the peptide, whether carboxyl-terminal or side chain, may be provided in the form of a salt of a pharmaceutically-acceptable cation or esterified to form a C_1–C_{16} ester, or converted to an amide of formula NR_1R_2 wherein R_1 and R_2 are each independently H or C_1–C_{16} alkyl, or combined to form a heterocyclic ring, such as 5- or 6-membered. Amino groups of the peptide, whether amino-terminal or sidechain, may be in the form of a pharmaceutically-acceptable acid addition salt, such as the HCl, HBr, acetic, benzoic, toluene sulphonic, maleic, tartaric and other organic salts, or may be modified to C_1–C_{16} alkyl or dialkyl amino or further converted to an amide. Hydroxyl groups of the peptide sidechain may be converted to C_1–C_{16} alkoxy or to a C_1-C_{16} ester using well-recognised techniques. Phenyl and phenolic rings of the peptide sidechain may be substituted with one or more halogen atoms, such as fluorine, chlorine, bromine or iodine, or with C_1-C_{16} alkyl, C_1-C_{16} alkoxy, carboxylic acids and esters thereof, or amides of such carboxylic acids. Methylene groups of the peptide sidechains can be extended to homologous C_2 –C_4 alkylenes. Thiols can be protected with any one of a number of well-recognised protecting groups, such as acetamide groups.

Those skilled in the art will also recognise methods for introducing cyclic structures into the peptides of this invention to select and provide conformational constraints to the structure that result in enhanced binding and/or stability. For example, a carboxyl-terminal or amino-terminal cysteine residue can be added to the peptide, so that when oxidised the peptide will contain a disulphide bond, thereby generating a cyclic peptide. Other peptide cyclising methods include the formation of thioethers and carboxyl- and amino-terminal amides and esters.

Peptidomimetic and organomimetic embodiments are also hereby explicitly declared to be within the scope of the present invention, whereby the three-dimensional arrangement of the chemical constituents of such peptido-and organomimetics mimic the three-dimensional arrangement of the peptide backbone and component amino acid sidechains in the peptide, resulting in such peptido- and organomimetics of the peptides of this invention having substantial biological activity. It is implied that a pharmacophore exists for each of the described activities of the I-TAC monomer and/or dimer. A pharmacophore is an idealised, three-dimensional definition of the structural requirements for biological activity. Peptido- and organomimetics can be designed to fit each pharmacophore with current computer modelling software (computer aided drug design). The degree of overlap between the specific activities of pharmacophores remains to be determined.

In addition to peptides consisting only of naturally occurring amino acids, peptidomimetics or peptide analogues are also provided. Peptide analogues are

commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" (Luthman, et al., A Textbook of Drug Design and Development, 14:386-406, 2d Ed., Harwood Academic Publishers (1996); Grante (1994) Angew. Chem. Int. Ed. Engl. 33:1699-1720; Fauchere (1986) Adv. Drug Res. 15:29; Veber and Freidinger (1985) TINS, p.392; and Evans, et al. (1987) J. Med. Chem. 30:1229). Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent or enhanced therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biological or pharmacological activity), such as naturally-occurring receptor-binding polypeptide, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: --CH_2NH--, --CH_2S--, --CH_2—CH_2 --, --CH=CH-- (cis and trans), --$COCH_2$ --, --$CH(OH)CH_2$ --, and –CH_2SO--, by methods known in the art and further described in the following references: Spatola, A. F. in Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., Vega Data (March 1983), Vol. 1, Issue 3, Peptide Backbone Modifications (general review); Morley (1980) Trends Pharm. Sci. pp. 463-468, (general review); Hudson, et al. (1979) Int. J. Pept. Prot. Res., 14:177-185 (--CH_2NH--, CH_2CH_2 --); Spatola, et al. (1986) Life Sci., 38:1243-1249 (--CH_2--S); Hann (1982) Chem. Soc. Perkin Trans. I, 307-314 (--CH=CH--, cis and trans); Almquist, et al. (1980) J. Med. Chem., 23:1392-1398, (--$COCH_2$ --); Jennings-White, et al, (1982) Tetrahedron Lett. 23:2533, (--$COCH_2$ --); Szelke, et al. (1982) European Appln. EP 45665 (--$CH(OH)CH_2$ --); Holladay, et al. (1983) Tetrahedron Lett., 24:4401-4404 (--$C(OH)CH_2$ --); and Hruby (1982) Life Sci., 31:189-199 (--CH_2--S--). Such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others.

Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) may be used to generate more stable peptides. In addition, constrained peptides comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods known in the art (Rizo, et al. (1992) Ann. Rev. Biochem., 61:387).

Synthetic or non-naturally occurring amino acids refer to amino acids which do not naturally occur *in vivo* but which, nevertheless, can be incorporated into the peptide structures described herein. Preferred synthetic amino acids are the D-α-amino acids of

naturally occurring L-α-amino acid as well as non-naturally occurring D- and L-α-amino acids represented by the formula H_2NCHR^5COOH where R^5 is 1) a lower alkyl group, 2) a cycloalkyl group of from 3 to 7 carbon atoms, 3) a heterocycle of from 3 to 7 carbon atoms and 1 to 2 heteroatoms selected from the group consisting of oxygen, sulphur, and nitrogen, 4) an aromatic residue of from 6 to 10 carbon atoms optionally having from 1 to 3 substituents on the aromatic nucleus selected from the group consisting of hydroxyl, lower alkoxy, amino, and carboxyl, 5) -alkylene-Y where alkylene is an alkylene group of from 1 to 7 carbon atoms and Y is selected from the group consisting of (a) hydroxy, (b) amino, (c) cycloalkyl and cycloalkenyl of from 3 to 7 carbon atoms, (d) aryl of from 6 to 10 carbon atoms optionally having from 1 to 3 substituents on the aromatic nucleus selected from the group consisting of hydroxyl, lower alkoxy, amino and carboxyl, (e) heterocyclic of from 3 to 7 carbon atoms and 1 to 2 heteroatoms selected from the group consisting of oxygen, sulphur, and nitrogen, (f) $--C(O)R^2$ where R^2 is selected from the group consisting of hydrogen, hydroxy, lower alkyl, lower alkoxy, and $–NR^3R^4$ where R^3 and R^4 are independently selected from the group consisting of hydrogen and lower alkyl, (g) $--S(O)_nR^6$ where n is an integer from 1 to 2 and R^6 is lower alkyl and with the proviso that R^5 does not define a side chain of a naturally occurring amino acid.

Other preferred synthetic amino acids include amino acids wherein the amino group is separated from the carboxyl group by more than one carbon atom such as β-alanine, γ-aminobutyric acid, and the like.

"Detectable label" refers to materials, which when covalently attached to the peptides and peptide mimetics of this invention, permit detection of the peptide and peptide mimetics *in vivo* in the patient to whom the peptide or peptide mimetic has been administered. Suitable detectable labels are well known in the art and include, by way of example, radioisotopes, fluorescent labels (e.g., fluorescein), and the like. The particular detectable label employed is not critical and is selected relative to the amount of label to be employed as well as the toxicity of the label at the amount of label employed. Selection of the label relative to such factors is well within the skill of the art.

Covalent attachment of the detectable label to the peptide or peptide mimetic is accomplished by conventional methods well known in the art. For example, when the ^{125}I radioisotope is employed as the detectable label, covalent attachment of ^{125}I to the peptide or the peptide mimetic can be achieved by incorporating the amino acid tyrosine into the peptide or peptide mimetic and then iodinating the peptide (see, e.g., Weaner, et al., Synthesis and Applications of Isotopically Labelled Compounds, pp. 137-140 (1994)). If tyrosine is not present in the peptide or peptide mimetic, incorporation of tyrosine to the N or C terminus of the peptide or peptide mimetic can be achieved by well-known

chemistry. Likewise, ^{32}P can be incorporated onto the peptide or peptide mimetic as a phosphate moiety through, for example, a hydroxyl group on the peptide or peptide mimetic using conventional chemistry.

Validation of MCRA Peptide Activity

Characterization of MCRA peptides can be carried out using *in vitro* assays, such as determination of chemokine activity by chemotaxis assay and intracellular calcium induction. The affinity of peptides to chemokine receptors can be determined using receptor binding assays. The receptor specificity of each peptide is tested using a binding assay, in which the candidate MCRA peptide is competing with the native chemokine for receptor binding, and calcium desensitization assay, which tests the usage of each receptor. The antagonist activity of peptide can be determined by inhibition of target cell migration (chemotaxis) or by calcium response. These assays are conducted using cell lines which express single receptors, for example mouse B-lymphoma cell line 300-19, as target cells. Since each target cell expresses only one type of chemokine receptor, such as only CCR3, the results shown are highly receptor specific (without cross reaction with other receptors). The details of exemplary methods are described herein.

N-terminal fragments of various chemokine have been synthesized, dimerized, and screened for binding on chemokine receptors (Table 1). The *in vitro* study shows that MCRA peptides are antagonists for multiple chemokine receptors, including CCR3, CCR5, CXCR4, CCR2 and CCR1. The MCRA peptides bind to these receptors with varying levels of potency (IC_{50} ranges from 4 μM to 100 μM), however, binding of these peptides does not result in receptor activation (Figure 2). MCRA peptides exhibit very weak binding to their parental receptor, CXCR3 (250 μM, table 2 and Fig. 16) and very low activity. Figure 1 shows that at very high concentration (10 μM), MCRA peptides induced calcium response. The activity was 10,000-fold lower than that of native ligand MCRA. Further experiments indicated that MCRA peptide exhibit a dose-dependent inhibition of the binding and activity of various chemokines via their receptors (Figures 3-16). MCRA peptides inhibited binding and activity of Eotaxin, MIP-1β, RANTES, SDF-1, to lesser degree MCP-1, MIP-1α via CCR3, CCR5, CXCR4, CCR2, CCR1 receptors.

Further studies demonstrated that MCRA peptides can be modified in many ways without loss of their antagonist properties. For example the MCRA can be monomer and dimer (Figures 1-18). Another example is that the disulfide bridge can be replaced by lysine

(Figures 4a, 7, and 10a). And another example is that the N-terminal residue phenylalanine replaced by lysine (Figures 4b and 10b).

Uses of MCRA Peptide *In Vivo*

It is well known that chemokine receptors are expressed by various leukocytes, including eosinophils, monocytes, Th2 lymphocytes. These cells play important roles in the pathogenesis of inflammatory diseases, such as asthma, allergic reaction, inflammatory diseases. It is also known that each above mentioned cells carry different receptors, and each receptor binds to multiple chemokines. Thus the MCRA peptides of the present invention are useful for treatment of inflammatory diseases and disorders. It is also well known that various chemokine receptors act as HIV-1 coreceptors (e.g. CCR5, CXCR4, CCR3). Inhibition of these receptors can inhibit HIV entry. Thus MCRA peptides of the present invention are useful in AIDS therapy.

i) Therapeutic Applications

One aspect of the present invention provides the use of the MCRA peptide of the present invention in the treatment and prevention of conditions which involve chemokine receptor activities, including, but not limited to, inflammatory diseases such as asthma, allergy and atopic dermatitis, and HIV-1 infection. The MCRA peptides of the present invention are useful as inhibitors to prevent the infiltration of eosinophils, T-lymphocytes and monocytes and in the reduction of HIV-1 infection through blocking the receptors of the M-tropic and T-tropic strains.

In an alternative aspect of the present invention the MCRA peptides are useful for cancer therapy by inhibiting blood vessel formation.

ii) Pharmaceutical Compositions

One embodiment of the present invention provides pharmaceutical and therapeutic compositions comprising an MCRA peptide or combination of peptides.

The pharmaceutical compositions of the present invention may be administered orally, topically (including ophthalmic, vaginal, rectal, intranasal, epidermal, and transdermal), parenterally, by inhalation or spray in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques. One or more protease inhibitor may be present in association with one or more non-toxic pharmaceutically acceptable carriers

and/or diluents and/or adjuvants and, if desired other active ingredients. The pharmaceutical compositions containing one or more protease inhibitor may be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion hard or soft capsules, or syrups or elixirs.

Compositions intended for oral use may be prepared according to any known to the art for the manufacture of pharmaceutical compositions and such compositions may contain one or more agents selected from the group consisting of sweetening agents, flavouring agents, colouring agents and preserving agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients which are suitable for the manufacture of tablets. These excipients may be for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate: granulating and disintegrating agents for example, corn starch, or alginic acid: binding agents, for example starch, gelatin or acacia, and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate may be employed.

Pharmaceutical compositions for oral use may also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methyl cellulose, hydropropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia: dispersing or wetting agents may be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethyene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example hepta-decaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives, for example ethyl, or *n*-

propyl *p*-hydroxy-benzoate, one or more colouring agents, one or more flavouring agents or one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions may be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions may contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents such as those set forth above, and flavouring agents may be added to provide palatable oral preparations. These compositions may be preserved by the addition of an anti-oxidant such as ascorbic acid.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavouring and colouring agents, may also be present.

Pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions. The oils phase may be a vegetable oil, for example olive oil or arachis oil, or a mineral oil, for example liquid paraffin or mixtures of these. Suitable emulsifying agents may be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monoleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monoleate. The emulsions may also contain sweetening and flavouring agents.

Syrups and elixirs may be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol or sucrose. Such formulations may also contain a demulcent, a preservative and flavouring and colouring agents. The pharmaceutical compositions may be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension may be formulation according to known art using those suitable dispersing or wetting agents and suspending agents which have been mentioned above. The sterile injectable preparation may also be sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, lactated Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this

purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

When administered by nasal aerosol or by inhalation, these compositions are prepared according to techniques well known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art.

Any common topical formulation such as a solution, suspension, gel, ointment, or salve and the like may be used to administer the MCRA peptide(s) of the present invention. Preparation of such topical formulations are well described in the art of pharmaceutical formulations as exemplified, for example, Remington's Pharmaceutical Science, Edition 17, Mack Publishing Company, Easton, Pa. Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Other medicaments can be added to such topical formulation for such secondary purposes as treating skin dryness; providing protection against light; other medications for treating dermatoses; medicaments for preventing infection, reducing irritation and the like.

A useful therapeutic or prophylactic concentration will vary from condition to condition and in certain instances may vary with the severity of the condition being treated and the patient's susceptibility to treatment. Accordingly, no single concentration will be uniformly useful, but will require modification depending on the particularities of the disease being treated.

iii) Gene Therapy

One aspect of the present invention provides the use of nucleotides encoding the MCRA peptide for expression of such peptides *in vivo*, which is often referred to as "gene therapy."

In one embodiment, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding the MCRA peptide.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing the MCRA peptide may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering the MCRA peptide by such methods should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium Rosenfeld et al. (1992) cited supra), endothelial cells (Lemarchand et al. (1992) Proc. Natl. Acad. Sci. USA 89:6482-6486), hepatocytes (Herz and Gerard (1993) Proc. Natl. Acad. Sci. USA 90:2812-2816) and muscle cells (Quantin et al. (1992) Proc. Natl. Acad. Sci. USA 89:2581-2.

The genome of an adenovirus can be manipulated such that it encodes and expresses a peptide compound of the invention, but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) BioTechniques 6:616; Rosenfeld etal. (1991) Science 252:431-434; and Rosenfeld et al. (1992) Cell 68:143-155.

Defective retroviruses are well characterised for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) Blood 76:271). Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F. M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses incluse pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) Science 230:1395-1398; Danos and Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464; Wilson et al. (1988) Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano et al. (199) Proc. Natl. Acad. Sci. USA 87:6141:6145; Huber et al. (1991) Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry et al. (1991) Proc. Natl. Acad. Sci. USA 88:8377-8381; Chowdhury et al. (1991) Science 254:1802-1805; van

Beusechem et al. (1992) Proc. Natl. Acad. Sci. USA 89:7640-7644; Kay et al. (1992) Human Gene therapy 3:641-647; Dai et al. (1992) Proc. Natl. Acad. Sci. USA 89:10892-10895; Hwu et al. (1993) J. Immunol. 150:4104-4115; U.S. Pat. No. 4,868,116; U.S. Pat. No.4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; PCT Application WO 92/07573.

Retroviruses, from which the retroviral plasmid vectors mentioned herein, may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumour virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

The nucleic acid sequence encoding the MCRA peptide of the present invention is placed under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the β-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the genes encoding the polypeptides.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, ψ-2, ψ-AM, PA12, T19-14X, VT-19-17-H2, ψCRE, ψCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or PTH to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide.

Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

A nucleic acid of the invention can be delivered to cells *in vivo* using method known in the art, such as direct injection of DNA, receptor-mediated DNA uptake or viral-mediated transfection. Direct injection has been used to introduce naked DNA into cells *in vivo* (see e.g., Acsadi et al. (1991) Nature 332:815-818; Wolff et al. (1990) Science 247:1465-1468). A delivery apparatus (e.g., a "gene gun") for injecting DNA into cells in vivo can be used. Such apparatus is commercially available (e.g., from Biorad). Naked DNA can also be introduced into cells by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell surface receptor (see example Wu, G. and Wu, C.H. (1988) J. Biol. Chem. 263-14621; Wilson et al. (1992) J. Biol. Chem. 267:963-967; and U.S. Pat. No. 5,166,320). Binding of the DNA –ligand complex to the receptor facilitates uptake the DNA by receptor-mediated endocytosis. Additionally, a DNA-ligand complex linked to adenovirus capsids which naturally disrupt endosomes, thereby releasing material into the cytoplasm can be used to avoid degradation of the complex by intracellular lysosomes (see for example Curiel et al. (1991) Proc. Natl. Acad. Sci. USA 88:8850; Cristiano et al., (1993) Proc. Natl. Acad. Sci. USA 90:2122-2126).

Methods of delivery of foreign DNA are known in the art, such as containing the DNA in a liposome and infusing the preparation into an artery (LeClerc G. et al., (1992) J Clin Invest. 90: 936-44), transthoracic injection (Gal, D. et al., (1993) Lab Invest. 68: 18-25.). Other methods of delivery may include coating a balloon catheter with polymers impregnated with the foreign DNA and inflating the balloon in the region of arteriosclerosis, thus combining balloon angioplasty and gene therapy (Nabel, E.G. et al., (1994) Hum Gene Ther. 5: 1089-94.)

To gain a better understanding of the invention described herein, the following examples are set forth. It should be understood that these examples are for illustrative purposes only. Therefore, they should not limit the scope of this invention in any way.

EXAMPLES

All chemokines or peptides used in the following examples were chemically synthesised with tBoc chemistry using an Applied Biosystems 430 Peptide Synthesiser and purified by reverse phase HPLC according to standard procedures.

EXAMPLE I: METHOD FOR STUDYING THE INHIBITOR AFFECT OF MCRA AND MCRA(1-8)$_2$K IN AN ASTHMA MOUSE MODEL

C57/BL6 mice, 6 – 10 weeks old, male and female were used for this study. The mice were sensitised with i.p. injection of ovalbumin mixed with aluminium hydroxide on day 1 and day 12. On days 24, 25, 26 and 27 the animals were exposed to aerosolised ovalbumin.

i) Treatment and Analysis

The mice were systemically treated with a control peptide (an inactive peptide) or MCRA dimer (or MCRA(1-8)$_2$K) or an alternative chemokine receptor antagonist. The injection was i.p., twice a day, starting immediately after the lung challenging, for a total of five days.

On day 29, the mice were sacrificed. The bronchoalveolar lavage (BAL) fluid was collected. The number of cells in the BAL fluid was counted. MG-Giemsa stains identified the subtypes of cells. The lungsection was stained. The number of cells accumulated around the bronchus in the lung was scored. A reduction in the number of cells is indicative of therapeutic effect.

ii) Results

Figures 17, 18, 19 demonstrate that systemic treatment of both the MCRA dimer and MCRA(1-8)$_2$K, at a dose of 12mg/kg, reduced the number of cells which infiltrate into both BAL and lung of the asthmatic mice. Simultaneous treatment with MCRA peptides and MCP-1 antagonist reduce the infiltration more significantly (more than 50 %). This indicates that these peptides have significant therapeutic potential in the treatment of inflammatory diseases, including asthma.

EXAMPLE II: EFFECT OF MCRA(1-8)$_2$K AND MCRA DIMER BINDING ON CCR3 RECEPTOR.

Methods:

Chemokine receptor carried target cells: For the *in vitro* functional assay chemokine receptor transfected 300-19 cell lines (transfectants) were used as cell targets. Each of these transfectants carries one type of chemokine receptor, for example only CCR3 receptor, and , therefore, provided a reliable result of the receptor specificity.

Receptor binding assay : ^{125}I-labeled Eotaxin, in the presence of unlabeled Eotaxin or peptides, was incubated with CCR3 receptor transfectants at 4 °C for 30 min. Cell-bound and free ^{125}I-Eotaxin was separated by centrifugation of the cells through a column.

Bound ^{125}I-Eotaxin was measure by gamma counting. Non-specific binding was determined in the presence of a 100-fold concentration of unlabeled ligand and was subtracted from the total.

Results:

Figure 3 shows that both MCRA monomer and dimer competed with ^{125}I-Eotaxin for binding on CCR3 receptor, whereby the potency of the dimer was approximately 10-fold higher than that of the monomer.

Figure 4a shows that MCRA $(1\text{-}8)_2K$ competed with Eotaxin for binding on CCR3 receptor, with slightly better potency than that of MCRA dimer.

EXAMPLE III. INHIBITION OF CCR3+ CELL MIGRATION BY MCRA,DIMER AND PEPTIDES

Methods:

Chemotaxis assy: Cell migration was evaluated using transwell trays (Costar). Chemokines, with or without various MCRA peptides, were added to the lower well, and target cells were added in the upper well. After 3 h, cells that had migrated to the lower well were counted. To determine the inhibition by the peptides, the number of migrated cells in the presence of peptides plus chemokine was compared to that of chemokine alone.

Calcium desensitization assay: This assay was used to detect the usage of chemokine receptor by peptide. The fluorescence of Fluo-3AM loaded cells was monitored with a spectrofluorometer. The desensitization assay was performed by sequential addition of MCRA peptide, followed by a chemokine. If a peptide blocks a chemokine's response, it indicates that the peptide is sharing the same receptor as this chemokine.

Results:

Figure 5 shows that MCRA dimer significantly inhibited cells (CCR3+) migration. The inhibition was dose dependent (IC_{50} was 2 μM). Figure 2 shows that 10 μM of MCRA dimer completely blocked 50 nM of Eotaxin induced calcium response.

EXAMPLE IV. MCRA PEPTIDES BINDING ON CCR5 RECEPTOR, AND INHIBITING OF MIP-1β RANTES ACTIVITY.

Methods: The methods used were the same as outlined above.

Results:

Figure 6, 7 and 2 show that MCRA monomer, dimer and MCRA(1-8)$_2$K competed with RANTES or MIP-1β for binding on CCR5 transfectants. The dimer had higher potency than the monomer and MCRA(1-8)2K.

Figure 8 and 2 show MCRA dimer inhibited RANTES induced cell (CCR5+) migration and calcium response (IC_{50} was 3 μM) in a dose dependent manner.

EXAMPLE V: EFFECT OF MCRA PEPTIDES BINDING ON CXCR4 RECEPTOR, AND INHIBITING OF SDF-1 ACTIVITY.

Methods: The methods used were the same as outlined above.

Results: Figure 9, 10a, 10b show that MCRA monomer, dimer, MCRA(1-8)$_2$K and MCRA(1-9)K1 dimer competed with SDF-1 for binding on CXCR4 transfectants. The dimer had the same potency as that of MCRA(1-8)$_2$K and MCRA(1-9)K1 dimer, and higher potency than the monomer.

Figure 11 and 2 show MCRA dimer dose dependently inhibited SDF-1 induced cell (CXCR4+) migration and calcium response (IC_{50} of 1 μM).

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

Table 1

Ligands/Receptors	CCR1	CCR2	CCR3	CCR5	CXCR4
N-SDF-1, dimer ***	-	-	-	-	3
N-SDF, P2G dimer***	-	-	-	-	50
N-MCP-1	-**	-	-	-	-
N-MCP-1, dimer		-	-	-	-
N-vMIP-2C	-	-	200	-	20
N-vMIP-2C, dimer	-	-	60	-	5
N-MIP-1α	-	-	-	-	-
N-MIP-1β	-	-	-	-	-
N-MIP-1β, dimer	-		-	-	-
N-RANTES	-	-	-	-	-
N-RANTES, dimer	-	-	-	-	-
N-IP10	-	-	-		-
N-IP10, dimer	-	-	-		-
N-Eotaxin	-	-	-	-	-
N-Eotaxin-2	-	-	-	-	-
N-MIP-3a	-	-	-	-	
N-ROa		-	-		-
N-ROa, dimer		-	-		-
N-NTN	-	-	-	-	100
N-NTN, dimer	-	-			20
N-NAP-2		-	-	-	-
N-TECK	-	-	-		-
MCRA	40	100	40	60	30
MCRA,dimer	40	100	4	6	3
N-BCA		-	-		
N-BCA, dimer		-	-		
N-I309		-			
N-I309, dimer		-			

Table 2

Chemokine Receptors (nature chemokine)	CCR1 (MIP-1α)	CCR2 (MCP-1)	CCR3 (Eotaxin)	CCR5 (RANTES)	CXCR1 (IL-8)	CXCR2 (IL-8)	CXCR3 (MCRA)	CXCR4 (SDF-1)
Inhibition of Receptor binding	40 μM	100μM	4 μM	6 μM	-- **	--	250 μM	3 μM
Inhibition of Chemotaxis	1 μM ***	6 μM ****	3 μM	2 μM	--	--		2 μM

Table 3

Treatment	24 hr	48 hr	52 hr
Control peptide	10 ± 1	11 ± 1	12 ± 1
MCRA dimer	8 ± 1	6 ± 1	8 ± 1

Table 4

Treatment	Total	Eosinophils	Monocytes	Lymphocytes	n
Control peptide	294 ± 42	228 ± 21	36 ± 9	14 ± 4	2
MCRA(1-8)$_2$K	151 ± 17	115 ± 13	9 ± 9	11 ± 4	3
MCP1 antagonist	249 ± 26	180 ± 56	33 ± 30	22 ± 3	2
MCRA(1-8)$_2$K + MCP1 antagonist	50 ± 31	31 ± 18	4 ± 2	11 ± 5	3

Table 5

Treatment	48 hr	n
Control peptide	9.7 ± 2	3
MCRA(1-8)$_2$K	7.3 ± 3	3
MCP1 antagonist	5.7 ± 3.8	3
MCRA(1-8)$_2$K + MCP1 antagonist	5.5 ± 3.8	3

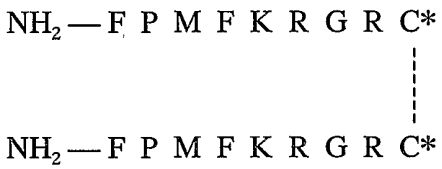
THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A peptide having antagonist activity toward multiple chemokine receptors, with the structure of Formula I:

$$NH_2 — F\ P\ M\ F\ K\ R\ G\ R — X \qquad (I)$$

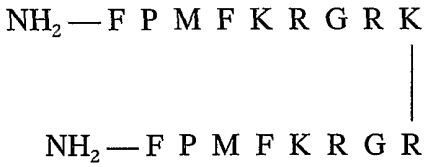
wherein X is: C; K; a covalent linker; C* --- C* R G R K F M P F — NH_2, wherein C*--- C* represents a disulphide bridge; K — R G R K F M P F — NH_2, wherein K — R represents an amide bond between the ε-amino group of K and the carboxyl group of R; or a covalent linker additionally bound to a second NH_2 — F P M F K R G R at its C-terminal R, and wherein NH_2 represents the N-terminus.

2. The peptide according to claim 1 having the structure of Formula II:



(II).

3. The peptide according to claim 1 having the structure of Formula (III):



(III).

4. The peptide according to claim 1, wherein said covalent linker is selected from the group consisting of ornithine, L-amino-N_butyric acid and benzene.

5. A peptide variant of the peptide according to any one of claims 1, 2 or 3, having antagonist activity toward multiple chemokine receptors.

6. The peptide variant according to claim 5, wherein an N-terminal K is replaced with lysine.

7. A composition comprising the peptide according to any one of claims 1, 2, 3 or 4 and a pharmaceutically acceptable diluent or excipient.

8. A composition comprising the peptide variant according to claim 5 or 6 and a pharmaceutically acceptable diluent or excipient.

9. A method of inhibiting the activity of at least two chemokine receptors in a mammal comprising administering the peptide according to any one of claims 1, 2, 3 or 4.

10. A method of inhibiting at least two chemokine receptors in a mammal comprising administering the peptide variant according to claim 5 or 6.

11. The method according to claim 9 or 10, wherein said method is used to treat an inflammatory disease or disorder.

12. The method according to claim 11, wherein said inflammatory disorder is asthma.

13. The method according to claim 9 or 10, wherein said method is used to prevent or reduce HIV infection in the mammal.

14. The method according to any one of claims 9, 10, 11, 12 or 13, wherein said mammal is a human.

15. Use of the peptide according to any one of claims 1, 2, 3 or 4 in the preparation of a medicament for the treatment of an inflammatory or immunoregulatory disease or disorder or for the prevention or reduction of HIV infection in a mammal.

16. Use of the peptide variant according to claim 5 or 6 in the preparation of a medicament for the treatment of an inflammatory or immunoregulatory disease or disorder or for the prevention or reduction of HIV infection in a mammal.

17. A peptide for use in inhibition of at least two chemokine receptors in a mammal, wherein the peptide has the structure of Formula I:

$$NH_2 — F\ P\ M\ F\ K\ R\ G\ R — X \qquad (I)$$

wherein X is: C; K; a covalent linker; C* --- C* R G R K F M P F — NH_2, wherein C*--- C* represents a disulphide bridge; K — R G R K F M P F — NH_2,

wherein K — R represents an amide bond between the ε-amino group of K and the carboxyl group of R; or a covalent linker additionally bound to a second NH_2 — F P M F K R G R at its C-terminal R, and wherein NH_2 represents the N-terminus.

18. A nucleic acid encoding the peptide according to claim 1, wherein X is C or K.

19. A method of inhibiting the activity of at least two chemokine receptors in a mammal comprising administering the nucleic acid according claim 18.

20. The method according to claim 19, wherein said method is used to treat an inflammatory disease or disorder.

21. The method according to claim 20, wherein said inflammatory disorder is asthma.

22. The method according to claim 19, wherein said method is used to prevent or reduce HIV infection in the mammal.

23. The method according to any one of claims 19, 20, 21 or 22, wherein said mammal is a human.

24. Use of the nucleic acid according to claim 18 in the preparation of a medicament for the treatment of an inflammatory or immunoregulatory disease or disorder or for the prevention or reduction of HIV infection in a mammal.

Figure 1

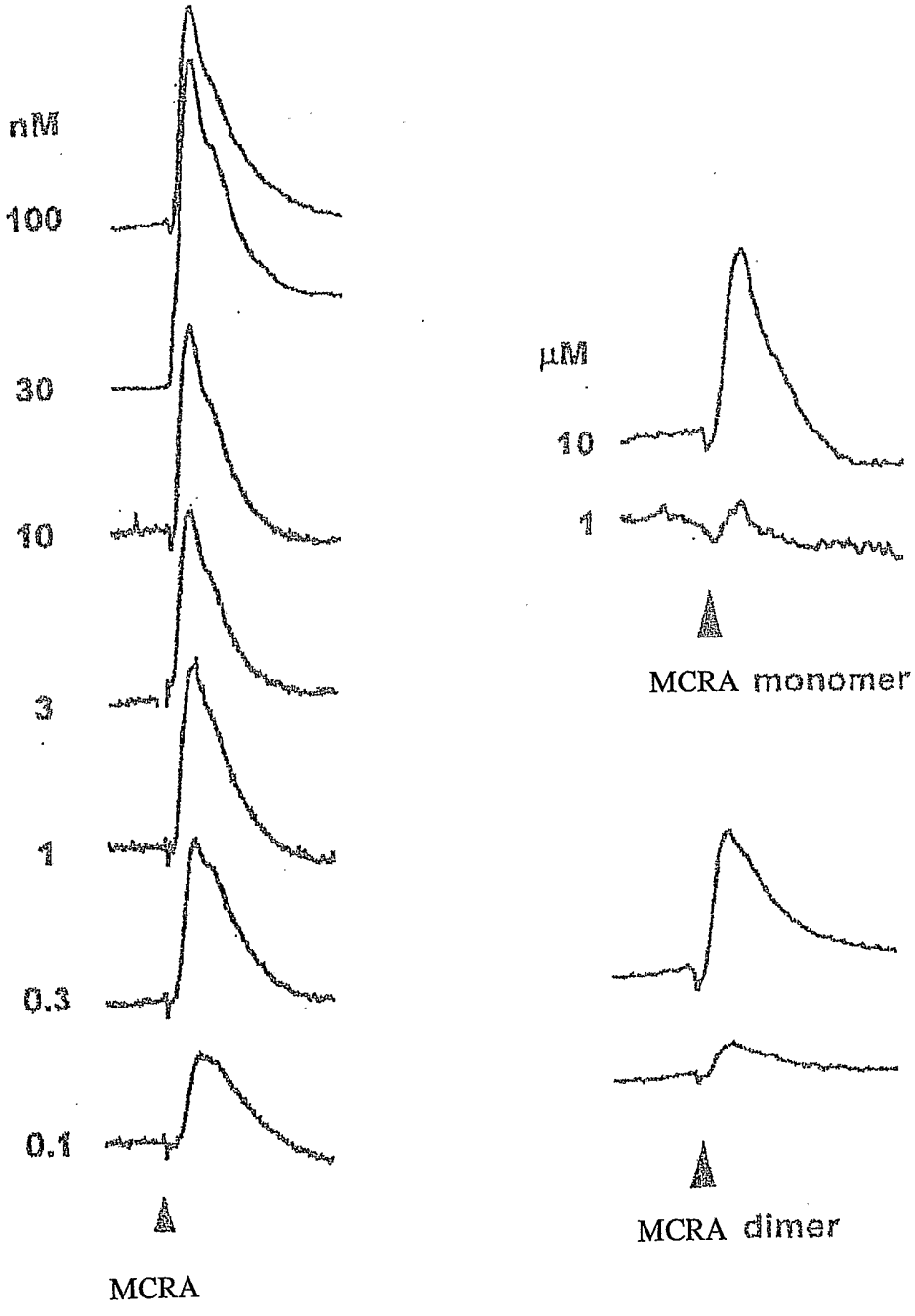


Figure 2

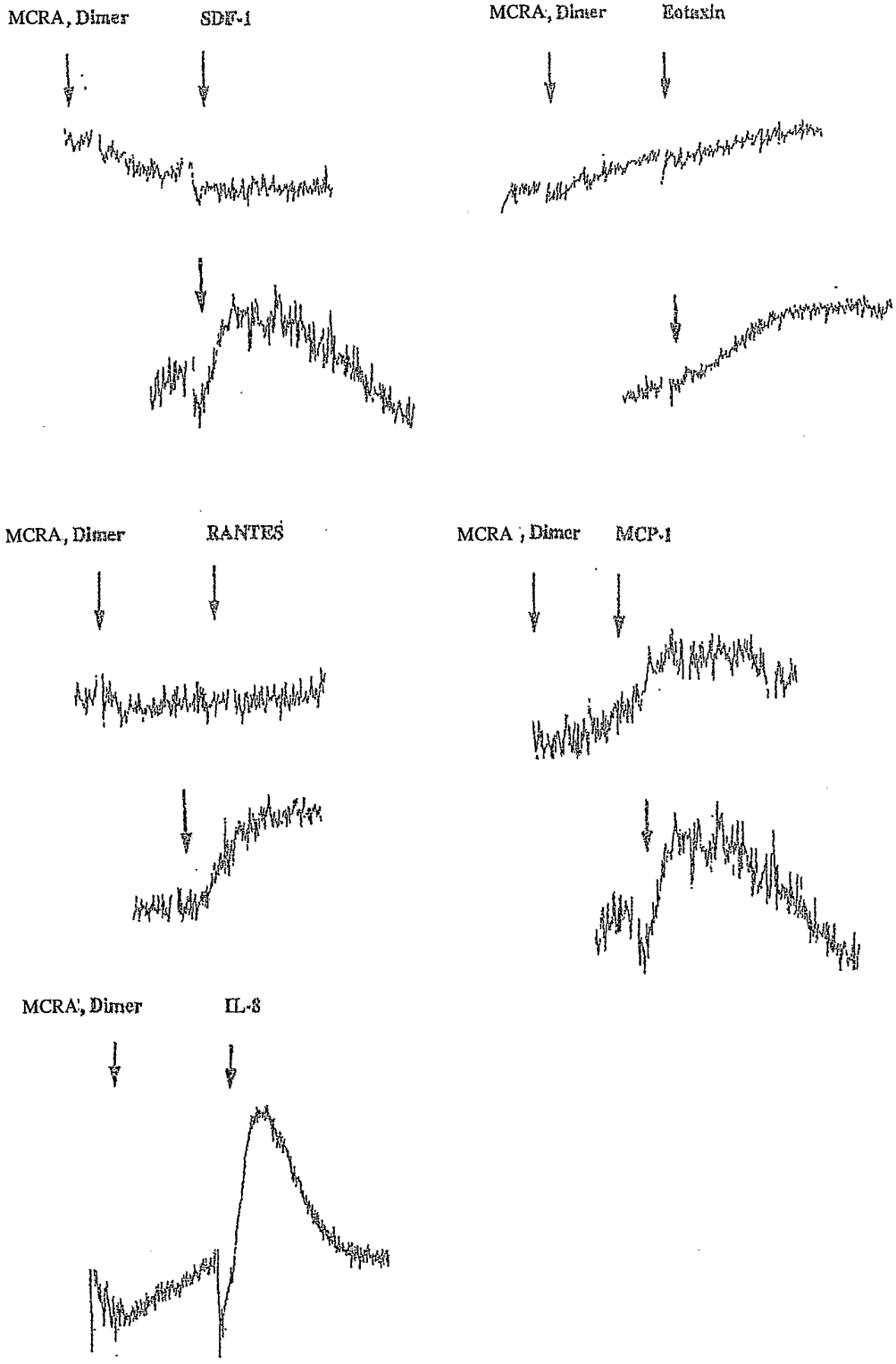

MCRA, Dimer
SDF-1
MCRA, Dimer
Eotaxin
MCRA, Dimer
RANTES
MCRA, Dimer
MCP-1
MCRA, Dimer
IL-8

Figure 3

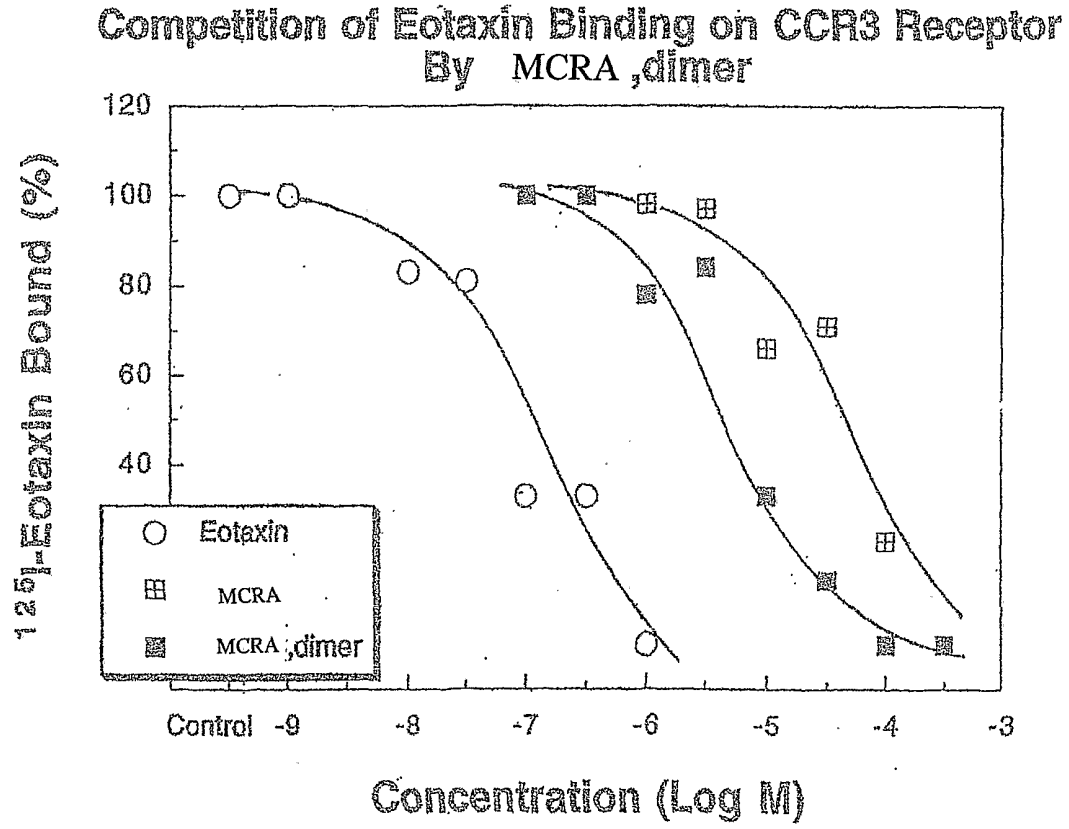
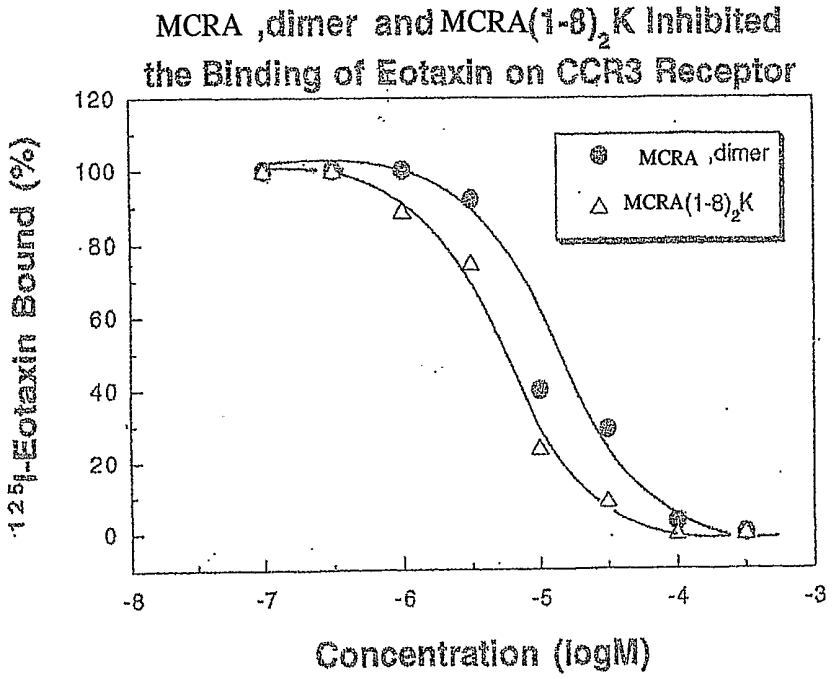

Competition of Eotaxin Binding on CCR3 Receptor By MCRA ,dimer
125I-Eotaxin Bound (%)
120
100
80
60
40
Eotaxin
MCRA
MCRA ;dimer
Control -9 -8 -7 -6 -5 -4 -3
Concentration (Log M)

Figure 4

A


MCRA ,dimer and MCRA(1-8)2K Inhibited
the Binding of Eotaxin on CCR3 Receptor
125I-Eotaxin Bound (%)
120
100
80
60
40
20
0
-8
-7
-6
-5
-4
-3
Concentration (logM)
MCRA ,dimer
MCRA(1-8)2K

B

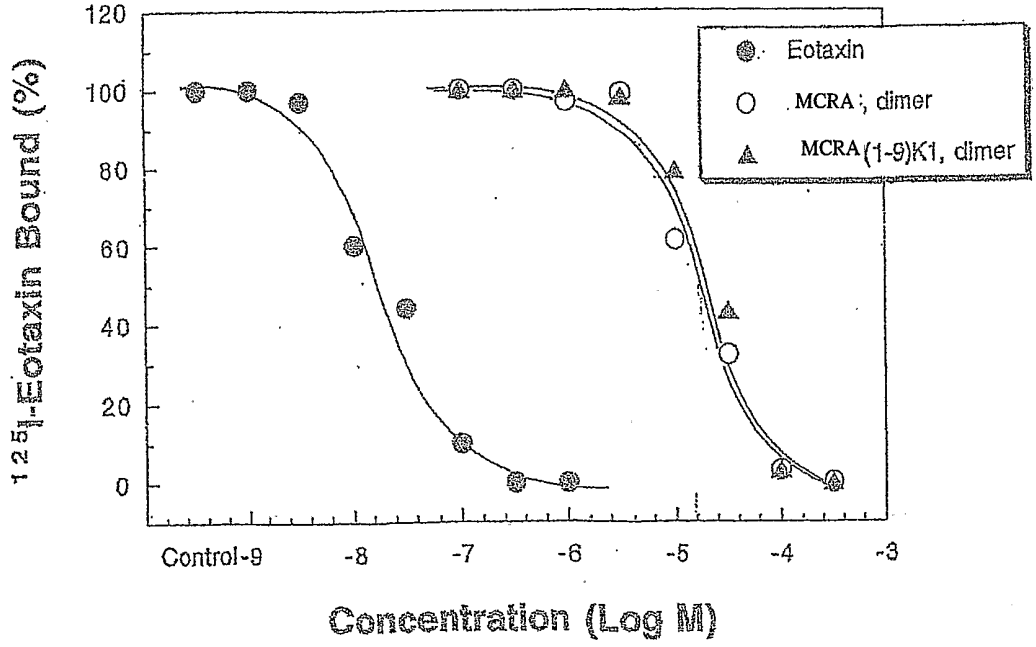

Binding on CCR3 Receptor
Sept. 13, 1999
125I-Eotaxin Bound (%)
120
100
80
60
40
20
0
Control-9
-8
-7
-6
-5
-4
-3
Concentration (Log M)
Eotaxin
MCRA, dimer
MCRA(1-9)K1, dimer

Figure 5

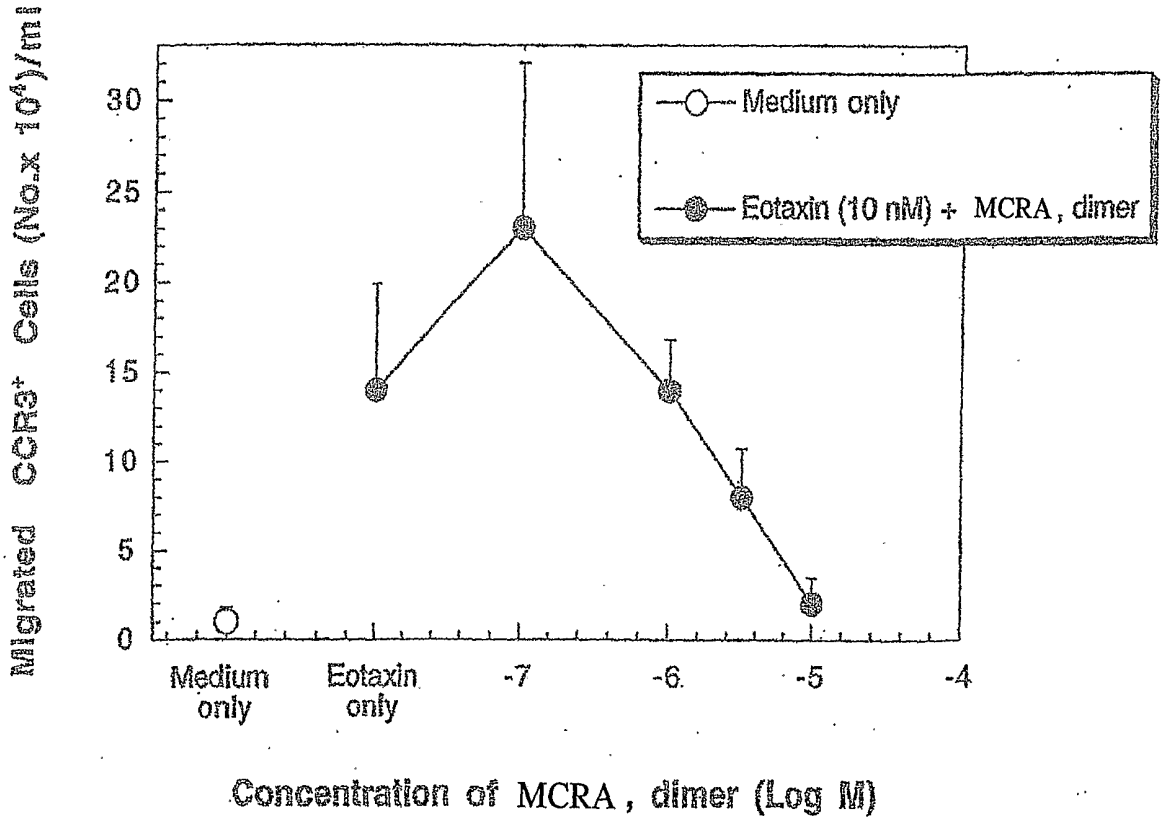

Migrated CCR3+ Cells (No.x 10⁴)/ml
30
25
20
15
10
5
0
Medium only
Eotaxin (10 nM) + MCRA, dimer
Medium only
Eotaxin only
-7
-6
-5
-4
Concentration of MCRA, dimer (Log M)

Figure 6

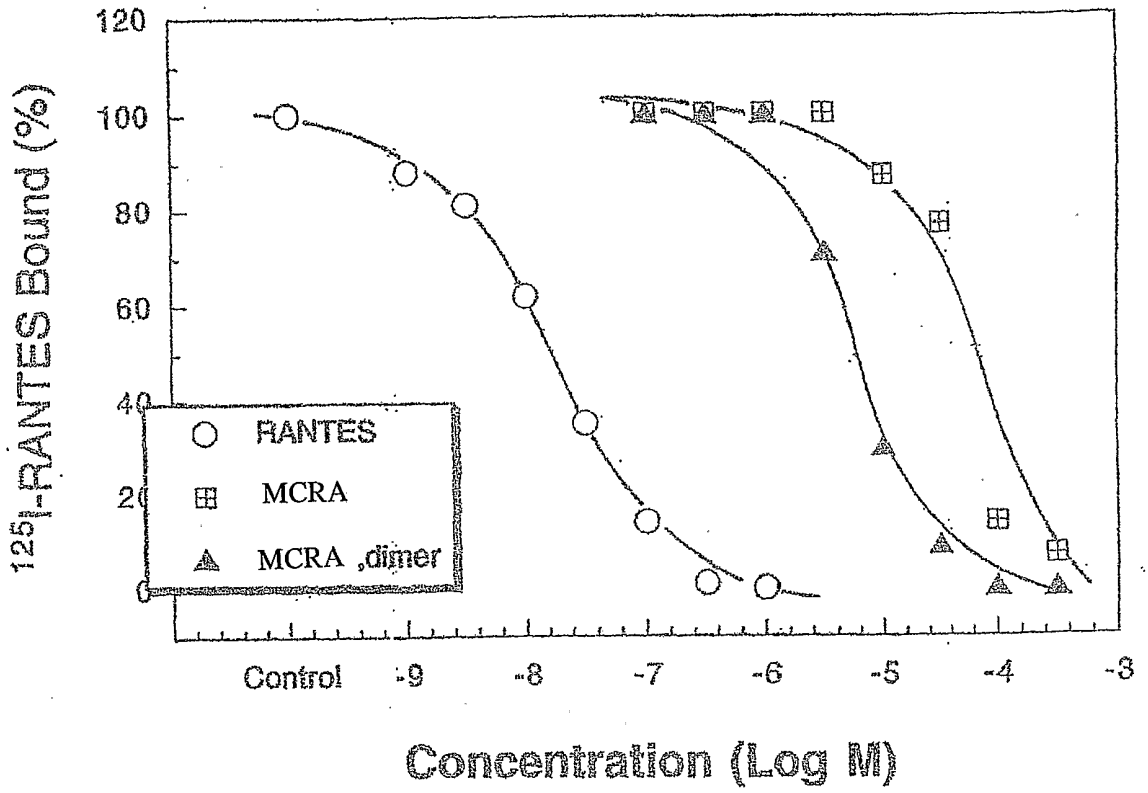

120
100
80
60
40
20
0
125I-RANTES Bound (%)
RANTES
MCRA
MCRA ,dimer
Control
-9
-8
-7
-6
-5
-4
-3
Concentration (Log M)

Figure 7

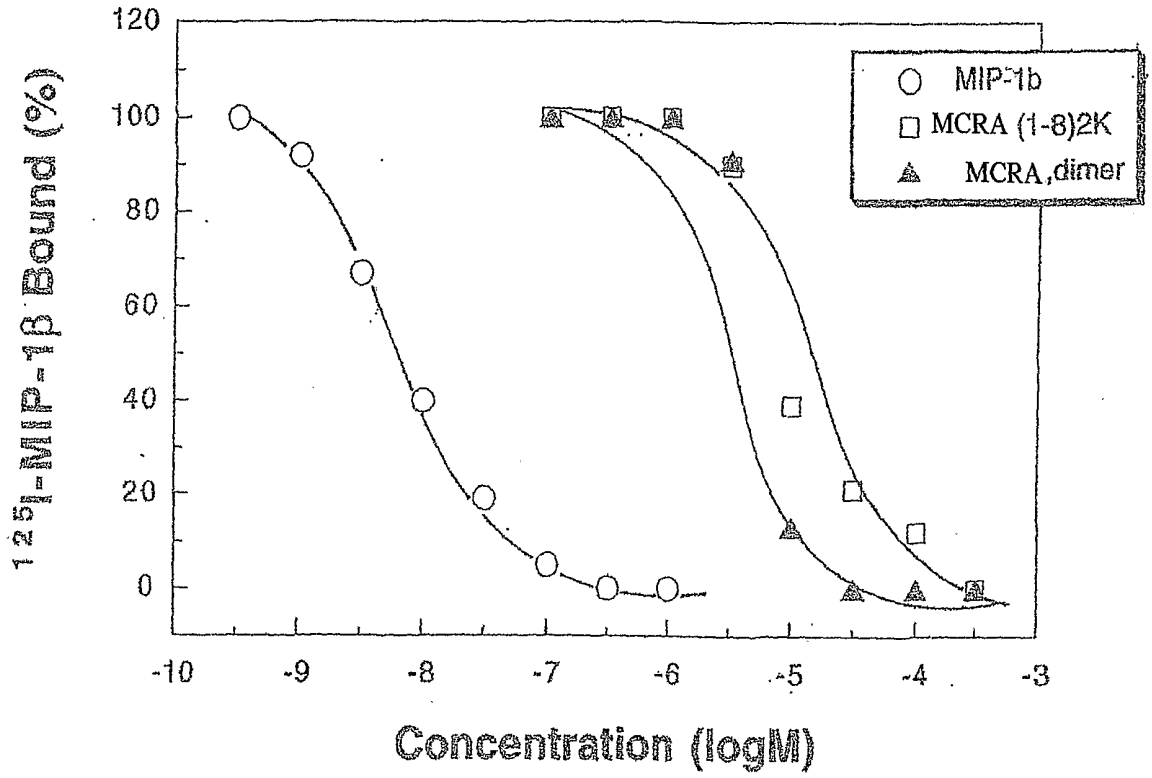

120
100
80
60
40
20
0
125-MIP-1β Bound (%)
-10
-9
-8
-7
-6
-5
-4
-3
Concentration (logM)
MIP-1b
MCRA (1-8)2K
MCRA,dimer

Figure 8

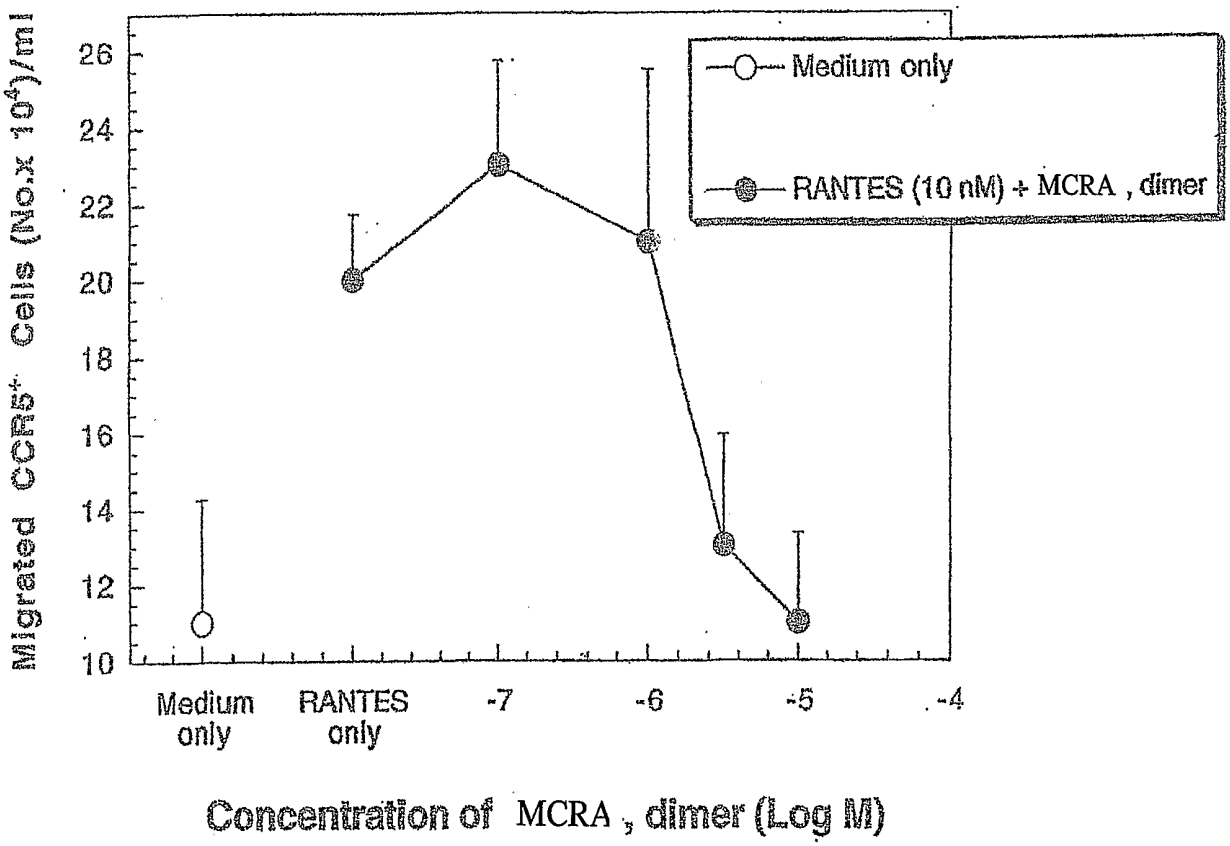

Migrated CCR5+ Cells (No.x 10⁴)/ml
26
24
22
20
18
16
14
12
10
Medium only
RANTES only
-7
-6
-5
-4
Medium only
RANTES (10 nM) + MCRA , dimer
Concentration of MCRA , dimer (Log M)

Figure 9

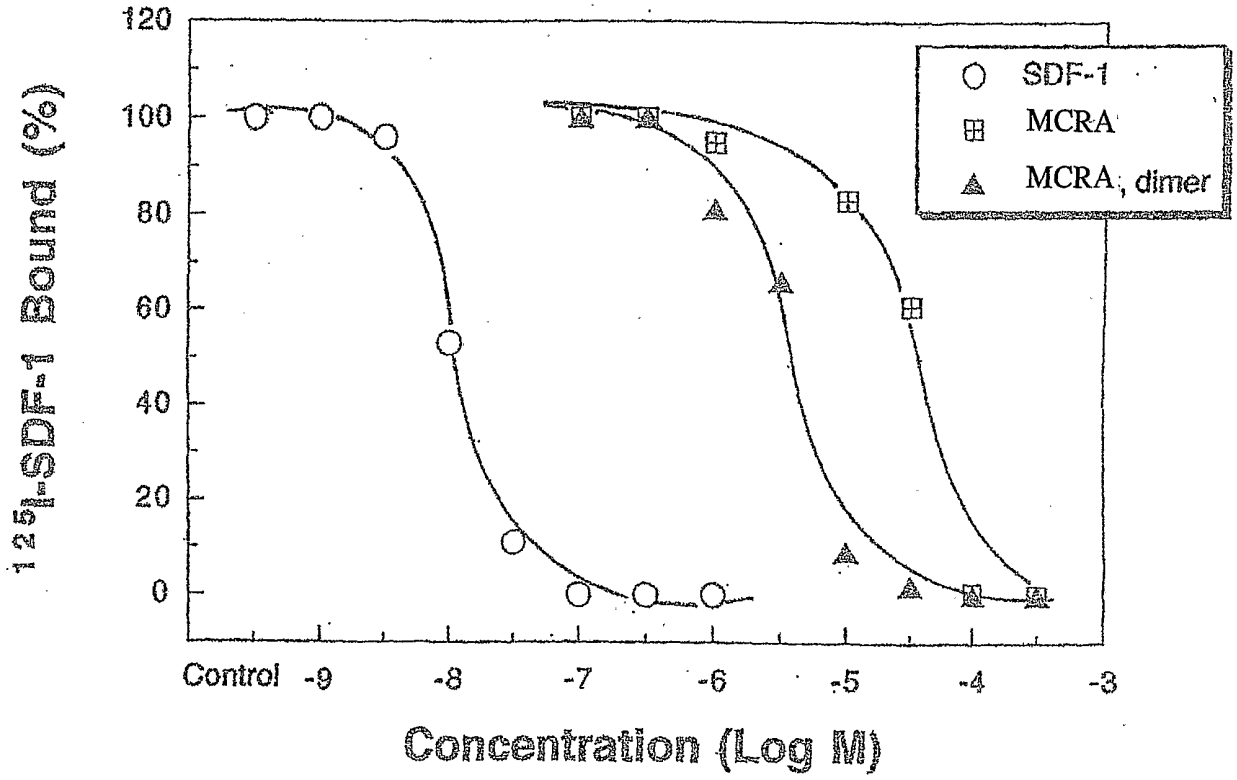
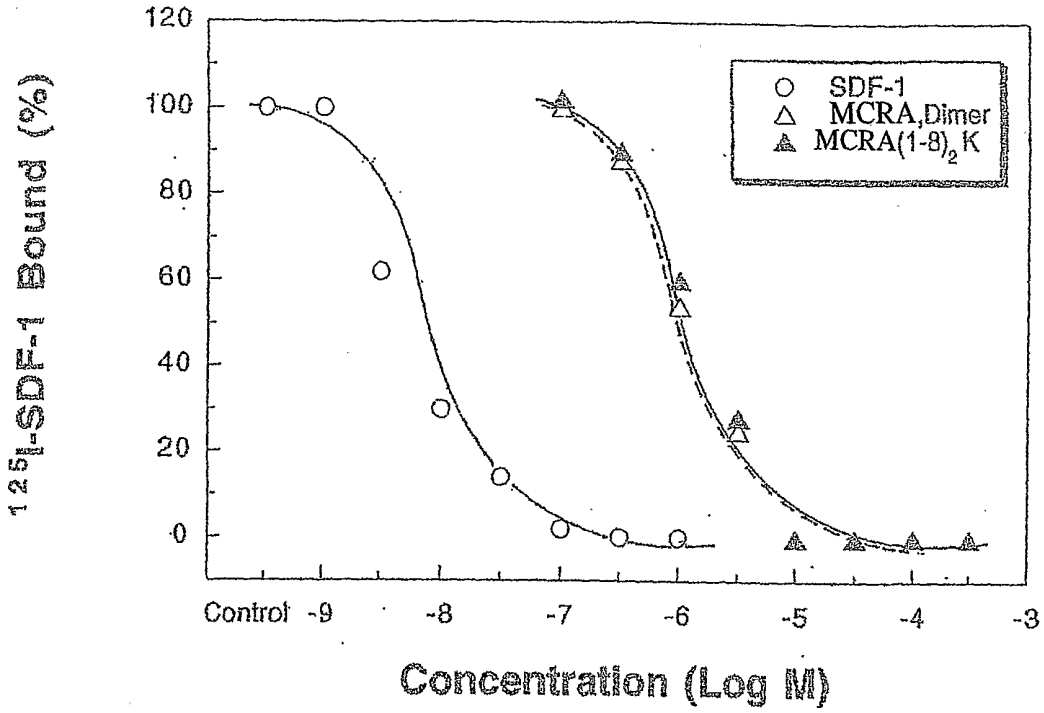

120
100
80
60
40
20
0
^{125}I-SDF-1 Bound (%)
SDF-1
MCRA
MCRA, dimer
Control
-9
-8
-7
-6
-5
-4
-3
Concentration (Log M)

Figure 10

A


120
100
80
60
40
20
0
^{125}I-SDF-1 Bound (%)
SDF-1
MCRA,Dimer
MCRA(1-8)$_2$K
Control -9 -8 -7 -6 -5 -4 -3
Concentration (Log M)

B

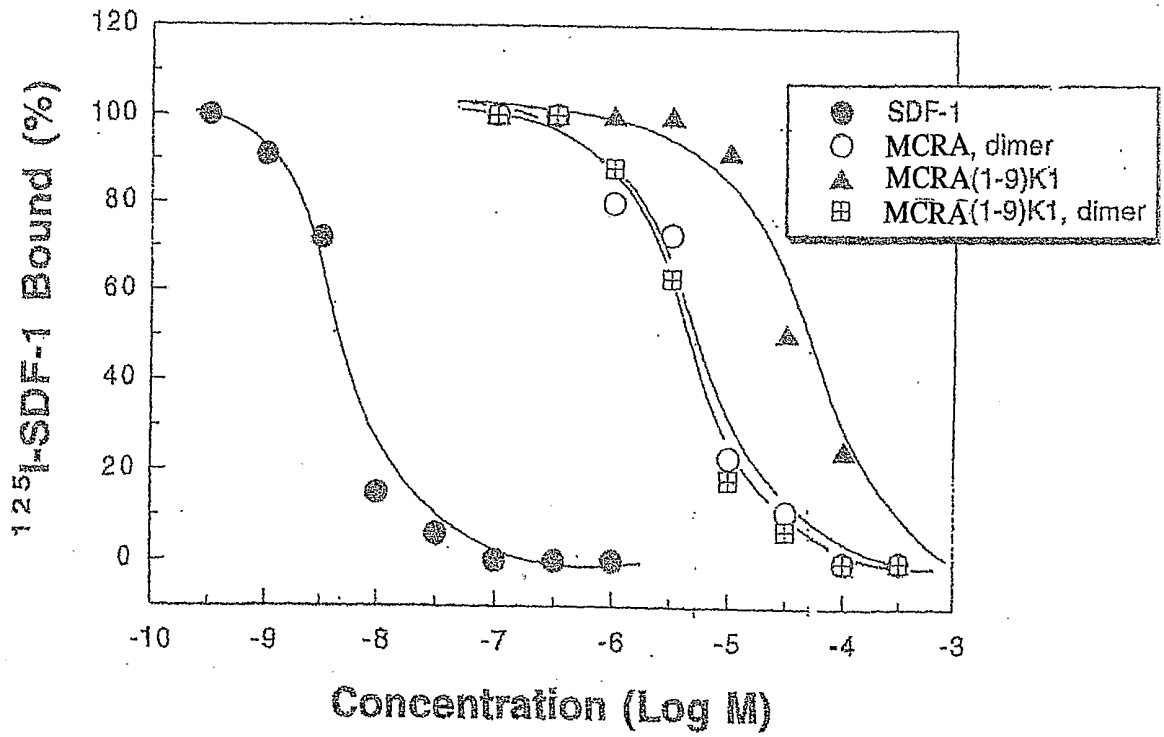

120
100
80
60
40
20
0
^{125}I-SDF-1 Bound (%)
SDF-1
MCRA, dimer
MCRA(1-9)K1
MCRA(1-9)K1, dimer
-10 -9 -8 -7 -6 -5 -4 -3
Concentration (Log M)

Figure 11

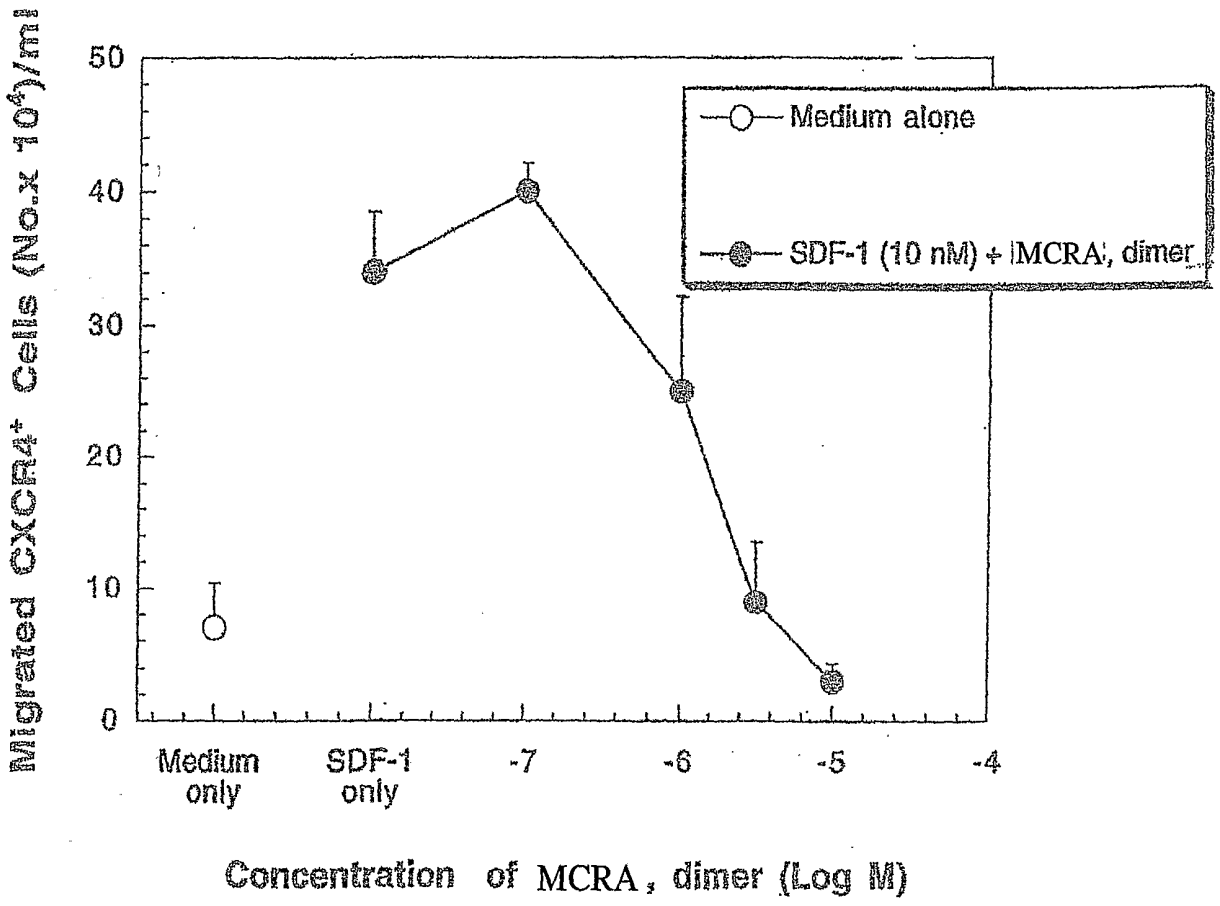

Migrated CXCR4+ Cells (No.x 10⁴)/ml
50
40
30
20
10
0
Medium alone
SDF-1 (10 nM) + MCRA, dimer
Medium only
SDF-1 only
-7
-6
-5
-4
Concentration of MCRA, dimer (Log M)

Figure 12

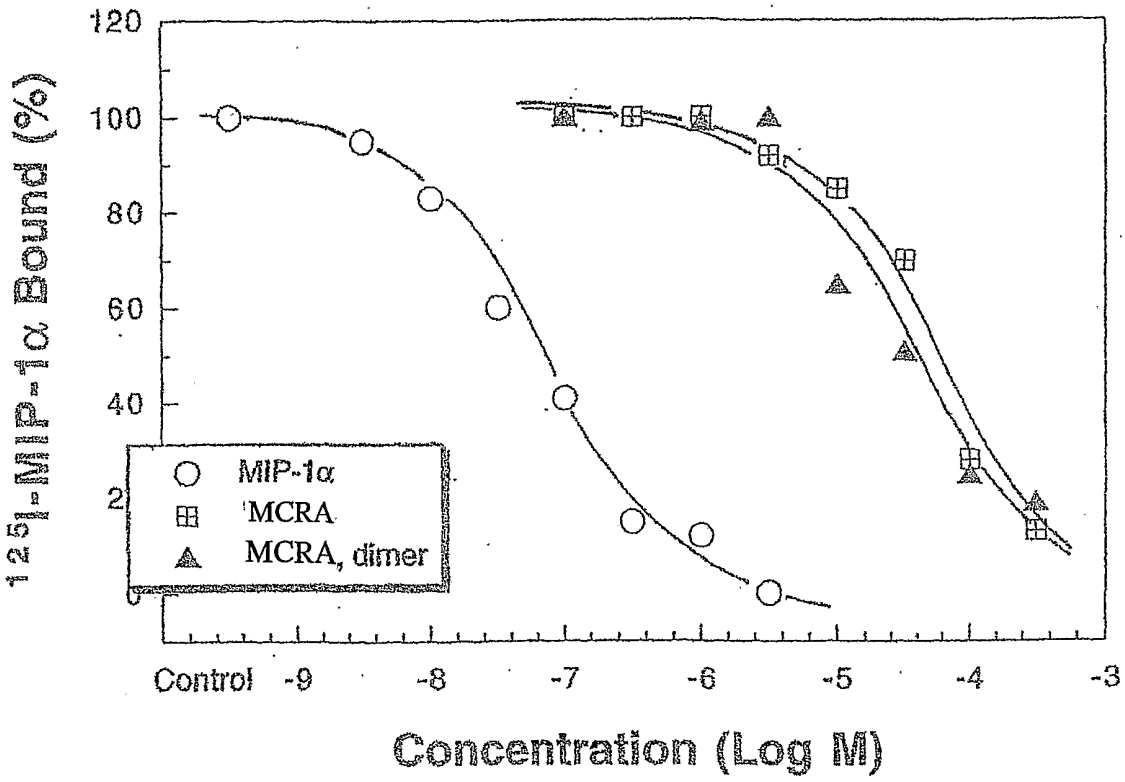

120
100
80
60
40
2
0
125I-MIP-1α Bound (%)
MIP-1α
MCRA
MCRA, dimer
Control
-9
-8
-7
-6
-5
-4
-3
Concentration (Log M)

Figure 13

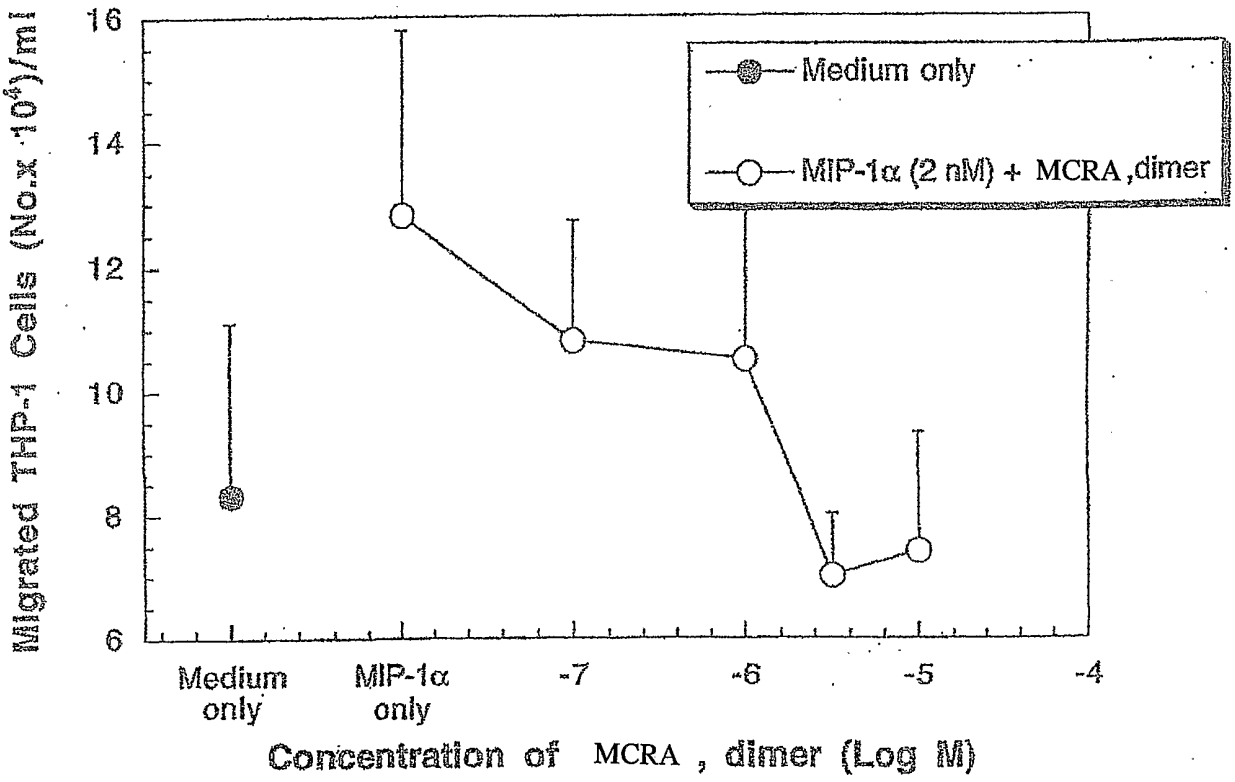

Migrated THP-1 Cells (No.x 10⁴)/ml
16
14
12
10
8
6
Medium only
MIP-1α (2 nM) + MCRA ,dimer
Medium only
MIP-1α only
-7
-6
-5
-4
Concentration of MCRA , dimer (Log M)

Figure 14

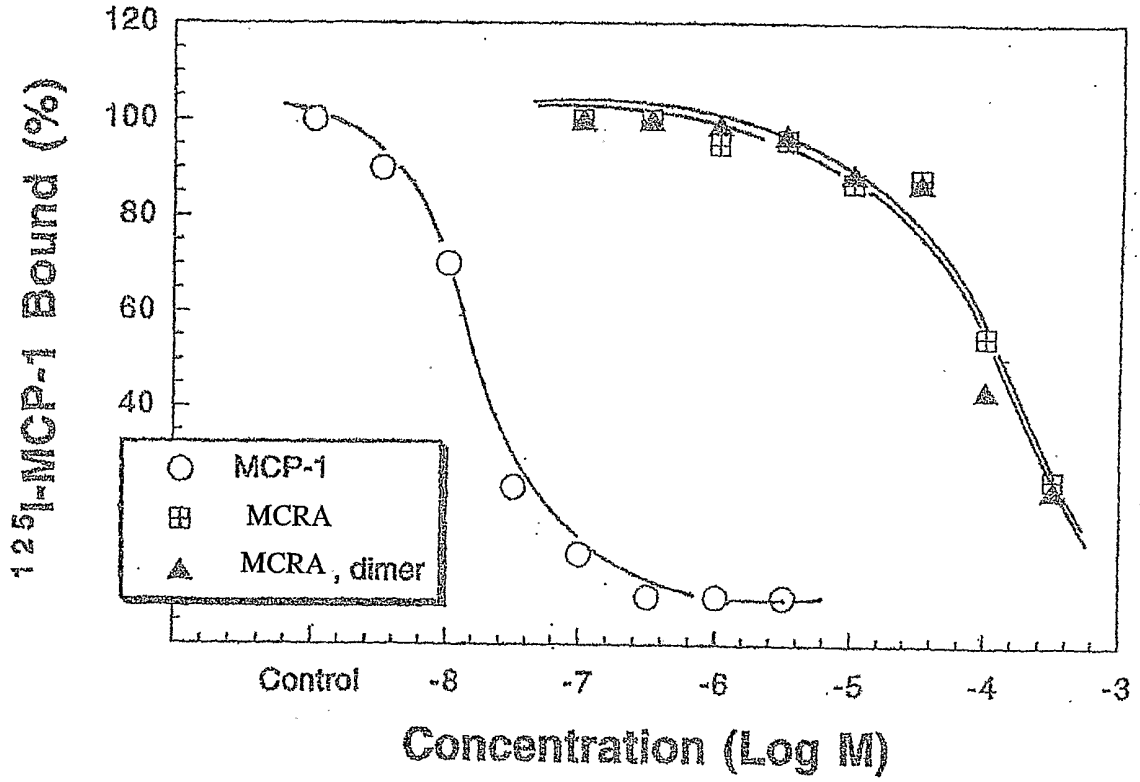

120
100
80
60
40
125I-MCP-1 Bound (%)
MCP-1
MCRA
MCRA , dimer
Control
-8
-7
-6
-5
-4
-3
Concentration (Log M)

Figure 15

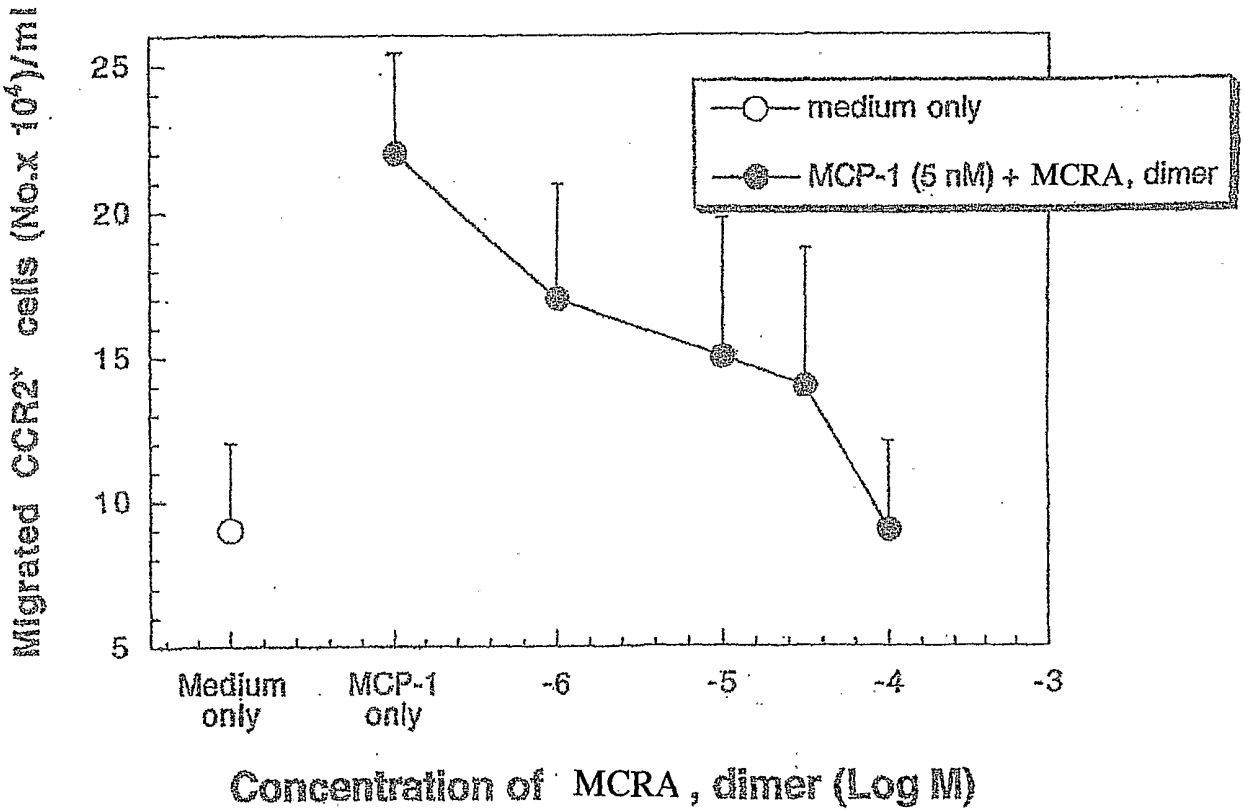

Migrated CCR2+ cells (No.x 10⁴)/ml
25
20
15
10
5
medium only
MCP-1 (5 nM) + MCRA, dimer
Medium only
MCP-1 only
-6
-5
-4
-3
Concentration of MCRA, dimer (Log M)

Figure 16

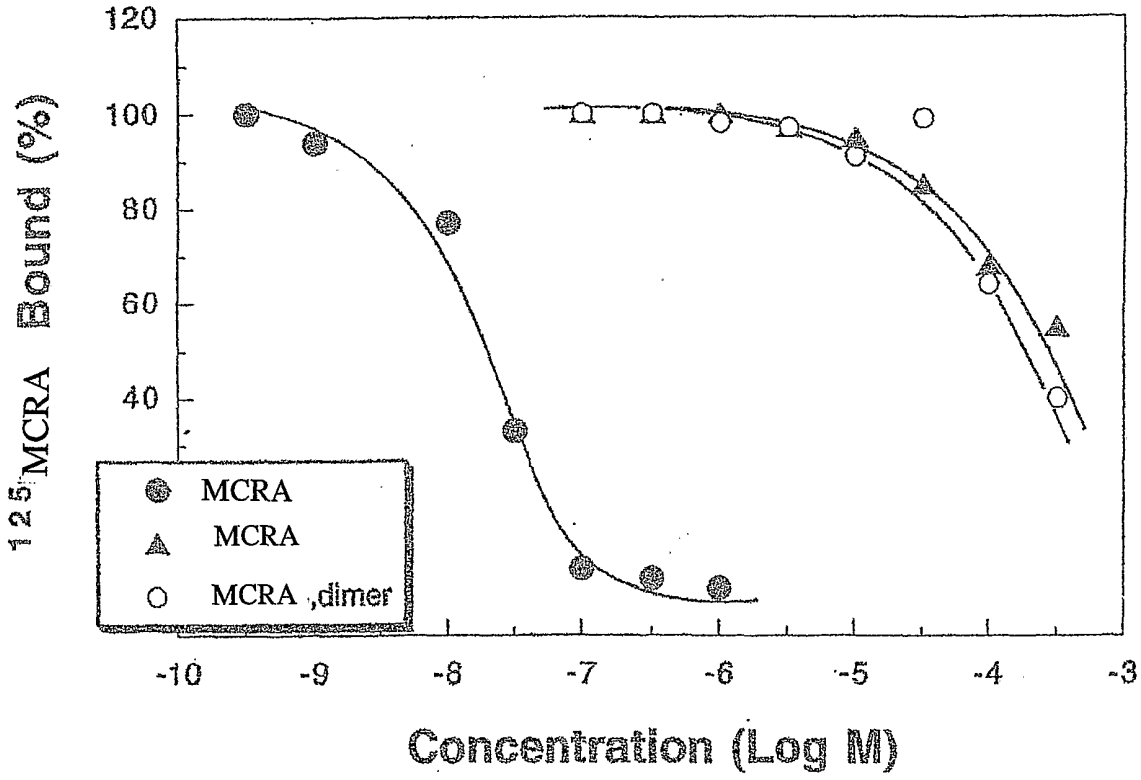

120
100
80
60
40
^{125}MCRA Bound (%)
MCRA
MCRA
MCRA ,dimer
-10
-9
-8
-7
-6
-5
-4
-3
Concentration (Log M)

Figure 17

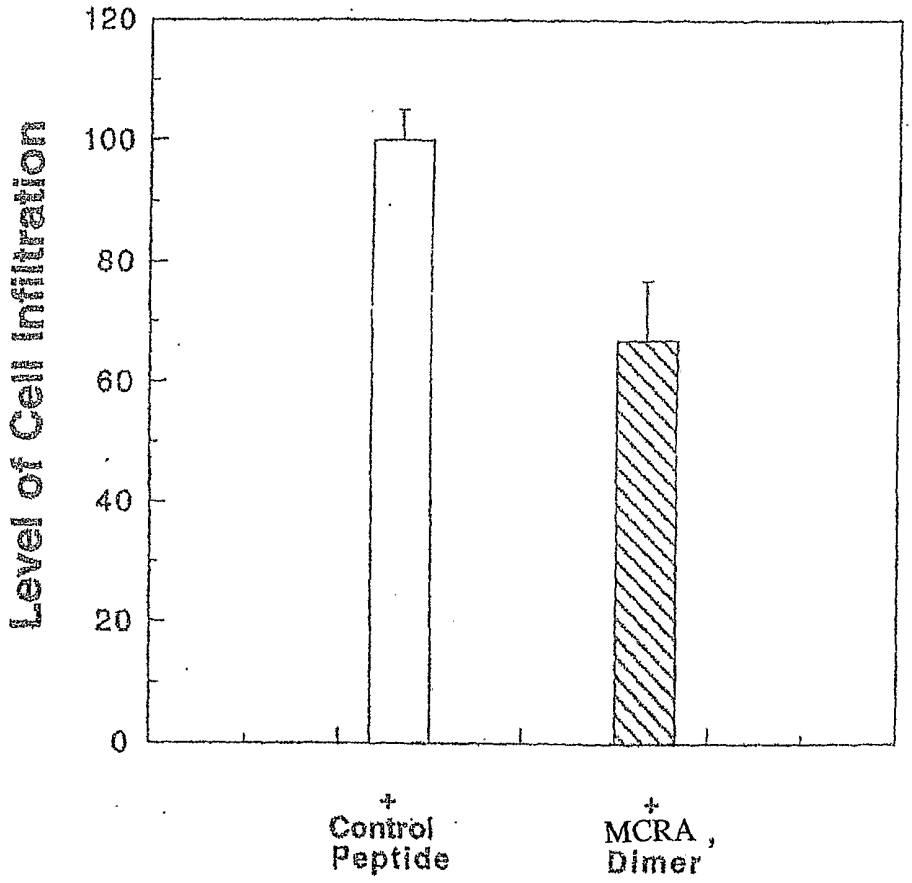

Level of Cell Infiltration
120
100
80
60
40
20
0
+
Control
Peptide
+
MCRA ,
Dimer

Figure 18

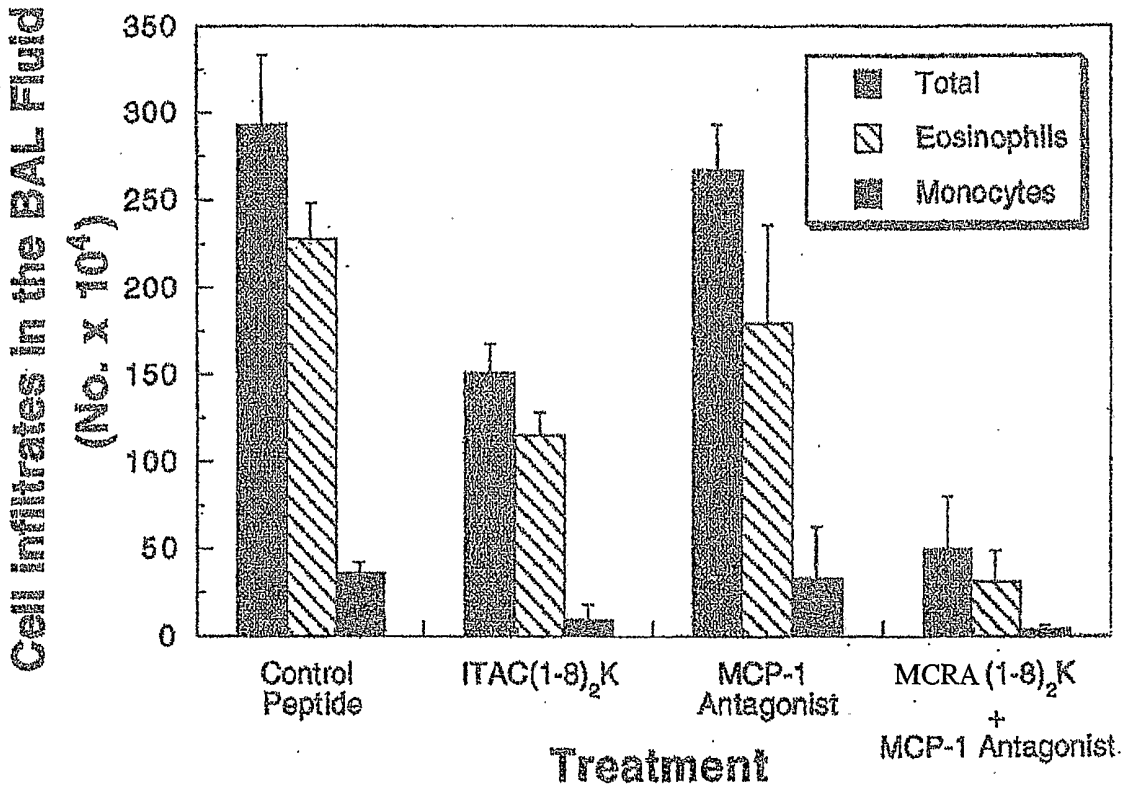
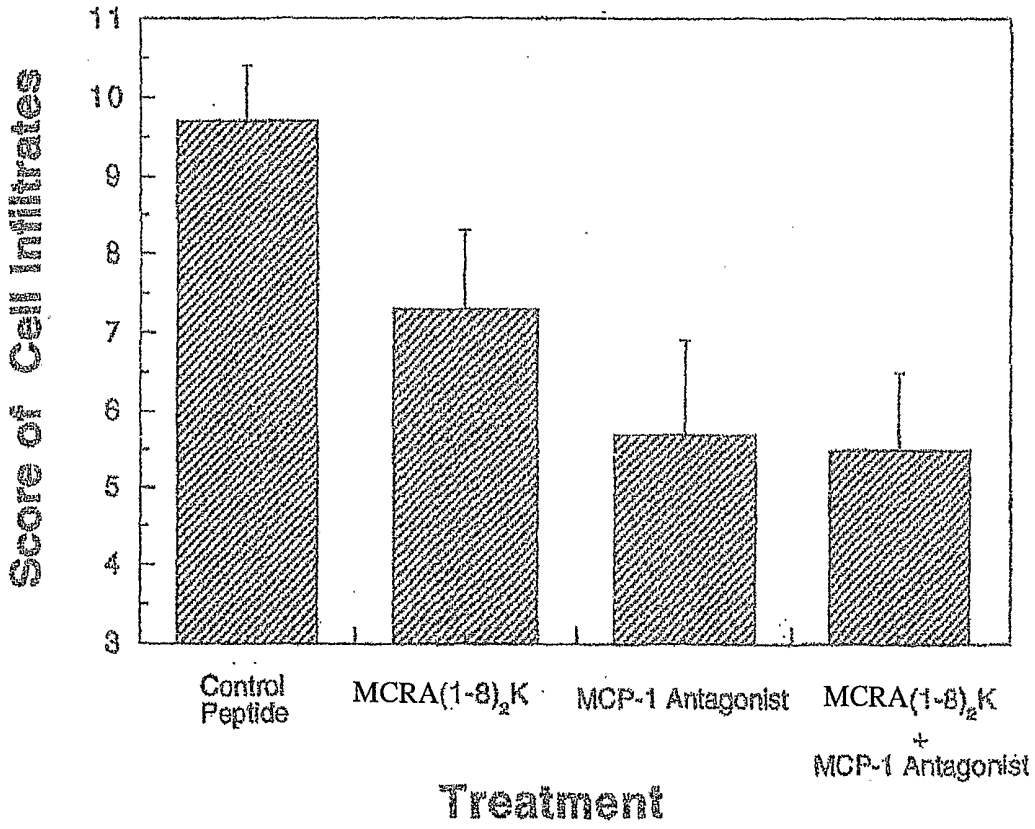

Cell infiltrates in the BAL Fluid
(No. x 10⁴)
350
300
250
200
150
100
50
0
Total
Eosinophils
Monocytes
Control Peptide
ITAC(1-8)2K
MCP-1 Antagonist
MCRA (1-8)2K + MCP-1 Antagonist
Treatment

Figure 19


Score of Cell Infiltrates
11
10
9
8
7
6
5
4
3
Control Peptide
MCRA(1-8)2K
MCP-1 Antagonist
MCRA(1-8)2K + MCP-1 Antagonist
Treatment