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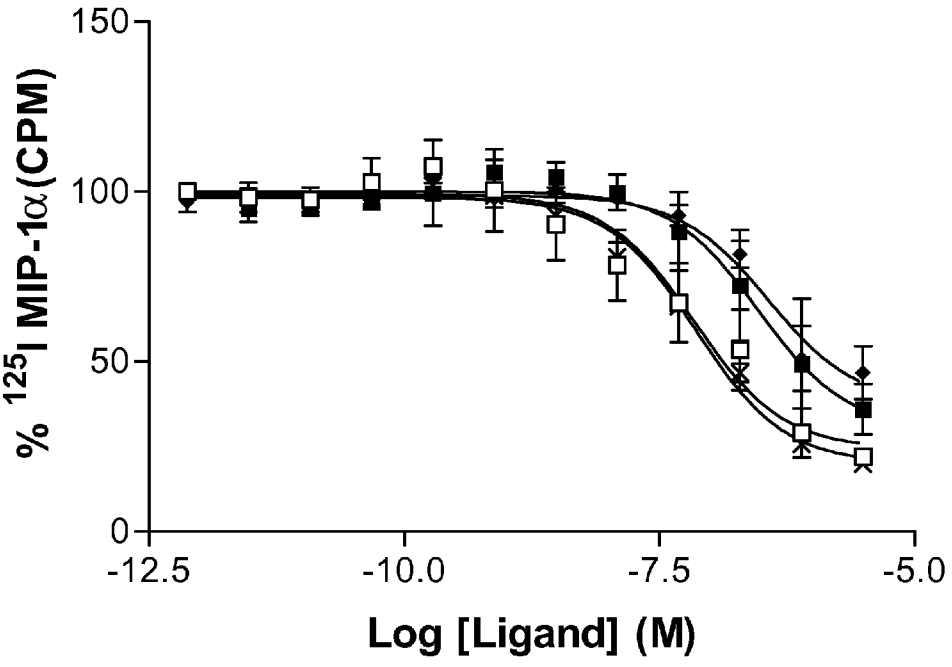

(57) Abstract: New CC-chemokine fusion proteins acting as dual CC-chemokine antagonists are provided. Compounds prepared in accordance with the present invention can be used as anti-inflammatory and immunomodulatory compounds and in the treatment or prevention of CC-chemokine-related diseases.

WO 2008/015199 A1

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CHEMOKINE ANTAGONISTS

FIELD OF THE INVENTION

The invention relates to novel CC-chemokine fusion proteins acting as dual CC-chemokine antagonists.

BACKGROUND OF THE INVENTION

Chemokines are secreted pro-inflammatory proteins of small dimensions (70-130 amino acids) mostly involved in the directional migration and activation of cells, especially the extravasation of leukocytes from the blood to tissue localizations needing the recruitment of these cells (Baggiolini M et al., 1997). Usually chemokines are produced at the site of an injury, inflammation, or other tissue alteration in a paracrine or autocrine fashion, triggering cell-type specific migration and activation.

Depending on the number and the position of the conserved cysteines in the sequence, chemokines are classified into C, CC, CXC and CX_3C chemokines. Inside each of these families, chemokines can be further grouped according to the homology of the entire sequence, or of specific segments.

A series of heptahelical G-protein coupled membrane receptors, are the binding partners that allow chemokines to exert their biological activity on the target cells, which present specific combinations of receptors according to their state and/or type. An unified nomenclature for chemokine ligands and receptors, which were originally named by the scientists discovering them in a very heterogeneous manner, has been proposed to associate each of these molecules to a systematic name including a progressive number: CCL1, CCL2, etc. for CC chemokines; CCR1, CCR2, etc. for CC chemokines receptors, and so on.

The physiological effects of chemokines result from a complex and integrated system of concurrent interactions. The receptors often have overlapping ligand specificity, so that a single receptor can bind different chemokines, as well as a single chemokine can bind different receptors. In particular, the N-terminal domain of chemokines is involved in receptor binding and N-terminal processing can either activate chemokines or render chemokines completely inactive.

Chemokine analogues offer the possibility for therapeutic intervention in pathological conditions associated to such processes, in particular by inhibiting/antagonizing specific chemokines and their receptors at the scope to preventing the excessive recruitment and activation of cells, in

particular leukocytes, for a variety of indications related to inflammatory and autoimmune diseases, cancers, and bacterial or viral infections (Proudfoot A et al., 2000).

Amongst all the chemokines characterized so far, CC-chemokines, such as CCL5 (also known as RANTES; Appay V and Rowland-Jones SL, 2001) or CCL2 (also known as MCP-1) have been intensively studied to identify therapeutically useful molecules. RANTES binds to three CC chemokine receptors, CCR1, CCR3 and CCR5 whilst MCP-1 only binds to CCR2 (Zlotnik, A. and Yoshie, O., 2000). Variants of CC-chemokines, missing up to nine N-terminal amino acids, have been tested for their activity as inhibitors or antagonists of the naturally occurring forms. These molecules are inactive on monocytes and are useful as receptor antagonists (WO 99/16877). Alternatively, N-terminal extension of the mature CC-chemokine with one Methionine results in almost complete inactivation of the molecule, which also behaves as an antagonist for the authentic one (WO 96/17935).

Moreover, in order to perform structure-function analysis of CC-chemokines, variants containing substitutions or chemical modifications in different positions, as well as CC-chemokine derived peptides, have been tested for the interactions with receptors or other molecules, such as Glycosaminoglycans (GAGs). Some of these variants have been disclosed as having significantly altered binding properties, and sometimes they are active as CC-chemokine antagonists, having potential therapeutic applications in the treatment of HIV infection and inflammatory or allergic diseases (WO 99/33989).

However, there is a need for novel and improved CC-chemokine antagonists that may be used in the treatment of inflammatory and autoimmune diseases.

SUMMARY OF THE INVENTION

It has been surprisingly found that fusion proteins between MCP-1 and RANTES, having Methionine at the amino-terminus of the fusion protein, have dual antagonistic activity. They are antagonistic to the receptors of both MCP-1 and RANTES. The fusion proteins may be in the form of Methionine-RANTES/MCP-1 or Methionine-MCP-1/RANTES, hence the C-terminus of RANTES is either operatively linked to the N-terminus of MCP-1 or the C-terminus of MCP-1 is operatively linked to the N-terminus of RANTES.

Such dual antagonists are highly useful since the chemokines RANTES and MCP-1 are often both involved in inflammatory disorders.

The antagonists can be used in the treatment of inflammatory and autoimmune diseases, cancers, and bacterial or viral infections, in particular wherein the involvement of RANTES and/or MCP-1 has been shown.

Other features and advantages of the invention will be apparent from the following detailed description.

DESCRIPTION OF THE FIGURES

Figure 1. Equilibrium competition binding of chemokine fusion proteins to CCR1

The binding assay was performed by monitoring the displacement of [^{125}I]-MIP-1α from CCR1-expressing CHO membranes consequent to the addition, as chemokine, of RANTES (□), Met-RANTES (X), Met-RANTES/MCP-1 (■) or Met-MCP-1/RANTES (◆). The IC_{50} values are 69 nM for RANTES, 72 nM for Met-RANTES, 295 nM for Met-RANTES/MCP-1 and 371 nM for Met-MCP-1/RANTES.

Figure 2. Equilibrium competition binding of chemokine fusion proteins to CCR2

The binding assay was performed by monitoring the displacement of [^{125}I]-MCP-1 from CCR2-expressing CHO membranes consequent to the addition, as chemokine, of hMCP-1 (○), Met-RANTES/MCP-1 (■) or Met-MCP-1/RANTES (◆). The IC_{50} values are 0.17 nM for hMCP-1, 136 nM for Met-RANTES/MCP-1 and 200 nM for Met-MCP-1/RANTES.

Figure 3. Equilibrium competition binding of chemokine fusion proteins to CCR5

The binding assay was performed by monitoring the displacement of [^{125}I]-MIP-1α from CCR5-expressing CHO membranes consequent to the addition, as chemokine, of RANTES (□), Met-RANTES (X), Met-RANTES/MCP-1 (■) or Met-MCP-1/RANTES (◆). The IC_{50} values are 1.2 nM for RANTES, 17 nM for Met-RANTES, 126 nM for Met-RANTES/MCP-1 and 79 nM for Met-MCP-1/RANTES.

Figure 4. Ability to induce chemotaxis of monocytes

A graph representing the results of the transwell chemotaxis assay performed using freshly isolated monocytes and, as chemotactic agent, recombinant human MCP-1 (○), recombinant human RANTES (□), Met-RANTES/MCP-1 (■), Met-MCP-1/RANTES (◆) and (X) Met-RANTES.

Figure 5. Ability to of Met-MCP-1/RANTES to inhibit RANTES-induced chemotaxis

A graph representing the inhibition of 1 nM RANTES-induced chemotaxis of L1.2/CCR5 transfectants in the transwell chemotaxis assay. Dose response using recombinant human RANTES (□), inhibition of 1 nM RANTES by Met-MCP-1/RANTES (◆), inhibition of 1 nM RANTES by Met- RANTES (X).

Figure 6. Ability to inhibit MCP-1 induced chemotaxis of THP-1 cells

A graph representing the inhibition of 1 nM MCP-1 induced chemotaxis of the monocytic THP-1 cell line in the transwell chemotaxis assay by Met-MCP-1/RANTES (◆) and Met-RANTES/MCP-1 (■).

Figure 7. Ability to inhibit MCP-1 induced chemotaxis of human monocytes

A graph representing the inhibition of 1 nM MCP-1 induced chemotaxis of the human monocytes in the transwell chemotaxis assay by Met-MCP-1/RANTES (◆) and Met-RANTES/MCP-1 (■).

DETAILED DESCRIPTION OF THE INVENTION

On the basis of equilibrium competition binding and chemotaxis assays, we have now found that chemokine fusion proteins having a methionine residue at their N-terminus and wherein a MCP-1 polypeptide and a RANTES polypeptide are operatively linked, may act as dual antagonists of the receptors for both MCP-1 and RANTES.

Fusion proteins according to this invention have reduced receptor binding activity for CCR1 and CCR5 compared to RANTES and for CCR2 compared to MCP-1, as shown in Example 4 and Figures 1 to 3.

Fusion proteins according to the invention may have CCR1 antagonistic activity.

Fusion proteins according to the invention may have CCR2 antagonistic activity.

Fusion proteins according to the invention may have CCR5 antagonistic activity.

Fusion proteins according to the invention do not induce chemotaxis of monocytes, which express CCR1, CCR2 and CCR5 receptors, as shown in Example 4 and Figure 4.

Fusion proteins according to the invention may have MCP-1 antagonistic activity, as shown in Example 4 and Figures 6 and 7.

Fusion proteins according to the invention may have RANTES antagonistic activity, a shown in Example 4 and Figure 5.

Preferably, fusion proteins according to the invention have MCP-1 and RANTES dual antagonistic activity.

Such dual antagonists are highly useful since the chemokines RANTES and MCP-1 are often both involved in inflammatory disorders. The chemokine fusion proteins according to this invention are therefore useful in the treatment of inflammatory and autoimmune diseases,

cancers, and bacterial or viral infections in particular wherein the involvement of RANTES and/or MCP-1 has been shown.

Therefore the main object of the present invention is to provide chemokine fusion proteins having a methionine residue at its N-terminus wherein a MCP-1 polypeptide and a RANTES polypeptide are operatively linked.

In a preferred embodiment, the C-terminus of RANTES polypeptide is operatively linked to the N-terminus of MCP-1 polypeptide.

In another preferred embodiment, the C-terminus of MCP-1 polypeptide is operatively linked to the N-terminus of RANTES polypeptide.

In the context of a fusion protein, the expression "operatively linked" indicates that the MCP-1 polypeptide and RANTES polypeptide are associated through peptide linkage(s), either directly or via spacer residues (e.g., a linker). In this manner, the fusion protein can be produced recombinantly, by direct expression in a host cell of a nucleic acid molecule encoding the same, as will be discussed below.

The design of the linkers, as well as methods and strategies for the construction, purification, detection, maturation, and use of fusion proteins are widely discussed in the literature (Nilsson J et al., 1997; “Applications of chimeric genes and hybrid proteins“ Methods Enzymol. Vol. 326-328, Academic Press, 2000).

In one embodiment, the linker is comprised of amino acids linked together by peptide bonds, wherein the amino acids are selected from the twenty naturally occurring amino acids. In various embodiments the linker can comprise 1-5 amino acids, 1-10 amino acids, 1-20 amino acids, 10-50 amino acids, 50-100 amino acids or 100-200 amino acids. In one embodiment the amino acids are selected from glycine, alanine, proline, asparagine, glutamine and lysine. In one embodiment a linker is made up of a majority of amino acids that are sterically unhindered, such as glycine and alanine.

Non-peptide linkers are also possible. For example, alkyl linkers could be used. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl, lower acyl, halogen, CN, NH2, phenyl etc. An exemplary non-peptide linker is a PEG (polyethylene glycol) linker.

In one embodiment, the C-terminus of RANTES is directly operatively linked to the N-terminus of MCP-1. The fusion protein according to this embodiment has preferably the amino acid sequence as defined in SEQ ID NO: 1. It is preferably coded for by a nucleic acid sequence identified by SEQ ID NO: 2.

In another embodiment, the C-terminus of MCP-1 is directly operatively linked to the N-terminus of RANTES. The fusion protein according to this embodiment has preferably the amino

acid sequence as defined in SEQ ID NO: 3. It is preferably coded for by a nucleic acid sequence identified by SEQ ID NO: 4.

MCP-1 of the invention includes for example native or wild-type MCP-1, in particular human MCP-1, bioactive variants thereof, such as bioactive allelic variants, and bioactive truncated forms of MCP-1. The present invention may relate to any variant or modified form of MCP-1 which retains the desired MCP-1 bioactivity as herein described, such as in particular its applicability in a fusion protein.

RANTES of the invention includes for example native or wild-type RANTES, in particular human RANTES, bioactive variants thereof, such as bioactive allelic variants, and bioactive truncated forms of RANTES. The present invention may relate to any variant or modified form of RANTES which retains the desired RANTES bioactivity as herein described, such as in particular its applicability in a fusion protein.

The chemokine fusion proteins of the present invention can be provided in alternative forms which can be preferred according to the desired method of use and/or production, for example as active fractions, precursors, salts, derivatives, conjugates or complexes.

The “precursors” are compounds, which can be converted into the fusion proteins of the present invention by metabolic, or enzymatic processing prior to or after the administration to the cells or to the organism.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the peptides, polypeptides, or analogs thereof, of the present invention. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid. Any of such salts should have substantially similar activity to the fusion proteins of the invention or their analogs.

The term "derivatives" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the C-terminal groups according to known methods. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alcanoyl- or aroyl-groups. Alternatively, the derivatives may contain sugars or phosphates groups linked to the functional groups present on the lateral chains of the amino acid moieties.

Such molecules can result from *in vivo* or *in vitro* processes which do not normally alter primary sequence, for example chemical derivatization of peptides (acetylation or carboxylation), phosphorylation (introduction of phosphotyrosine, phosphoserine, or phosphothreonine residues) or glycosylation (by exposing the peptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes).

Useful conjugates or complexes of the fusion proteins of the present invention can be generated, using molecules and methods known in the art for improving the detection of the interaction with other proteins (radioactive or fluorescent labels, biotin), therapeutic efficacy (cytotoxic agents, isotopes), or drug delivery efficacy.

In one embodiment, the present invention also encompasses a process for the preparation of a chemokine fusion protein according to this invention wherein the cDNAs of MCP-1 and RANTES are joined in tandem for recombinant protein expression.
In one embodiment, the 5' of the cDNA of RANTES is joined to the 3' of the cDNA of MCP-1. In another embodiment, the 5' of the cDNA of MCP-1 is joined to the 3' of the cDNA of RANTES.
Expression of any of the recombinant proteins of the invention as mentioned herein can be effected in Eukaryotic cells (e.g. yeasts, insect or mammalian cells) or Prokaryotic cells, using the appropriate expression vectors. Any method known in the art can be employed.
In particular, mammalian cells, such as human, monkey, mouse, and Chinese hamster ovary (CHO) cells in particular, are preferred because they provide post-translational modifications to protein molecules, including correct folding or glycosylation at correct sites. Also yeast cells can carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides). Alternatively, any of the specific protocols for the expression of chemokines in bacterial cells disclosed in the literature can be used. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector, may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

The vectors should allow the expression of the fusion protein of the invention in the Prokaryotic or Eukaryotic host cell under the control of transcriptional initiation/termination regulatory sequences, which are chosen to be constitutively active or inducible in said cell.

After the introduction of the vector(s), the host cells are grown in a selective medium, which selects for the growth of vector-containing cells.

Expression of the cloned gene sequence(s) results in the production of the desired proteins. A cell line substantially enriched in such cells can be then isolated to provide a stable cell line.

For Eukaryotic hosts (e.g. yeasts, insect or mammalian cells), different transcriptional and translational regulatory sequences may be employed, depending on the nature of the host. They may be derived form viral sources, such as adenovirus, bovine papilloma virus, Simian virus or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Examples are the TK promoter of the Herpes virus, the SV40 early promoter, the yeast gal4 gene promoter, etc. Transcriptional initiation regulatory signals may be selected which allow for repression and activation, so that expression of the genes can be modulated. The cells, which have been stably transformed by the introduced DNA, can be selected by also introducing one or more markers that allow for selection of host cells which contain the expression vector. The marker may also provide for phototrophy to an auxotropic host, biocide resistance, e.g. antibiotics, or heavy metals such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection.

The chemokine fusion proteins of the invention may be prepared by any other well known procedure in the art, in particular, by the well established chemical synthesis procedures, which can be efficiently applied on these molecule given the short length. Totally synthetic chemokines, also containing additional chemical groups, are disclosed in the literature (Brown A et al., 1996; Vita C et al., 2002).

Examples of chemical synthesis technologies are solid phase synthesis and liquid phase synthesis. As a solid phase synthesis, for example, the amino acid corresponding to the carboxy-terminus of the peptide to be synthesized is bound to a support which is insoluble in organic solvents, and by alternate repetition of reactions, one wherein amino acids with their amino groups and side chain functional groups protected with appropriate protective groups are condensed one by one in order from the carboxy-terminus to the amino-terminus, and one where the amino acids bound to the resin or the protective group of the amino groups of the peptides are released, the peptide chain is thus extended in this manner. Solid phase synthesis methods are largely classified by the tBoc method and the Fmoc method, depending on the type of protective group used. Typically used protective groups include tBoc (t-butoxycarbonyl), Cl-Z (2-chlorobenzyloxycarbonyl), Br-Z (2-bromobenzyloxycarbonyl), Bzl (benzyl), Fmoc (9-fluorenylmethoxycarbonyl), Mbh (4,4'-dimethoxydibenzhydryl), Mtr (4-methoxy-2,3,6-trimethylbenzenesulphonyl), Trt (trityl), Tos (tosyl), Z (benzyloxycarbonyl) and Cl2-Bzl (2,6-

dichlorobenzyl) for the amino groups; NO_2 (nitro) and Pmc (2,2,5,7,8-pentamethylchromane-6-sulphonyl) for the guanidino groups; and tBu (t-butyl) for the hydroxyl groups.

After synthesis of the desired peptide, it is subjected to the de-protection reaction and cut out from the solid support. Such peptide cutting reaction may be carried out with hydrogen fluoride or tri-fluoromethane sulfonic acid for the Boc method, and with TFA for the Fmoc method. Finally, the intact full-length peptides are purified and chemically or enzymatically folded (including the formation of disulphide bridges between cysteines) into the corresponding chemokine fusion proteins of the invention.

Purification of the natural, synthetic or recombinant proteins is carried out by any one of the methods known for this purpose, i.e. any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like. A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using monoclonal antibodies, heparin, or any other suitable ligand which can bind the target protein at high efficiency and can be immobilized on a gel matrix contained within a column. Impure preparations containing the proteins are passed through the column. The protein will be bound to the column by means of this ligand while the impurities will pass through. After washing, the protein is eluted from the gel by a change in pH or ionic strength. Alternatively, HPLC (High Performance Liquid Chromatography) can be also used.

Another object of the present invention is the use of the chemokine fusion proteins as above defined as medicaments, in particular as the active ingredients in pharmaceutical compositions (and formulated in combination with pharmaceutically acceptable carriers, excipients, stabilizers, adjuvants, or diluents). As explained above, the chemokine fusion proteins of the invention are dual antagonists to the receptors of both RANTES and MCP-1 and they are highly useful since the chemokines RANTES and MCP-1 are often both involved in inflammatory disorders. Still another object is the use of the chemokine fusion proteins of the invention to produce a pharmaceutical composition for treating or preventing disorders in which the antagonistic properties of said molecules can provide beneficial effects such as autoimmune and inflammatory diseases, cancers, as well as bacterial and viral infections. A non-limitative list of specific disorders includes arthritis, rheumatoid arthritis (RA), psoriatic arthritis, osteoarthritis, systemic lupus erythematosus (SLE), systemic sclerosis, scleroderma, polymyositis, glomerulonephritis, melanoma, carcinoma, leukaemia, lymphoblastoma, liver fibrosis, skin fibrosis, lung fibrosis, allergic or hypersensitvity diseases, dermatitis, Type IV hypersensitivity also called delayed-type hypersensitivity or DTH, asthma, chronic obstructive pulmonary disease (COPD), inflammatory bowel disease (IBD), Crohn's diseases, ulcerative colitis, multiple sclerosis, septic shock, HIV–infection, transplantation, graft-versus-host disease (GVHD), atherosclerosis.

Another object of the present invention is, therefore, the method for treating or preventing any of the above mentioned diseases by administering an effective amount of the chemokine fusion proteins of the invention together with a pharmaceutically acceptable excipient, and/or with another therapeutic composition which acts synergistically or in a coordinated manner with the chemokine fusion proteins of the invention. For example, synergistic properties of chemokine antagonists have been demonstrated in combination with cyclosporin (WO 00/16796).

An "effective amount" refers to an amount of the active ingredients that is sufficient to affect the course and the severity of the disease, leading to the reduction or remission of such pathology. The effective amount will depend on the route of administration and the condition of the patient.

A further object of the present invention are the pharmaceutical compositions containing the chemokine fusion proteins of the invention, in the presence of one or more pharmaceutically acceptable carriers, for treating or preventing any of the above mentioned diseases. The pharmaceutical compositions may be formulated in any acceptable way to meet the needs of the mode of administration. For example, the use of biomaterials and other polymers for drug delivery, as well the different techniques and models to validate a specific mode of administration are disclosed in literature (Luo B and Prestwich GD, 2001; Cleland JL et al., 2001).

"Pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with the effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which it is administered. Carriers can be selected also from starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the various oils, including those of petroleum, animal, vegetable or synthetic origin (peanut oil, soybean oil, mineral oil, sesame oil). For example, for parenteral administration, the above active ingredients may be formulated in unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution.

The administration of such active ingredient may be by intravenous, intramuscular or subcutaneous route. Other routes of administration, which may establish the desired blood levels of the respective ingredients, are comprised by the present invention. For example, administration may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, oral, or buccal routes. The pharmaceutical compositions of the present invention can also be administered in sustained or controlled release dosage forms, including depot injections, osmotic pumps, and the like, for the prolonged administration of the polypeptide at a predetermined rate, preferably in unit dosage forms suitable for single administration of precise dosages.

Parenteral administration can be by bolus injection or by gradual perfusion over time.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain auxiliary agents or excipients known in the art, and can be prepared according to routine methods. In addition, suspension of the active compounds as appropriate oily injection suspensions may be administered.

Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions that may contain substances increasing the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Pharmaceutical compositions include suitable solutions for administration by injection, and contain from about 0.01 to 99.99 percent, preferably from about 20 to 75 percent of active compound together with the excipient.

The optimal dose of active ingredient may be appropriately selected according to the route of administration, patient conditions and characteristics (sex, age, body weight, health, size), extent of symptoms, concurrent treatments, frequency of treatment and the effect desired. Adjustment and manipulation of established dosage ranges are well within the ability of those skilled.

Usually a daily dosage of active ingredient can be about 0.01 to 100 milligrams per kilogram of body weight. Ordinarily 1 to 40 milligrams per kilogram per day given in divided doses or in sustained release form is effective to obtain the desired results. Second or subsequent administrations can be performed at a dosage, which is the same, less than, or greater than the initial or previous dose administered to the individual.

The present invention has been described with reference to the specific embodiments, but the content of the descriptions comprises all modifications and substitutions, which can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention. The examples will refer to the Figures specified here below.

EXAMPLES

1. Generation of Met-RANTES/MCP-1 fusion

The RANTES ORF is contained in plasmid 13844 .The MCP-1 ORF is contained in plasmid 11711. The cDNAs encoding the mature protein coding sequence of RANTES and MCP-1 were amplified by PCR using plasmids 13844 and 11711 as templates, respectively.

The PCR products from each reaction were mixed and subjected to a ligation to fuse the 2 cDNAs together. The resultant chimeric DNA was then subcloned into Nde 1 (5') and EcoR1 (3') sites of the E. coli expression vector pET 20b (+) (Novagen).

PCR primers

The sequences of PCR primers used to construct the RANTES/ MCP-1 fusion are given in Table 1. The RANTES (no stop) RP and MCP-1 FP were kinased at the 5' end using T4 polynucleotide kinase (PNK).

PCR 1:

Plasmid 13844 was used as PCR template to generate the mature RANTES coding sequence with a start codon (ATG) and a 5' Nde I site. The PCR reaction, (in a final volume of 50 µl) contained: 1 µl of plasmid 13844 (25 ng), 4.0 µl dNTPs (10 mM), 5 µl of 10X Pwo polymerase buffer, 1.0 µl each of gene specific primer (80 pico-moles) (RANTES (Nde I) FP and kinased RANTES (without stop) RP), and 0.5 µl Pwo DNA polymerase (Roche). The PCR reaction was performed using an initial denaturing step of 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec; 58°C for 30 sec and 72 °C for 30 sec; a final extension cycle of 72 °C for 5 minutes and a holding cycle at 4 °C. An aliquot of amplification product was visualized on 1.6% agarose gel in 1 X TAE buffer in order to verify that the product was of the expected size (222 bp). The PCR amplified product was purified using the QIAquick Gel Extraction Kit (Qiagen). A 1 µl aliquot of the purified product was visualized on 1.6 % agarose gel.

PCR 2:

Plasmid ID. 11711 was used as PCR template to generate the mature MCP-1 coding sequence with a 3' EcoRI site and stop codon. The PCR reaction, (in a final volume of 50 µl) contained: 1 µl of plasmid 11711 (25 ng), 4.0 µl dNTPs (10 mM), 5 µl of 10X Pwo polymerase buffer, 1.0 µl each of gene specific primer (80 pico-moles) [Kinased MCP-1 FP and MCP-1 (EcoRI) RP], and 0.5 µl Pwo DNA polymerase (Roche). The PCR reaction was performed using an initial denaturing step of 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec; 58°C for 30 sec and 72 °C for 30 sec; a final extension cycle of 72 °C for 5 minutes and a holding cycle of 4 °C. An aliquot of amplification product was visualized on 1.6% agarose gel in 1 X TAE buffer in order to verify that the product was of the expected size (243 bp). The PCR amplified product was purified using the QIAquick Gel Extraction Kit (Qiagen). A 1 µl aliquot of purified PCR product was visualized on 1.6 % agarose gel.

Ligation Reaction:

The gel purified PCR 1 and PCR 2 products were ligated in a molar ratio of 1:1. The ligation reaction (in a final volume of 20 µl) contained: 1 µl (10 pmole) of each PCR product, 2 µl of 10X T4 DNA ligase buffer NEB, 1 µl of T4 DNA ligase NEB. The ligation mix was incubated at 16 °C overnight.

PCR 3:

The ligation mix was used as a template to generate the RANTES/MCP-1 fusion. The PCR reaction, (in a final volume of 50 µl) contained: 5 µl of ligation mix, 4.0 µl dNTPs (10 mM), 5 µl of 10X Pwo polymerase buffer, 1.0 µl of each gene specific primer (80 pmoles) (RANTES (NdeI) FP and MCP-1 (EcoRI) RP), and 0.5 µl Pwo DNA polymerase (Roche). The PCR reaction was performed using an initial denaturing step of 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec; 58°C for 30 sec and 72 °C for 45 sec; a final extension cycle of 72 °C for 5 minutes and a holding cycle of 4 °C. An aliquot of the amplification product was visualized on 1.6% agarose gel in 1 X TAE buffer in order to verify that the product was of the expected size (222 + 243 = 465 bp). The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen). A 1 µl aliquot of the purified PCR product was visualized on 1.6 % agarose gel.

Restriction Digestion:

The gel purified RANTES/MCP-1 fusion was digested with NdeI and EcoRI restriction enzymes. The restriction digest (in a final volume of 100 µl) contained: 45 µl (2 µg) of the gel purified DNA, 10 µl of 10X NEB buffer 2, 1 µl (10 U) each of NdeI and EcoRI enzymes. The digestion was carried out for 1 h at 37 °C. The digestion products were directly gel purified using the QIAquick Gel Extraction Kit (Qiagen). A 1 µl aliquot of the purified PCR product was visualized on 1.6% agarose gel.

Vector Preparation:

pET20b(+) vector was digested with NdeI and EcoRI enzymes. The restriction digest reaction (in a final volume of 250 µl) contained: 20 µl (5 µg) of the vector pET20b(+), 25 µl of 10X NEB buffer 2, 1 µl (10 U) each of NdeI and EcoRI enzymes. The digest was carried out for 1 h at 37 °C. The digestion products were directly gel purified using the QIAquick Gel Extraction Kit (Qiagen). A 1 µl aliquot was of the purified PCR product visualized on 1.6% agarose gel.

Dephosphorylation:

The NdeI/EcoRI cut pET20b(+) vector was dephosphorylated using Shrimp alkaline Phosphatase (SAP).

The reaction (in a final volume of 30 µl) contained: 18 µl (900 ng) of NdeI/EcoRI cut pET20b(+) DNA, 3 µl of 10X SAP buffer, and 3 µl (1 U/µl) of SAP. The reaction mix was incubated at 37 °C for 1 h and the enzyme was heat inactivated by incubating at 65 °C for 20 min.

Ligation of pET20b(+) and RANTES / MCP-1 fusion:

The ligation was carried out at a molar ratio of 1:5. The ligation reaction (in a final volume of 20 µl) contained: 2 µl (45 ng) of RANTES/MCP-1 fusion, 1 µl (30 ng) of pET20b(+), 2 µl of 10X NEB T4 DNA ligase buffer, 1 µl of NEB T4 DNA Ligase enzyme diluted 1:4. The ligation mix was incubated at 16 °C overnight.

Transformation:

The ligation mix was transformed into E. coli DH5α strain as follows: a 50 µl aliquot of DH5α cells was thawed on ice and 10 µl of ligation mixture was added. The mixture was incubated for 30 min on ice and then heat shocked by incubation at 42 °C for exactly 2 min. Samples were returned to ice for 2 min and 300 µl of warm SOC media (room temperature) was added. Samples were incubated with shaking (250 rpm) for 1 h at 37 °C. The transformation mixture was then plated on Luria agar plates containing Ampicillin (100 µg/ml) and incubated overnight at 37 °C.

Screening:

Ten ampicillin resistant colonies were picked and patched onto LB agar plates containing ampicillin (100 µg/ml) and incubated overnight at 37 °C. A scoop of the grown culture from the patched plate was resuspended in 50 µl of water and boiled for 5 minutes to lyse the cells. The cell lysate was centrifuged to remove the cell debris and the supernatant obtained was used as a template for colony PCR screening.

The PCR mixture (in a final volume of 25 µl) contained 10 µl of the centrifuged cell lysate, 2.0 µl dNTPs (10 mM), 2.5 µl of Taq polymerase buffer, 0.5 µl of screening primers (100 pmoles) (T7P and MCP-1 (EcoRI) RP) and 0.5 µl of Taq DNA polymerase.

The conditions for the screening PCR reaction were: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 sec; 58 °C for 30 sec and 72 °C for 45 sec; a final extension cycle of 72 °C for 5 minutes and a holding cycle of 4 °C. The PCR products were loaded onto a 1.6 % agarose gel to verify the size of the cDNA insert.

Plasmid DNA preparation and sequencing:

One positive clone was selected and plasmid mini-prep DNA was prepared from 5 ml cultures using QIAprep Spin Miniprep kit (Qiagen). Plasmid DNA (150-200 ng) was subjected to DNA sequencing with T7P and T7T primers using the CEQ Dye Terminator Cycle sequencing Quick Start Kit (Beckman Coulter P/N 608120) according to the manufacturer's instructions. The primer sequences are shown in Table 1. Sequencing reactions were analyzed on CEQ 2000 XL DNA analysis system (Beckman Coulter P/N 608450).

A sequence confirmed clone was designated as pET20b(+) / RANTES/MCP-1 fusion (plasmid 16347).

Table 1

PCR primers

RANTES Nde I FP SEQ ID NO: 5	5' TCC GCA AAA CAT ATG TCC CCA TAT TCC TCG GAC ACC 3'
RANTES (without stop) RP SEQ ID NO: 6	5' GCT CAT CTC CAA AGA GTT GAT 3'
MCP1 FP SEQ ID NO: 7	5' CAG CCA GAT GCA ATC AAT GCC CCA 3'
MCP1 EcoRI RP SEQ ID NO: 8	5' GAA GAA TTC TCA AGT CTT CGG AGT TTG GGT TTG CTT 3'
T7P SEQ ID NO: 9	5' TAA TAC GAC TCA CTA TAG GG 3'
T7T SEQ ID NO: 10	5' GCT AGT TAT TGC TCA GCG G 3'

The Met-RANTES/MCP-1 fusion protein has the following amino acid sequence:

MSPYSSDTTPCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKKWVR
EYINSLEMSQPDAINAPVTCCYNFTNRKISVQRLASYRRITSSKCPKEAVIFKTIVAKEI
CADPKQKWVQDSMDHLDKQTQTPKT

It is herein defined as SEQ ID NO: 1.

The cDNA coding for Met-RANTES/MCP-1 in the plasmid 16347 has the following nucleic acid sequence:

ATGTCCCCATATTCCTCGGACACCACACCCTGCTGCTTTGCCTACATTGCCCGCCCACTG
CCCCGTGCCCACATCAAGGAGTATTTCTACACCAGTGGCAAGTGCTCCAACCCAGCAGTC
GTCTTTGTCACCCGAAAGAACCGCCAAGTGTGTGCCAACCCAGAGAAGAAATGGGTTCGG
GAGTACATCAACTCTTTGGAGATGAGCCAGCCAGATGCAATCAATGCCCCAGTCACCTGC
TGTTATAACTTCACCAATAGGAAGATCTCAGTGCAGAGGCTCGCGAGCTATAGAAGAATC
ACCAGCAGCAAGTGTCCCAAAGAAGCTGTGATCTTCAAGACCATTGTGGCCAAGGAGATC
TGTGCTGACCCCAAGCAGAAGTGGGTTCAGGATTCCATGGACCACCTGGACAAGCAAACC
CAAACTCCGAAGACT

It is herein defined as SEQ ID NO: 2.

2. Generation of Met-MCP-1/RANTES fusion

The MCP-1 ORF is contained in plasmid 11711. The RANTES ORF is contained in plasmid 13844. The cDNAs encoding the mature protein coding sequence of MCP-1 and RANTES were amplified by PCR using plasmids 11711 and 13844 as templates respectively. The PCR products from each reaction were mixed and subjected to a ligation to fuse the 2 cDNAs together. The resultant chimeric DNA was then subcloned into Nde 1 (5') and EcoR1 (3') sites of the E coli expression vector pET 20b (+) (Novagen).

PCR primers

The sequences of PCR primers used to construct the MCP-1/RANTES fusion are given in Table 2. The MCP-1 (no stop) RP and RANTES FP were kinased at the 5' end using T4 polynucleotide kinase (PNK).

PCR 1:

Plasmid ID 11711 was used as PCR template to generate the MCP-1 with 5' NdeI site. The PCR reaction, (in a final volume of 50 µl) contained: 1 µl of plasmid 11711 (25 ng), 4.0 µl dNTPs (10 mM), 5 µl 10X Pwo polymerase buffer, 1.0 µl of each gene specific primer (80 pmoles) (MCP-1 (Nde I) FP and kinased MCP-1 (without stop) RP), and 0.5 µl Pwo DNA polymerase (Roche). The PCR reaction was performed using an initial denaturing step of 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec; 59°C for 30 sec and 72 °C for 30 sec; and a final extension cycle of 72 °C for 5 minutes and a holding cycle of 4 °C. An aliquot of the amplification product was visualized on a 1.6% agarose gel in 1 X TAE buffer in order to verify that the product was of the expected molecular weight (243 bp). The PCR amplified product was purified using the QIAquick Gel Extraction Kit (Qiagen). A 1 µl aliquot of the purified DNA was visualized on 1.6 % agarose gel.

PCR 2:

Plasmid 13844 was used as a PCR template to generate the mature RANTES coding sequence with a stop codon and a 3' EcoRI site. The PCR reaction, (in a final volume of 50 µl) contained respectively: 1 µl of plasmid 13844 (25 ng), 4.0 µl dNTPs (10 mM), 5 µl of 10X Pwo polymerase buffer, 1.0 µl of each gene specific primer (80 pmoles) (Kinased RANTES FP and RANTES (EcoRI) RP), and 0.5 µl Pwo DNA polymerase (Roche). The PCR reaction was performed using an initial denaturing step of 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec; 57°C for 30 sec and 72 °C for 30 sec; a final extension cycle of 72 °C for 5 min and a holding cycle of 4 °C. An aliquot of amplification product was visualized on 1.6% agarose gel in 1 X TAE buffer in order to verify that the product was of the expected size (219 bp). The PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen). A 1 µl aliquot of the purified product was visualized on 1.6 % agarose gel.

Ligation Reaction:

The gel purified PCR 1 and PCR 2 products were ligated in a molar ratio of 1:1. The ligation reaction (in a final volume of 20 µl) contained: 1 µl (10 pmole) of each PCR product, 2 µl of 10X T4 DNA ligase buffer NEB (New England Biolabs), and 1 µl of T4 DNA Ligase NEB. The ligation mix was incubated at 16 °C overnight.

PCR 3:

The ligation mix was used as a template to generate the MCP-1/RANTES fusion. The PCR reaction, (in a final volume of 50 µl) contained: 5 µl of ligation mix, 4.0 µl dNTPs (10 mM), 5 µl of 10X Pwo polymerase buffer, 1.0 µl of each gene specific primer (80 pmoles) [MCP-1 (NdeI) FP and RANTES (EcoRI) RP], and 0.5 µl Pwo DNA polymerase (Roche). The PCR reaction was performed using an initial denaturing step of 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec; 57°C for 30 sec and 72 °C for 45 sec; a final extension cycle of 72 °C for 5 minutes and a holding cycle of 4 °C. An aliquot of amplification product was visualized on 1.6% agarose gel in 1 X TAE buffer in order to verify that the product was of the expected molecular weight (243 + 219 = 462 bp). The PCR amplified product was purified using the QIAquick Gel Extraction Kit (Qiagen). A 1 µl aliquot was visualized on 1.6 % agarose gel.

Restriction Digestion:

The gel purified MCP-1/RANTES fusion product was digested with NdeI and EcoRI restriction enzymes. The restriction digestion reaction (in a final volume of 100 µl) contained: 45 µl (2 µg) of the gel purified DNA, 10 µl of 10X NEB buffer 2,1 µl (10 U) each of NdeI and EcoRI enzyme. The digestion was carried out for 1 h at 37 °C.

The digestion products were directly gel purified using the QIAquick Gel Extraction Kit (Qiagen). A 1 µl aliquot of the purified product was visualized on 1.6% agarose gel.

Vector Preparation:

pET20b(+) vector was digested with NdeI and EcoRI enzymes. The restriction digest (in a final volume of 250 µl) contained: 20 µl (5 µg) of pET20b(+), 25 µl of 10X NEB buffer 2,1 µl (10 U) each of NdeI and EcoRI enzymes. The digest was carried out for 1 h at 37 °C. The reaction products were directly gel purified using the QIAquick Gel Extraction Kit (Qiagen). A 1 µl aliquot of the purified NdeI/EcoRI cut pET20b(+) product was visualized on 1.6% agarose gel.

Dephosphorylation:

The NdeI/EcoRI cut pET20b(+) vector was dephosphorylated using Shrimp alkaline Phosphatase (SAP). The reaction (in a final volume of 30 µl) contained: 18 µl (900 ng) of NdeI/EcoRI cut pET20b(+) vector DNA, 3 µl of 10X SAP buffer, and 3 µl (1 U/µl) of SAP enzyme. The reaction mix was incubated at 37 °C for 1 h and the enzyme was heat inactivated by incubating at 65 °C for 20 min.

Ligation of pET20b(+) and MCP-1 / RANTES fusion:

The ligation was carried out at a molar ratio of 1:5. The ligation reaction (in a final volume of 20 µl) contained: 2 µl (45 ng) of MCP-1/RANTES fusion, 1 µl (30 ng) of pET20b(+), 2 µl of 10X NEB T4 DNA ligase buffer, 1 µl of T4 DNA Ligase (NEB) diluted 1:4. The ligation was incubated at 16 °C overnight.

Transformation:

The ligation mixture was transformed into E. coli DH5α strain as follows: a 50 µl aliquot of DH5α cells was thawed on ice and 10 µl of ligation mixture was added. The mixture was incubated for 30 min on ice and then heat shocked by incubation at 42 °C for exactly 2 min. Samples were returned to ice for 2 min then 300 µl of warm SOC media (room temperature) was added. Samples were incubated with shaking (250 rpm) for 1 h at 37 °C. The transformation mixture was then plated on Luria agar plates containing ampicillin (100 µg/ml) and incubated overnight at 37 °C.

Screening:

Ten ampicillin resistant colonies were picked and patched on LB agar plates containing ampicillin (100 µg/ml) and incubated overnight at 37 °C. A scoop of the grown culture from the patched plate was resuspended in 50 µl of water and boiled for 5 minutes to lyse the cells.

The cell lysate was centrifuged to remove the cell debris and the supernatant obtained was used as a template for colony PCR screening.

The PCR mixture (in a final volume of 25 µl) contained 10 µl of the centrifuged cell lysate, 2.0 µl dNTPs (10 mM), 2.5 µl of Taq polymerase buffer, 0.5 µl of screening primers (100 pmoles) (T7P and RANTES (EcoRI) RP) and 0.5 µl of Taq DNA polymerase.

The PCR reaction conditions were: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 sec; 57 °C for 30 sec and 72 °C for 45 sec; a final extension cycle of 72 °C for 5 minutes and a holding cycle of 4 °C. The PCR products were loaded onto a 1.6 % agarose gel to verify the presence of the fusion cDNA insert.

Plasmid DNA preparation and sequencing:

One positive clone was selected and plasmid mini-prep DNA was prepared from 5 ml cultures using QIAprep Spin Miniprep kit (Qiagen). Plasmid DNA (150-200 ng) was subjected to DNA sequencing with T7P and T7T primers using the CEQ Dye Terminator Cycle sequencing Quick Start Kit (Beckman Coulter P/N 608120) according to the manufacturer's instructions. The primer sequences are shown in Table 2. Sequencing reactions were analyzed on CEQ 2000 XL DNA analysis system (Beckman Coulter P/N 608450).

One sequence confirmed clone was designated as pET20b(+) / MCP-1/RANTES fusion (plasmid 16348).

Table 2

PCR primers

MCP1 Nde I FP SEQ ID NO: 11	5' TCC GCA AAA CAT ATG CAG CCA GAT GCA ATC AAT GC 3'
MCP1 (without stop) RP SEQ ID NO: 12	5' AGT CTT CGG AGT TTG GGT TTG CTT G 3'
RANTES FP SEQ ID NO: 13	5' TCC CCA TAT TCC TCG GAC ACC ACA 3'
RANTES EcoRI RP SEQ ID NO: 14	5' CAA GAA TTC TCA GCT CAT CTC CAA AGA GTT GAT 3'
T7P SEQ ID NO: 9	5' TAA TAC GAC TCA CTA TAG GG 3'
T7T SEQ ID NO: 10	5' GCT AGT TAT TGC TCA GCG G 3'

The Met-MCP-1/RANTES fusion protein has the following amino acid sequence:

MQPDAINAPVTCCYNFTNRKISVQRLASYRRITSSKCPKEAVIFKTIVAKEICADPKQKW
VQDSMDHLDKQTQTPKTSPYSSDTTPCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVT
RKNRQVCANPEKKWVREYINSLEMS

It is herein defined as SEQ ID NO: 3.

The cDNA coding for Met-MCP-1/RANTES in the plasmid 16348 has the following nucleic acid sequence:

ATGCAGCCAGATGCAATCAATGCCCCAGTCACCTGCTGTTATAACTTCACCAATAGGAAG
ATCTCAGTGCAGAGGCTCGCGAGCTATAGAAGAATCACCAGCAGCAAGTGTCCCAAAGAA
GCTGTGATCTTCAAGACCATTGTGGCCAAGGAGATCTGTGCTGACCCCAAGCAGAAGTGG
GTTCAGGATTCCATGGACCACCTGGACAAGCAAACCCAAACTCCGAAGACTTCCCCATAT
TCCTCGGACACCACACCCTGCTGCTTTGCCTACATTGCCCGCCCACTGCCCCGTGCCCAC
ATCAAGGAGTATTTCTACACCAGTGGCAAGTGCTCCAACCCAGCAGTCGTCTTTGTCACC
CGAAAGAACCGCCAAGTGTGTGCCAACCCAGAGAAGAAATGGGTTCGGGAGTACATCAAC
TCTTTGGAGATGAGC

It is herein defined as SEQ ID NO: 4.

3. Expression and purification of the Met-RANTES/MCP-1 and Met-MCP-1/RANTES fusion proteins

Both the Met-RANTES/MCP-1 and Met-MCP-1/RANTES fusion proteins were expressed and purified using the same protocol.

The vectors obtained by PCR as described above containing the two fusion proteins were used to retransform the BL21(DE3) Star *E.coli* strain. Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture. The *E. coli* cell pellets were suspended in 3 volumes per g (wet weight) of cells (mg/ml) with cell breakage buffer: 50 mM Tris-HCl pH 8.0 containing 10 mM $MgCl_2$, 5 mM Benzamidine/HCl, 1 mM DTT, 1 mM PMSF, 20 mg/l DNAse and polytroned. The cells were broken by two passages on the French Pressure cell and the solution was centrifuged 30 min at 13'000 rpm (27,500 x *g*).

The inclusion bodies from the pellet were solubilized with 100 ml/g of cells (wet weight) in extraction buffer: 0.1 M Tris-HCl pH 8.5 containing 6 M guanidine-HCl and 1 mM DTT, polytroned, heated for 1 h at 60°C, cooled to room temperature and filtered with a 22 µm filter. The amount of protein was calculated by a calorimetric assay and applied to a Reverse Phase Source 30 Chromatography (RPC) column previously equilibrated in 0.1 M Tris-HCl pH 7.5. After loading, the RPC column was washed with 5 column volumes (CV) of 0.1 M Tris-HCl pH 7.5, then with 5 CV of 0.1% TFA and eluted with a 0-90 % gradient of 90% acetonitrile in 0.1% TFA over 7 CV. The fractions were analysed by SDS-PAGE using NuPAGE 10% Bis-Tris gels run in MES buffer and pooled according to their chemokine quantity and purity.

The chemokine fusion proteins were renatured by infinite dilution. The pooled fractions from the RPC were adjusted to pH 8.0 with NaOH and added dropwise at 0.1 ml/min into the renaturation buffer: 0.1 M Tris/HCl pH8, containing 0.1 mM reduced glutathione, 0.01 mM oxidized glutathione at 4°C, to obtain a final protein concentration of 50 µg/ml. The renaturation solution was left overnight at 4°C under agitation. The solution was filtered with a 22 µm filter, quantified, adjusted to pH 6.8 with HCl, diluted until the conductivity was below 10 mS in order to concentrate the renatured chemokines by cation exchange chromatography. The column was freshly packed with SP Sepharose HP gel (Amersham®) and equilibrated with 50 mM potassium phosphate pH 6.8. After loading, the column was washed with 50 mM potassium phosphate pH 6.8 and eluted with a linear 0-2 M NaCl gradient in the same buffer over 10 CV. Fractions were analysed by SDS-PAGE as described above, pooled according to their chemokine content and purity, and the pool was quantified by UV spectra.

4. Characterization of the Met-RANTES/MCP-1 and Met-MCP-1/RANTES fusions

The authenticity of the proteins was verified by mass spectrometry using a MALDI-TOF. Their apparent molecular weight was estimated by size exclusion chromatography using a HiLoad 16/60 Superdex 200 HR column, previously equilibrated in PBS and calibrated with the standard proteins BSA (66 kDa), Ovalbumin (43 kDa), Chymotrypsinogen (25 kDa) and Ribonuclease (12.6 kDa). 50 µl of a 1 mg/ml solution of the fusion proteins in PBS were applied to the column.

Chemotaxis:

The chemotaxis assays were performed with L1.2/CCR5 transfectant cells, THP-1 cells or purified monocytes. Cells were cultured in the appropriate medium until a concentration of approximately $1x10^6$/ml. The L1.2/CCR5 transfectant cells were activated overnight prior to the chemotaxis experiment with 5 mM butyric acid. After washing with PBS, the cells were washed and incubated in the chemotaxis medium (RPMI 1640 without red phenol) containing heat

inactivated 5% FCS at a concentration of $1x10^6$/ml. Chemotaxis was assayed in ChemoTx 96 well plate (Neuro Probe Inc.®) with 5 µM pores for THP-1 cells and L1.2/CCR5 transfectants. The chemokines were placed in triplicate in the lower wells with appropriate dilutions in chemotaxis medium and covered with the membrane. The cells were placed on the upper surface of the membrane at 2.10^4 cells/well and the plates were incubated 2 h at 37°C with CO_2. The membrane was washed with PBS and the bottom wells were transferred into well flat bottom black plates (Costar®) using funnel adapters (Neuro Probe Inc.®). The black plates were frozen for a minimum of 1 h at -80°C, thawed and the number of migrated cells was measured using the CyQuant cell proliferation assay kit (Molecular Probes®).

For chemotaxis with human monocytes, cells were purified from a buffy coat using a MACS Monocyte Isolation Kit II human, LS MACS column and MACS separator (Miltenyi Biotech ®). The purified monocytes were suspended at $1x10^6$/ml in chemotaxis medium. The chemotaxis was carried out as described above but 3 µM membranes were used.

Competition equilibrium receptor binding assay:

The assay was carried out on membranes isolated from CHO transfectants expressing CCR1 and CCR5, or CHO membranes expressing CCR2b from Invitrogen® using a scintillation proximity assay (Alouani S., et al.). The unlabelled chemokines were prepared in triplicate by serial dilution in the range of 10^{-6} to 10^{-12} M in 50 mM HEPES pH 7.5, containing 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0,5% bovine serum albumin (binding buffer). Wheat germ scintillation proximity assay beads (Amersham ®) were solubilized at 50 mg/ml in PBS, diluted to 10 mg/ml in binding buffer and put at 250 µg/well. Membranes were diluted to 80 µg/ml in binding buffer and put at 2 µg/well in the assay plates. ^{125}I-chemokine (Amersham ®) was put at 0.1 nM/well and finally unlabelled chemokine was added into the plate. The plates were incubated for 3 h at room temperature with agitation and radioactivity was read with a Wallac counter.

Chemotaxis inhibition assays

To determine inhibition by the fusion proteins, chemotaxis was induced by a constant concentration (1 nM) of agonist in the presence of varying concentrations of the fusion protein as shown in Figures 5-7.

5. Results

The recombinant fusion proteins expressed at high levels after induction with IPTG, showing an intense band migrating at the expected MW around18 kDa. They were found as insoluble aggregates in the inclusion body fraction, and despite the fact that the two proteins were joined together, they were able to refold.

They were verified by mass spectroscopy using MALDI-TOF analysis, which confirmed that the initiating methionine was retained on both fusion proteins. Both RANTES and MCP-1 associate into higher order oligomers; in the case of RANTES, very large oligomers with a mass >600 kDa (Czaplewski, L.G. et al., 1999), and in the case of MCP-1, into dimers and tetramers (Lubkowski, J., et al. 1997). However the two fusion proteins do not associate into higher order oligomers since on size exclusion chromatography in PBS, the RANTES/MCP-1 fusion eluted at a volume corresponding to a molecular weight of 16 kDa and the MCP-1/RANTES fusion eluted at a volume corresponding to a molecular weight of 18.8 kDa.

Their ability to bind to both RANTES and MCP-1 receptors was ascertained by competition equilibrium binding assays. As shown in Fig.1, RANTES and Met-RANTES bind to CCR1 with an IC_{50} of 69 nM and 72 nM respectively, and both fusions retained CCR1 binding, but with approximately a 4- 5 fold drop in affinity, with an IC_{50} of 295 nM for Met-RANTES/MCP-1 and 371 nM for Met-MCP-1/RANTES. Again, binding to CCR5 was retained, with similar decreases in IC_{50} with Met-RANTES/MCP-1 showing 126 nM and Met-MCP-1/RANTES 79 nM compared to 1.2 nM for RANTES and 17 nM for Met-RANTES. Whilst binding to CCR2 was retained, the decrease in affinity was more pronounced since MCP-1 binds to CCR2 with an IC_{50} of 0.17 nM for hMCP-1, whereas the IC_{50} was 136 nM for Met-RANTES/MCP-1 and 200 nM for Met-MCP-1/RANTES.

Receptor activation was investigated via the fusion proteins ability to induce chemotaxis of cells expressing these receptors. Monocytes express all three receptors, although the levels of CCR5 are low on circulating monocytes, and the cells responded to both MCP-1 and RANTES (Fig.4). However neither fusion protein induced chemotaxis, as is the case for Met-RANTES.

Since Met-RANTES has been shown to have important anti-inflammatory properties in vivo in several disease models, and it retains receptor binding with abrogated receptor activation, these fusions will similarly have anti-inflammatory activities. As shown in Fig. 5, inhibition of RANTES-induced chemotaxis by Met-MCP-1/RANTES was observed, with a drop in potency compared to Met-RANTES in accordance with the drop observed in receptor binding. Both Met-RANTES/MCP-1 and Met-MCP-1/RANTES showed inhibition of the MCP-1 induced chemotaxis, again with potencies similar to those observed by the receptor binding studies.

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CLAIMS

1. A chemokine fusion protein having a methionine residue at its N-terminus wherein a MCP-1 polypeptide and a RANTES polypeptide are operatively linked.

2. A fusion protein according to claim 1 wherein MCP-1 and RANTES polypeptides are of human origin.

3. A fusion protein according to claim 1 or 2 wherein the C-terminus of RANTES polypeptide is operatively linked to the N-terminus of MCP-1 polypeptide.

4. A fusion protein according to any of claims 1 to 3 comprising or consisting of the amino acid sequence identified by SEQ ID NO: 1.

5. A nucleic acid sequence coding for the fusion protein according to any of claims 1 to 4.

6. A nucleic acid sequence according to claim 5 identified by SEQ ID NO: 2.

7. A fusion protein according to claim 1 or 2 wherein the C-terminus of MCP-1 polypeptide is operatively linked to the N-terminus of RANTES polypeptide.

8. A fusion protein according to claim 1, 2 or 7 having the amino acid sequence identified by SEQ ID NO: 3.

9. A nucleic acid sequence coding for the fusion protein according to claim 7 or 8.

10. A nucleic acid sequence according to claim 9 identified by SEQ ID NO: 4.

11. A fusion protein according to any of claims 1 to 3 or 7 wherein the MCP-1 polypeptide and RANTES polypeptide sequences are operatively linked via spacer residues.

12. A fusion protein according to any of claims 1 to 4, 7, 8 or 11 having at least MCP-1 antagonistic activity.

13. A fusion protein according to any of claims 1 to 4, 7, 8 or 11 having at least RANTES antagonistic activity.

14. A fusion protein according to claim 12 or 13 having dual MCP-1 and RANTES antagonistic activity.

15. A process for the preparation of a fusion protein according to any of claims 1 to 4, 7, 8, or 11 to 14 wherein the cDNAs of MCP-1 and RANTES are joined in tandem for recombinant protein expression.

16. A fusion protein according to any of claims 1 to 4, 7, 8, or 11 to 14 for use as a medicament.

17. Use of a fusion protein according to any of claims 1 to 4, 7, 8, or 11 to 14 as a dual antagonist for the receptors of RANTES and MCP-1.

18. Use of a fusion protein according to any of claims 1 to 4, 7, 8, or 11 to 14 in the preparation of a pharmaceutical composition for the treatment of autoimmune and inflammatory diseases, cancer, bacterial or viral infections.

19. Pharmaceutical composition for the treatment of autoimmune and inflammatory diseases, cancer, bacterial or viral infections comprising as active ingredient a fusion protein according to any of claims 1 to 4, 7, 8, or 11 to 14 together with a pharmaceutically acceptable carrier.

20. Method for the treatment or prevention of autoimmune and inflammatory diseases, cancer, bacterial or viral infections, comprising the administration of an effective amount of a fusion protein according to any of claims 1 to 4, 7, 8, or 11 to 14.

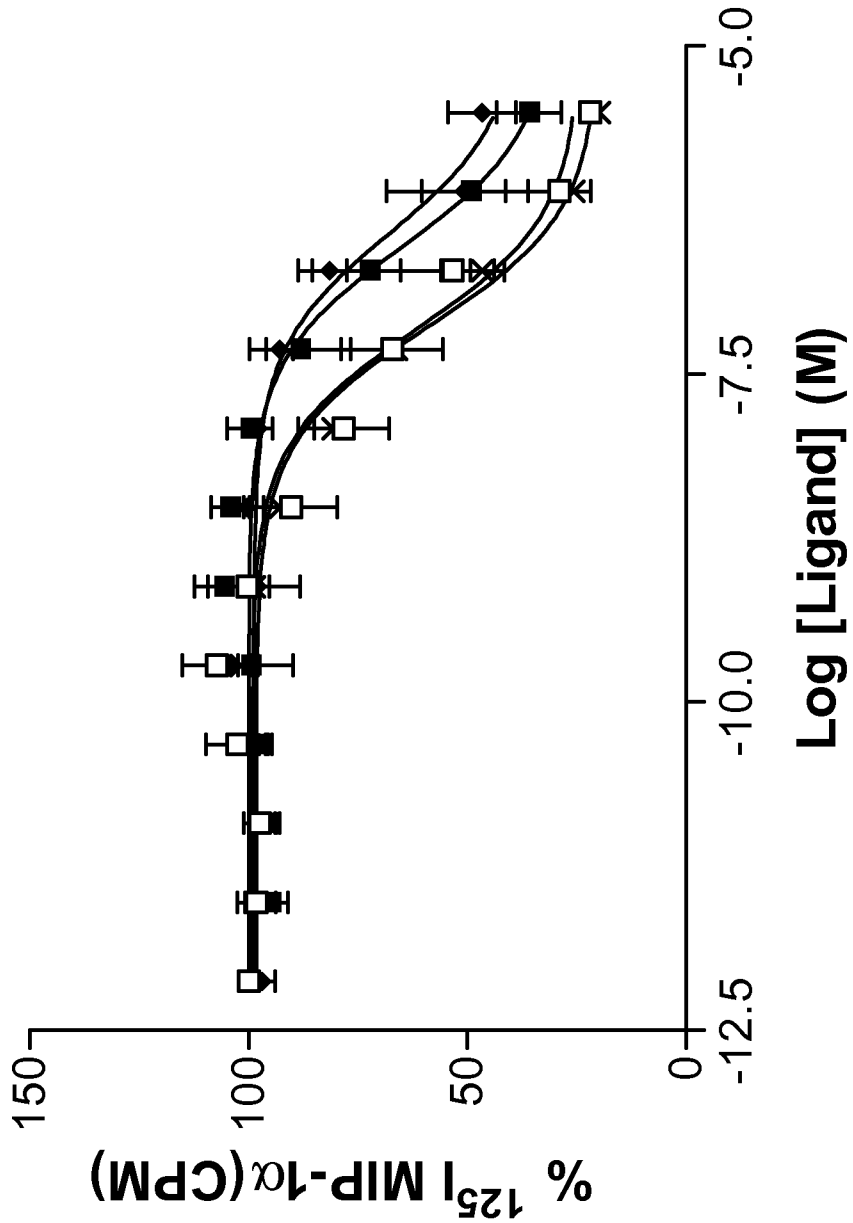

Fig. 1

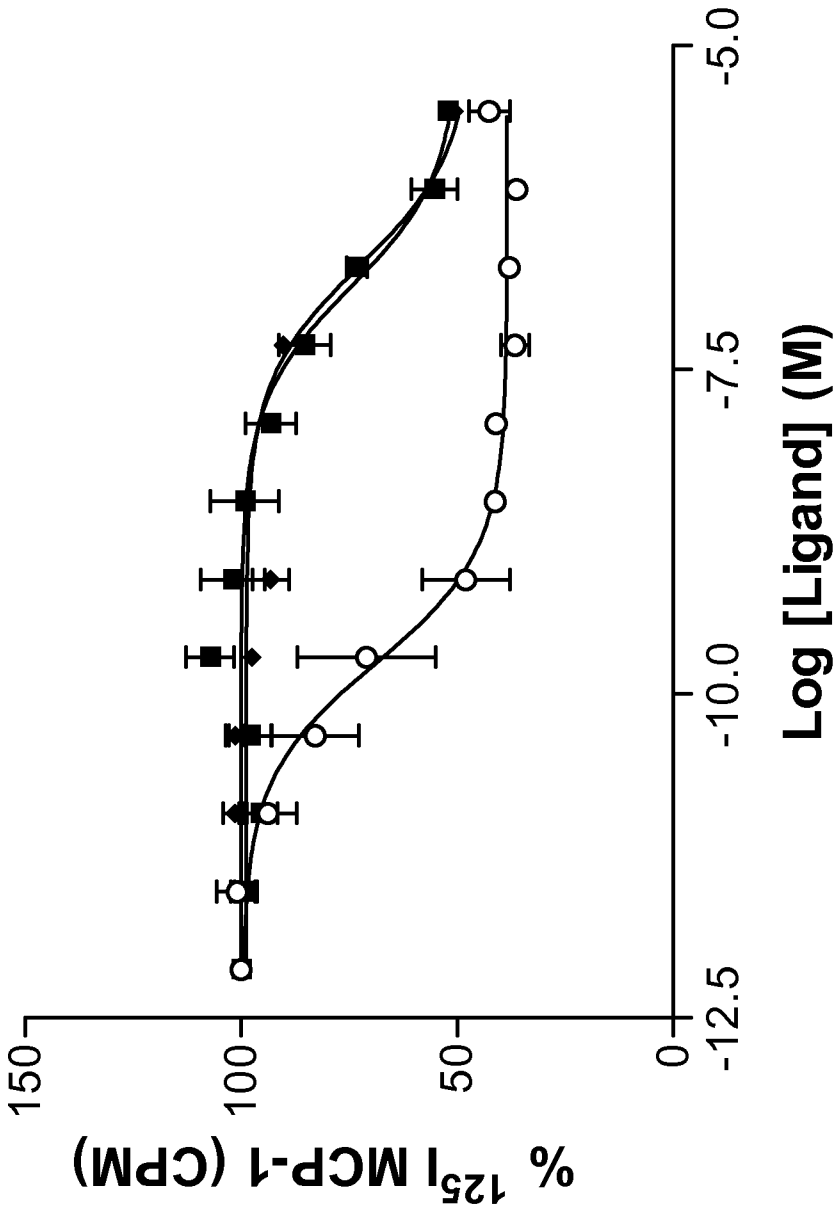

Fig. 2

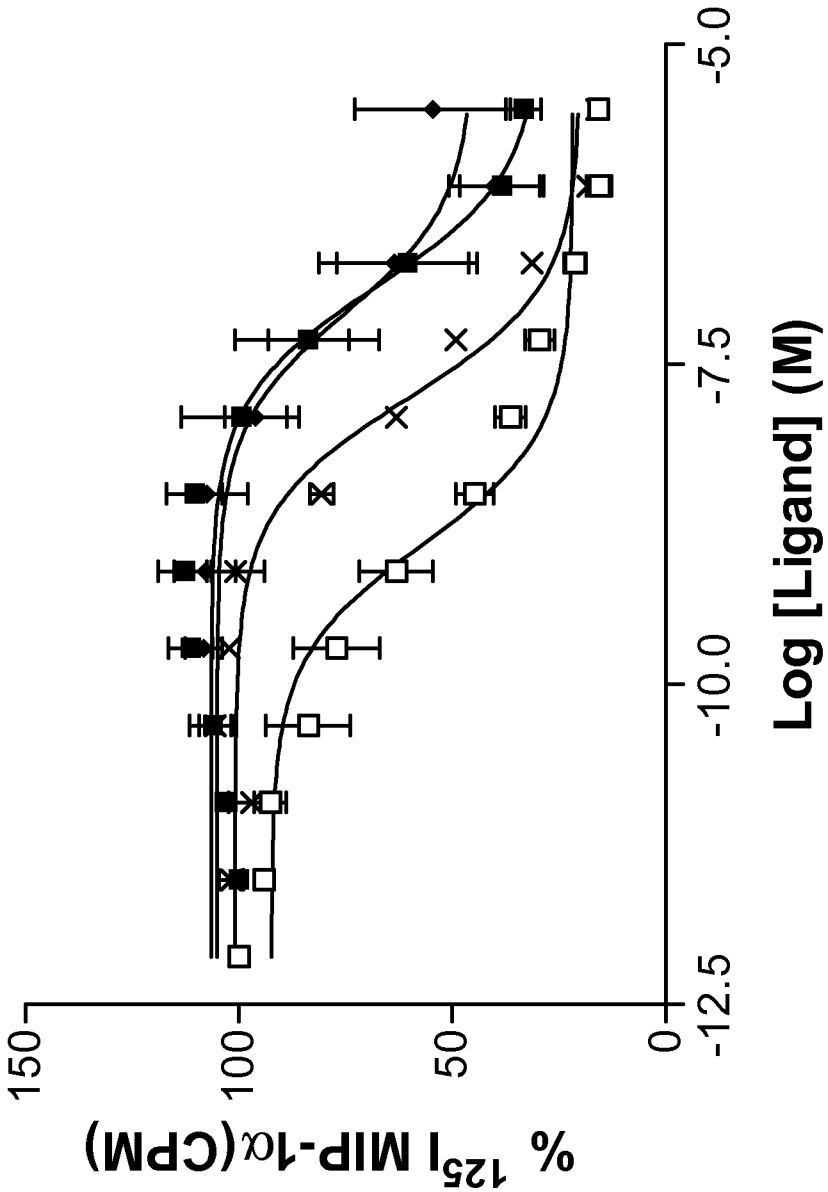

Fig. 3

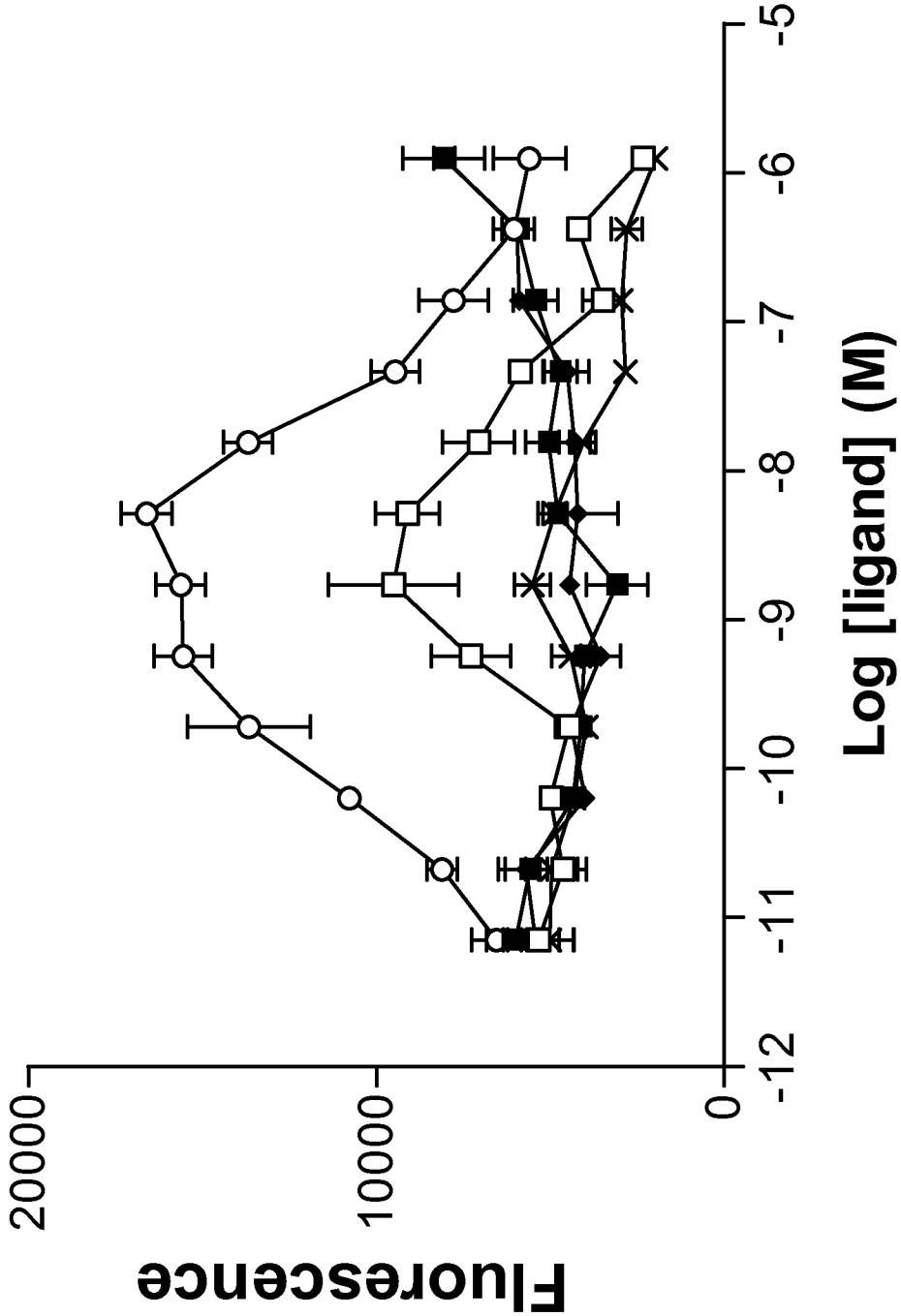

Fig. 4

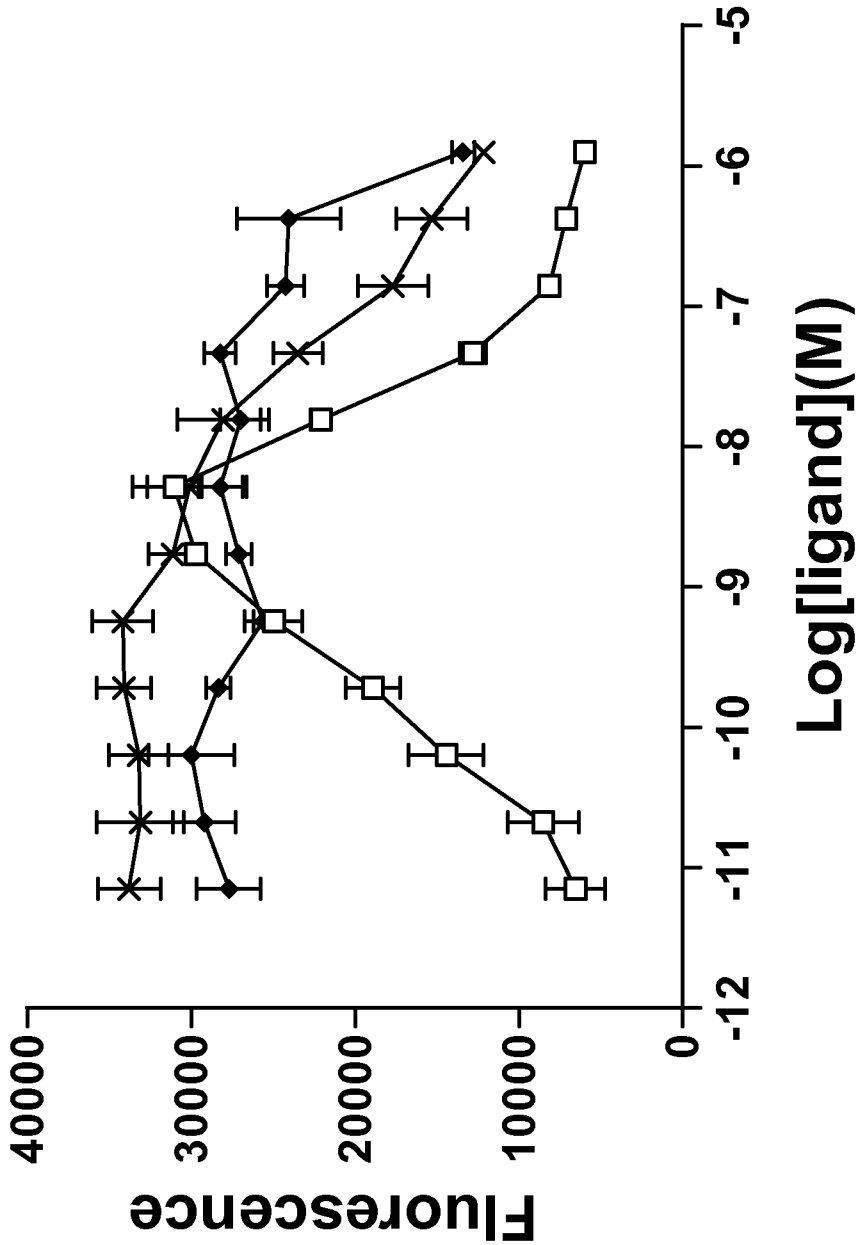
Fluorescence
40000
30000
20000
10000
0
-12
-11
-10
-9
-8
-7
-6
-5
Log[ligand](M)

Fig. 5

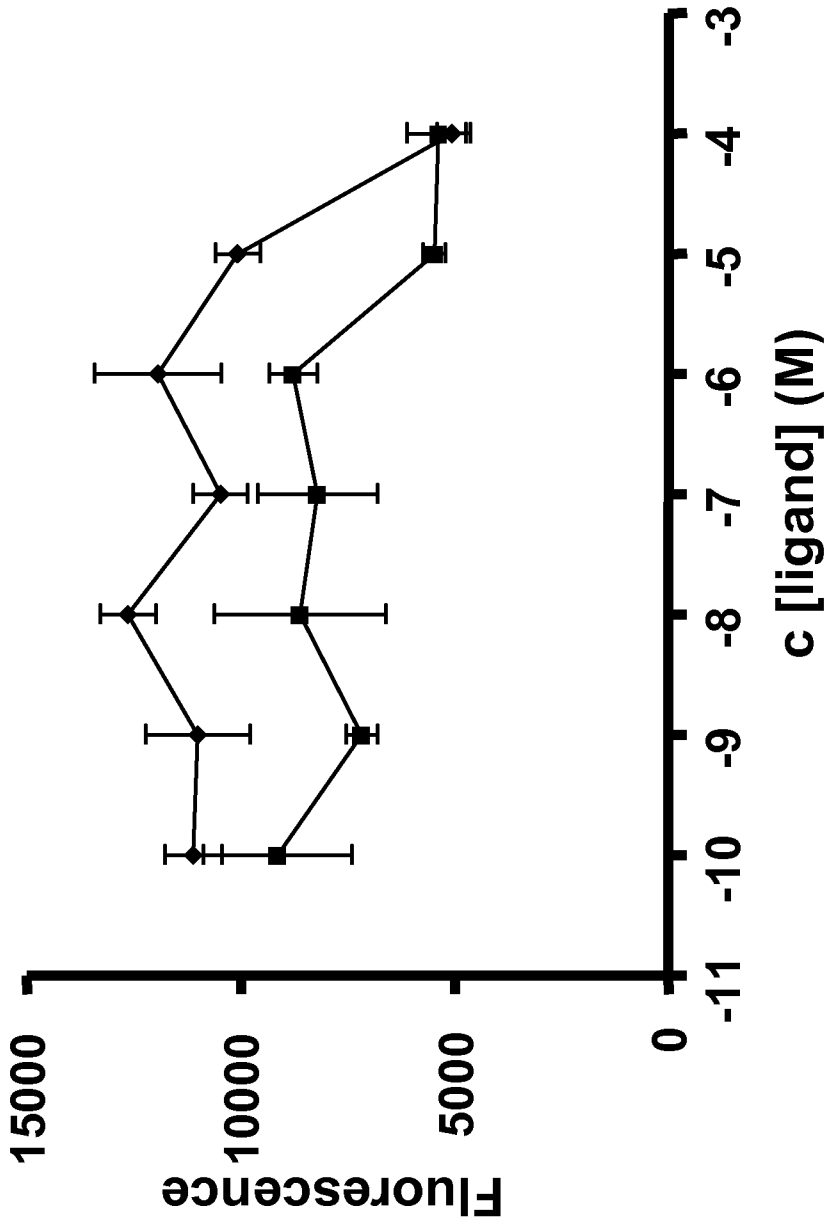

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/057856

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/52 C12N15/62 A61K38/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, EMBASE, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BHATIA M ET AL: "Treatment with Met-RANTES reduces lung injury in caerulein-induced pancreatitis." THE BRITISH JOURNAL OF SURGERY. JUN 2003, vol. 90, no. 6, June 2003 (2003-06), pages 698-704, XP002413115 ISSN: 0007-1323 abstract page 699, column 1, paragraph 3 -----	1-20
A	WELLS ET AL: "Chemokine blockers - therapeutics in the making?" TRENDS IN PHARMACOLOGICAL SCIENCES, ELSEVIER, HAYWARTH, GB, vol. 27, no. 1, January 2006 (2006-01), pages 41-47, XP005245875 ISSN: 0165-6147 the whole document -----	1-20

-/--

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 September 2007	01/10/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Voigt-Ritzer, Heike

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/057856

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZHANG Y J ET AL: "STRUCTURE/ACTIVITY ANALYSIS OF HUMAN MONOCYTE CHEMOATTRACTANT PROTEIN-1 (MCP-1) BY MUTAGENESIS. IDENTIFICATION OF A MUTATED PROTEIN THAT INHIBITS MCP-1 MEDIATED MONOCYTE CHEMOTAXIS" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOCHEMICAL BIOLOGISTS, BIRMINGHAM,, US, vol. 269, no. 22, 3 June 1994 (1994-06-03), pages 15918-15924, XP002017744 ISSN: 0021-9258 the whole document	1-20
A	DEEPIKA R PAKIANATHAN ET AL: "Distinct but overlapping epitopes for the interaction of a CC-Chemokinewith CCR1, CCR3, and CCR5" BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY. EASTON, PA, US, vol. 36, 12 August 1997 (1997-08-12), pages 9642-9648, XP002106002 ISSN: 0006-2960 page 9642, column 2, paragraph 3 the whole document	1-20
A	BAGGIOLINI M: "CHEMOKINES IN PATHOLOGY AND MEDICINE" JOURNAL OF INTERNAL MEDICINE, OXFORD, GB, vol. 250, no. 2, August 2001 (2001-08), pages 91-104, XP001021499 ISSN: 0954-6820 page 101, column 1, line 1 - page 102, column 1, paragraph 1	1-20
A	WO 96/17935 A2 (GLAXO GROUP LTD [GB]; WELLS TIMOTHY NIGEL CARL [CH]; PROUDFOOT AMANDA) 13 June 1996 (1996-06-13) cited in the application figures 5,7,9 page 14 - page 16	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2007/057856

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: partially 17, 20
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 17 and 20 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2007/057856

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9617935 A2	13-06-1996	AU 688641 B2	12-03-1998
		AU 4120896 A	26-06-1996
		BR 9509890 A	30-12-1997
		CA 2207036 A1	13-06-1996
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		CZ 9701719 A3	12-11-1997
		EP 0796330 A1	24-09-1997
		FI 972433 A	06-06-1997
		HU 77075 A2	02-03-1998
		JP 10510151 T	06-10-1998
		NO 972620 A	06-08-1997
		NZ 296570 A	25-11-1998
		PL 320565 A1	13-10-1997
		US 6555105 B1	29-04-2003
		US 6159711 A	12-12-2000