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(57) Abstract

The present invention relates to amino-terminally truncated MCP-2, lacking NH_2-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3, 1-4 or 1-5 of the naturally-occurring MCP-2 and having chemokine antagonistic activity, as well as cDNA sequences encoding them, their use in therapy and/or in diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required, and pharmaceutical compositions comprising them.

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AMINO-TERMINALLY TRUNCATED MCP-2 AS CHEMOKINE ANTAGONISTS

FIELD OF THE INVENTION

The present invention relates to amino-terminally truncated MCP-2, lacking NH_2-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3, 1-4 or 1-5 of the naturally-occurring MCP-2 and having chemokine antagonistic activity, as well as cDNA sequences encoding them, their use in therapy and/or in diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required, and pharmaceutical compositions comprising them.

BACKGROUND OF THE INVENTION

Chemokines constitute a family of small pro-inflammatory cytokines with leukocyte chemotactic and activating properties. Depending on the position of the first cysteines, the chemokine family can be divided in C-C, C-X-C and C-X_3-C chemokines (Baggiolini M. et al., 1994; Baggiolini M. et al., 1997 and Taub D. et al., 1996).

Many C-X-C chemokines such as interleukin-8 (IL-8) are chemotactic for neutrophils, while C-C chemokines, such as monocyte chemotactic protein-3 (MCP-3), are active on a variety of leukocytes including monocytes, lymphocytes, eosinophils, basophils, NK cells and dendritic cells.

The NH_2-terminal domain of chemokines is involved in receptor-binding and NH_2-terminal processing can either activate chemokines or render chemokines completely inactive.

The C-X-C chemokine platelet basic protein becomes a neutrophil chemotactic peptide (NAP-2) only after removal of the 24 NH_2-terminal residues (Walz A. et al., 1989 and Van Damme J. et al., 1990).

Deletion of up to 8 NH_2-terminal residues from IL-8 results in an enhanced chemotactic activity, but further cleavage of the Glu-Leu-Arg motif, which is located in front of the first Cys in all neutrophil chemotactic C-X-C chemokines, causes complete inactivation (Clark-Lewis I. et al., 1991).

CONFIRMATION COPY

Similar NH_2-terminal proteolysis (up to 8 amino acids) of another C-X-C chemokine, granulocyte chemotactic protein-2 (GCP-2), has no effect on the neutrophil chemotactic activity (Proost P.et al, 1993a).

The synthetical C-C chemokines MCP-1, MCP-3 and RANTES missing the 8 to 9 NH_2-terminal amino acids are inactive on monocytes and are useful as receptor antagonists (Gong J. et al., 1996; and Gong J. et al., 1995).

Extension of RANTES with one methionine results in complete inactivation of the molecule and Met-RANTES behaves as an antagonist for the authentic RANTES (Proudfoot A.E. et al., 1996).

The clone of human MCP-2 (Monocyte Chemoattractant Protein–2) has been isolated by differential library screening with cDNA probes derived from stimulated versus resting peripheral blood lymphocytes (PBL) (it was initially called "HC14", Chang H. C. et al., 1989). The cDNA-derived protein sequence was identical to that of purified natural MCP-2; however, a putative allelic variant has also been isolated, in which Gln 46 replaces Lys 46 (Van Coillie et al., 1997).

MCP-2 has also been synthesized by solid–phase chemistry (Proost P. et al., 1995).

DESCRIPTION OF THE INVENTION

The main object of the present invention are amino-terminally truncated MCP-2, lacking NH_2-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3, 1-4 or 1-5 of the naturally-occurring MCP-2 and having chemokine antagonistic activity

More particularly, one object of the present invention is MCP-2 (6-76), which is MCP-2 lacking the 1-5 NH_2-terminal amino acids, as shown in Figure 1 and in SEQ ID NO: 3 or SEQ ID NO: 4.

Such amino-terminally truncated MCP-2 of the invention can be in a glycosylated or non-glycosylated form.

The term "chemokine antagonist" means 'which acts as antagonist to the mature full-length naturally-occurring chemokines'.

Another object of the invention are the DNA molecules comprising the DNA sequences coding for the amino-terminally truncated MCP-2 of the invention, including nucleotide sequences substantially the same.

"Nucleotide sequences substantially the same" includes all other nucleic acid sequences which, by virtue of the degeneracy of the genetic code, also code for the given amino acid sequences.

The invention also includes expression vectors which comprise the above DNAs, host-cells transformed with such vectors and a process of preparation of such amino-terminally truncated MCP-2 of the invention, through the culture in appropriate culture media of said transformed cells.

The DNA sequence coding for the proteins of the invention can be inserted and ligated into a suitable plasmid. Once formed, the expression vector is introduced into a suitable host cell, which then expresses the vector(s) to yield the desired protein.

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.

For example the DNA molecules coding for the proteins obtained by any of the above methods are inserted into appropriately constructed expression vectors by techniques well known in the art (see Sambrook et al, 1989). Double stranded cDNA is linked to plasmid vectors by homopolymeric tailing or by restriction linking involving the use of synthetic DNA linkers or blunt-ended ligation techniques: DNA ligases are used to ligate the DNA molecules and undesirable joining is avoided by treatment with alkaline phosphatase.

In order to be capable of expressing the desired protein, an expression vector should also comprise specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding the desired protein in such a way as to permit gene expression and production of the protein. First in order for the gene to be transcribed, it must be preceded by a promoter recognizable by RNA polymerase, to which the polymerase binds and thus initiates the transcription process.

There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters).

For eukaryotic hosts, 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 DNA molecule comprising the nucleotide sequence coding for the protein of the invention is inserted into vector(s), having the operably linked transcriptional and translational regulatory signals, which is capable of integrating the desired gene sequences into the host cell.

The cells which have been stably transformed by the introduced DNA can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may also provide for phototrophy to a 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. Additional elements may also be needed for optimal synthesis of proteins of the invention.

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.

Once the vector(s) or DNA sequence containing the construct(s) has been prepared for expression the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation,

protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc.

Host cells may be either prokaryotic or eukaryotic. Preferred are eukaryotic hosts, e.g. mammalian cells, such as human, monkey, mouse, and Chinese hamster ovary (CHO) cells, 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).

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.

The amino-terminally truncated MCP-2 of the invention may be prepared by any other well known procedure in the art, in particular, by the well established chemical synthesis procedures, utilizing automated solid-phase peptide synthesizers followed by chromatographic purification.

The chemokines of the invention may, for example, be synthesized by Fmoc (9-fluorenylmethoxycarbonyl), tBoc (t-butoxycarbonyl) or any other comparable chemical synthesis with or without appropriate side-chain protection groups on the different amino acids. The amino acids with or without appropriate side-chain protection groups are preactivated - e.g. with HBTU/HOBt [2-(1H-Benzotriazole-1yl)-1,1,3,3-tetramethyl-uromium hexafluorophosphate/1-hydroxybenzotriazole) - and coupled to the growing peptide chain. Before the addition of the following residue, the protection group (e.g. Fmoc) is removed from the α-amino group. After synthesis, all protection groups are removed, the intact full length peptides are purified and chemically or enzymatically folded (including the formation of disulphide bridges between cysteines) into the corresponding chemokines 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 (see for example Proost P. et al., 1996). A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using monoclonal antibodies, or affinity for heparin, which bind the target protein and which are produced and 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 heparin or by the specific antibody while the impurities will pass through. After washing, the protein is eluted from the gel by a change in pH or ionic strength.

The amino-terminally truncated MCP-2 of the invention are useful in the therapy and/or diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required. Examples of such diseases include: inflammatory diseases, angiogenesis- and hematopoiesis-related diseases, tumors, infectious diseases, including HIV, auto-immune diseases, atherosclerosis, pulmonary diseases and skin disorders.

Therefore, in a further aspect, the present invention provides the use of the protein of the invention in the manufacture of a medicament for the treatment of the above-mentioned diseases.

The medicament is preferably presented in the form of a pharmaceutical composition comprising the proteins of the invention together with one or more pharmaceutically acceptable carriers and/or excipients. Such pharmaceutical compositions form yet a further aspect of the present invention.

A further embodiment of the invention is the method of treatment of the above-mentioned diseases comprising administering a pharmacologically active amount of the amino-terminally truncated MCP-2 of the invention to subjects at risk of developing such diseases or to subjects already showing such pathologies.

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.

DESCRIPTION OF THE FIGURES

Figure 1: it shows the amino acid sequence of MCP-2 and of its known variant. Signal sequences are reported in *italics*, whereas C -residues are in **bold**. Arrows indicate the

first amino acids of the amino-terminally truncated MCP-2(6-76) of the invention. Underlined is the amino acid, which is different in the MCP-2 variant.

Figure 2: SDS-PAGE of amino-terminally truncated MCP-2(6-76):

lane 1: natural MCP-2 (1-76, 100 ng/lane);

5 lane 2: natural MCP-2 (1-76, 30 ng/lane);

lane 3: natural MCP-2 (6-76, 30 ng/lane); and

lane 4: synthetic MCP-2 (1-76, 60 ng/lane).

Gels were run under reducing conditions and proteins were stained with silver.

Figure 3: it shows a comparison of the chemotactic potency of modified MCP-2 forms.

10 Intact natural (nat) and synthetic (syn) MCP-2(1-76), NH_2-terminally truncated natural MCP-2(6-76) and COOH-terminally truncated synthetic MCP-2(1-74) were tested for chemotactic activity on THP-1 cells. Results represent the mean CI ± SEM from four or more independent experiments.

Figure 4: Natural MCP-2 is a weaker agonist than MCP-1 to mobilize calcium in

15 monocytes. Intact MCP-2 (15, 50 and 150 ng/ml) dose-dependently increases the $[Ca^{2+}]_i$ in THP-1 cells. The result of one representative experiment out of two is shown.

EXAMPLES

EXAMPLE 1: Amino-terminally truncated MCP-2

20 *Materials and methods*

Chemokine and immunoassay

MCP-2 was synthesized and purified as described earlier (Proost P. et al., 1995).

Specific anti-human MCP-2 Ab were obtained from mice and affinity purified on a Sepharose column to which synthetic MCP-2 was coupled using the conditions

25 provided by the manufacturer (CNBr activated Sepharose 4B, Pharmacia, Uppsala, Sweden).

ELISA plates were coated with the affinity purified anti-human MCP-2 and biotinylated anti-MCP-2 was used as the capturing Ab. The detection was performed with peroxidase labeled streptavidine and TMB. The detection limit for the MCP-2

30 ELISA was about 0.1 ng/ml.

Production and purification of MCP-2

Monocyte chemotactic proteins were purified from peripheral blood mononuclear cell-derived conditioned medium from 132 blood donations obtained from Blood Transfusion Centers of Antwerp and Leuven (Proost P. et al., 1996).

5 Erythrocytes and granulocytes were removed by sedimentation in hydroxyethyl starch (Fresenius AG, Bad Homburg, Germany) and by gradient centrifugation in a sodium metrizoate solution (Lymphoprep; Nyegaard, Oslo Norway).

Mononuclear cells ($60x10^9$ cells) were incubated ($5x10^6$ cells/ml) with 10 μg/ml Con A and 2 μg/ml of LPS. After 48 to 120 h, conditioned medium was collected and
10 kept at -20 °C until purification.

Natural MCP-2 was purified in a four step purification procedure as previously described (Proost P. et al., 1996).

Briefly, the conditioned medium was concentrated on controlled pore glass or silicic acid and partially purified by affinity chromatography on a heparin-Sepharose
15 column (Pharmacia).

Fractions containing MCP-2 immunoreactivity were further purified by Mono S (Pharmacia) cation exchange chromatography and eluted in a NaCl gradient at pH 4.0.

Natural MCP-2 were purified to homogeneity through RP-HPLC on a C-8 Aquapore RP-300 column (Perkin Elmer, Norwalk CT) equilibrated with 0.1 %
20 trifluoroacetic acid TFA). Proteins were eluted in an acetonitrile gradient.

Biochemical characterization of MCP-forms by SDS-PAGE, amino acid sequence analysis and mass spectrometry

The purity of column fractions was examined by SDS-PAGE under reducing
25 conditions on Tris/tricine gels (Proost P. et al., 1996). Proteins were stained with silver and the following relative molecular (*Mr*) markers were used: OVA (*Mr* 45,000), carbonic anhydrase (*Mr* 31,000), soybean trypsin inhibitor (*Mr* 21,500), β-lactoglobulin (*Mr* 18,400), lysozyme (*Mr* 14,400) and aprotinin (*Mr* 6,500).

The NH_2-terminal sequence of purified chemokines was determined by Edman
30 degradation on a pulsed liquid 477A/120A protein sequencer (Perkin Elmer) with N-methylpiperidine as a coupling base. Blocked proteins were cleaved between Asp and

Pro in 75 % formic acid for 50 h. The formic acid digest was sequenced without further purification.

The *Mr* of MCP-2 was determined by matrix-assisted laser desorption ionization/time of flight-mass spectrometry (MALDI/TOF-MS) (Micromass TofSpec, Manchester, UK). Alpha-cyano-4-hydroxycinnamic acid and cytochrome C were used as matrix and internal standard, respectively.

Detection of chemotactic activity

MCP-2 was tested for its chemotactic potency on freshly purified monocytes ($2x10^6$ cells/ml) or monocytic THP-1 cells ($0.5x10^6$ cells/ml; 2 days after subcultivation) in the Boyden microchamber using polyvinylpyrrolidone-treated polycarbonate membranes with 5 µm pore size.

Samples and cells were diluted in HBSS (Life technologies/Gibco BRL, Paisley, Scotland) supplemented with 1 mg/ml human serum albumin (Red Cross Belgium). After 2 h incubation at 37 °C, the cells were fixed and stained with Diff-Quick staining solutions (Harleco, Gibbstown. NJ) and the cells that migrated through the membranes were counted microscopically in ten oil immersion fields at 500-fold magnification.

The chemotactic index (CI) of a sample (triplicates in each chamber) was calculated as the number of cells that migrated to the sample over the number of cells that migrated to control medium (Van Damme J. et al., 1992).

For desensitization experiments, cells were incubated with biologically inactive chemokine-variants for 10 min. at 37°C, before they were added to the upper well of the Boyden microchamber. The % inhibition of the CI was calculated using the CI from HBSS treated cells towards the sample as a reference value.

Detection of intracellular Ca^{2+} concentrations

Intracellular calcium concentrations ($[Ca^{2+}]_i$) were measured as previously described (Wuyts A. et al., 1997). Purified monocytes or THP-1 cells (10^7 cells/ml) were incubated in Eagle's Minimum Essential Medium (EMEM, Gibco) + 0.5 % FCS with the fluorescent indicator fura-2 (fura-2/AM 2.5 µM; Molecular Probes Europe BV, Leiden, The Netherlands) and 0.01 % Pluronic F-127 (Sigma, St Louis MO).

After 30 min at 37 °C the cells were washed twice and resuspended at 10^6 cells/ml in HBSS with 1 mM Ca^{2+} and 0.1 % FCS (buffered with 10 mM Hepes/NaOH at pH 7.4). The cells were equilibrated at 37 °C for 10 min before fura-2 fluorescence was measured in a LS50B luminescence spectrophotometer (Perkin Elmer).

5 Upon excitation at 340 and 380 nm, fluorescence was detected at 510 nm. The $[Ca^{2+}]_i$ was calculated from the Grynkiewicz equation (Grynkiewicz et al, 1985). In order to determine R_{max} the cells were lysed with 50 µM digitonin. Subsequently, the pH was adjusted to 8.5 with 20 mM Tris and R_{min} was obtained by addition of 10 mM EGTA to the lysed cells. The K_d used was 224 nM.

10 For desensitization experiments, monocytes or THP-1 cells were first stimulated with buffer, chemokine or chemokine antagonist at different concentrations. As a second stimulus, MCP-2 was used at a concentration inducing a significant increase in the $[Ca^{2+}]_i$ after prestimulation with buffer. The second stimulus was applied 2 min after addition of the first stimulus. The percentage inhibition of the $[Ca^{2+}]_i$ increase in response to the 15 second stimulus was calculated comparing the signal after prestimulation with chemokine or chemokine antagonist with the signal after addition of buffer.

Results

Isolation of post-translationally modified MCP-2 forms

20 A specific and sensitive ELISA was used to trace different MCP-2 forms produced by peripheral blood mononuclear cells stimulated with mitogen and endotoxin.

The conditioned medium was purified according to a standard isolation procedure (Proost P. et al., 1996), including adsorption to controlled pore glass and heparin Sepharose chromatography.

25 Subsequently, purification by FPLC mono S cation exchange chromatography was carried out and then a further purification step with C-8 RP HPLC was applied. Molecular masses were measured by SDS-PAGE and by MALDI/TOF-MS.

Different forms of MCP-2 were isolated: in addition to the authentic 7.5 kDa MCP-2(1-76), an NH_2-terminally truncated 7 kDa form of MCP-2 missing five residues 30 [MCP-2(6-76)] was purified to homogeneity by RP-HPLC and identified by amino acid sequence analysis (Fig. 2). MALDI/TOF-MS (Table I) yielded a molecular mass of 8881

Da for intact MCP-2 (theoretical *Mr* of 8893 Da), whereas for the MCP-2(6-76) a molecular mass of 8365 Da was measured, confirming the deletion of the five NH_2-terminal amino acids (theoretical *Mr* of 8384 Da). Functional comparison of these natural MCP-2 forms in the THP-1 chemotaxis assay showed that intact MCP 2 is still active at 5 ng/ml, whereas truncated MCP-2(6-76) remains devoid of chemotactic activity when tested at a concentration range from 0.6 to 60 ng/ml (Fig. 3). Intact natural MCP-2 was also compared in potency with the synthetical MCP-2(1-76) and a COOH-terminally truncated synthetical form (Proost P. et al., 1995) missing two residues [MCP-2(1-74)].

The minimal effective chemotactic concentration of these forms was also found to be 5 ng/ml (Fig. 3). Although in chemotaxis assays the specific activity of natural intact MCP-1 and MCP-2 is comparable (Van Damme J, et al., 1992), the calcium mobilizing capacity of MCP-2 is still a matter of debate.

However, in Ca^{2+} -mobilization experiments, the minimal effective dose for both natural or synthetic MCP-2(1-76) was 10-fold higher compared to that of natural intact MCP-1(1-76) (Fig. 4), whereas MCP-2(6-76) remained inactive.

Nevertheless, intact MCP-2 (50 ng/ml) was capable to desensitize for MCP-2 (15 ng/ml) and MCP-3 (10 ng/ml) yielding 52% and 45% inhibition of chemotaxis, respectively.

Due to this lower specific activity of MCP-2 in Ca^{2+} assays, desensitization of chemotaxis by MCP-2(6-76) was performed in the Boyden microchamber. Since intact MCP-2 is reported to cross-desensitize with active MCP-1, MCP-2 and MCP 3 in the monocyte chemotaxis assay (Sozzani S. et al., 1994), we investigated whether natural, inactive MCP-2(6-76) could also desensitize for MCP-1, MCP-2, MCP-3 and RANTES (Table II). Pre-incubation of THP-1 cells with 100 ng/ml of inactive MCP-2(6-76) could already significantly inhibit chemotaxis induced by 10 ng/ml of MCP-1 (63 %), 5 ng/ml of MCP-2 (75%), 30 ng/ml of MCP-3 (62 %) and 100 ng/ml of RANTES (75%). Moreover, chemotaxis in response to 3 times lower concentrations of the respective MCPs was completely (91-100 %) inhibited by 100 ng/ml MCP-2(6-76). Furthermore, at a concentration as low as 10 ng/ml, MCP-2(6-76) was still able to significantly inhibit the chemotactic activity induced by MCP-1 (3 ng/ml), MCP-2 (1.5 ng/ml) or

MCP 3 (10 ng/ml) and RANTES (30 ng/ml). Taken together, MCP-2(6-76) is produced naturally, is inactive as a chemoattractant and antagonizes several C-C chemokines, the effect being most predominant for MCP-3.

5

TABLE I

Biochemical characterization of natural forms of MCP-2. NH_2-terminal amino acid sequence analysis and comparison of the experimental (SDS-PAGE and MALDI/TOF-MS) and theoretical Mr of C-8 RP-HPLC purified natural MCP-isoforms.

10

MCP-form	NH_2-terminal sequence	*Mr* (Da)		
		theoretical unglycosylated	SDS-PAGE	MALDI/TOF-MS
MCP-2 (1-76)	blocked	8893	7500	8881
MCP-2 (2-76)	SIPITCC	8384	7000	8365

TABLE II

MCP-2(6-76) desensitizes the monocyte chemotactic responses of MCP-1, MCP-2 MCP-3 and RANTES in the microchamber.

Chemokine [a]	Concentration	Antagonization of chemotactic response [b,c]		% Inhibition of chemotaxis
		buffer	100 ng/ml MCP-2(6-76)	
MCP-1	10	22.3 ± 7.9	8.3 ± 3.8	63 ± 21
	3	15.0 ± 8.0	1.3 ± 0.3	99 ± 1.0
MCP-2	5	36.0 ± 12.6	10.8 ± 6.1	75 ± 8.0
	1.5	6.7 ± 1.4	1.5 ± 0.3	91 ± 7.0
MCP-3	30	13.2 ± 0.4	6.0 ± 4.0	62 ± 31
	10	3.0 ± 1.5	<1	100 ± 0.0
RANTES	100	6.3 ± 0.8	2.6 ± 1.3	75 ± 19
	30	4.0 ± 0.8	1.5 ± 0.3	77 ± 16
		buffer	10 ng/ml MCP-2 (6-76)	
MCP-1	10	12.7 ± 2.3	10.5 ± 3.8	24 ± 1.8
	3	7.5 ± 0.0	3.0 ± 0.3	69 ± 4.0
MCP-2	5	38.0 ± 5.3	27.2 ± 4.9	30 ± 6.0
	1.5	18.3 ± 4.6	9.2 ± 1.4	45 ± 23
MCP-3	30	13.2 ± 1.9	8.0 ± 1.0	37 ± 19
	10	7.7 ± 1.4	1.7 ± 0.3	90 ± 6.0
RANTES	100	5.5 ± 0.6	5.8 ± 0.9	17 ± 7.0
	30	3.2 ± 0.7	2.5 ± 0.5	39 ± 18

5 [a] MCP-1, MCP-2, MCP-3 or RANTES were added as chemoattractants to the lower wells.

[b] the upper wells of the microchamber were filled with THP-1 cells preincubated with MCP-2(6-76) or with buffer

[c] mean CI ± SEM of 3 independent experiments

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CLAIMS

1. Amino-terminally truncated MCP-2, lacking NH_2-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3, 1-4 or 1-5 of the naturally-occurring MCP-2 and having chemokine antagonistic activity.

2. Amino-terminally truncated MCP-2 according to claim 1, lacking NH_2-terminal amino acids corresponding to amino acid residues 1-5 of the naturally-occurring MCP-2 and having chemokine antagonistic activity.

3. Amino-terminally truncated MCP-2 according to claim 1, having the amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

4. Amino-terminally truncated MCP-2 according to one or more of the preceding claims, in a glycosylated form.

5. DNA molecules comprising the DNA sequences coding for the amino-terminally truncated MCP-2 of the invention according to one or more of the preceding claims, including nucleotide sequences substantially the same.

6. An expression vector which comprises the DNA molecule of any claim 5.

7. A host cell comprising the expression vector of claim 5.

8. A recombinant process for preparing any of the proteins from claim 1 to 4, comprising culturing in an appropriate culture medium the cells of claim 6.

9. A protein according to any of the claims from 1 to 4 for use as medicament.

10.Use of a protein according to any of the claims from 1 to 4, in the manufacture of a medicament for the therapy and/or diagnosis of diseases, in which an antagonistic activity of the chemokine effects is required.

5 11.Use according to claim 10, in the manufacture of a medicament for the treatment of inflammatory diseases, HIV-infection, angiogenisis- and hematopoiesis-related diseases, and tumors.

12.A pharmaceutical composition comprising the protein according to any of the claims
10 from 1 to 4 together with one or more pharmaceutically acceptable carriers and/or excipients.

MCP-2

```
-23                     1    ↓
MKVSAALLCL LLMAATFSPQ GLAQPDSVSI PITCCFNVIN RKIPIQRLES YTRITNIQCP
KEAVIFKTKR GKEVCADPKE RWVRDSMKHL DQIFQNLKP
                                       76
```

MCP-2 Variant

```
-23                     1    ↓
MKVSAALLCL LLMAATFSPQ GLAQPDSVSI PITCCFNVIN RKIPIQRLES YTRITNIQCP
KEAVIFQTKR GKEVCADPKE RWVRDSMKHL DQIFQNLKP
                                       76
```

Figure 1

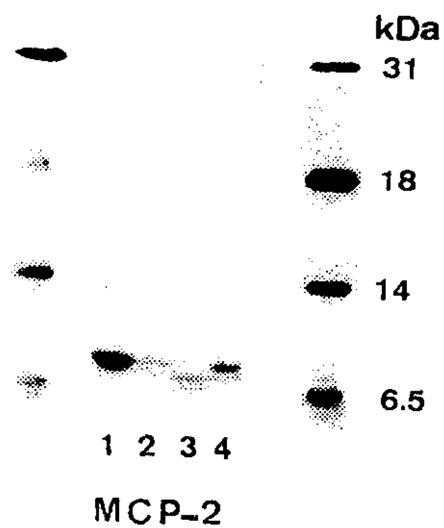


Figure 2

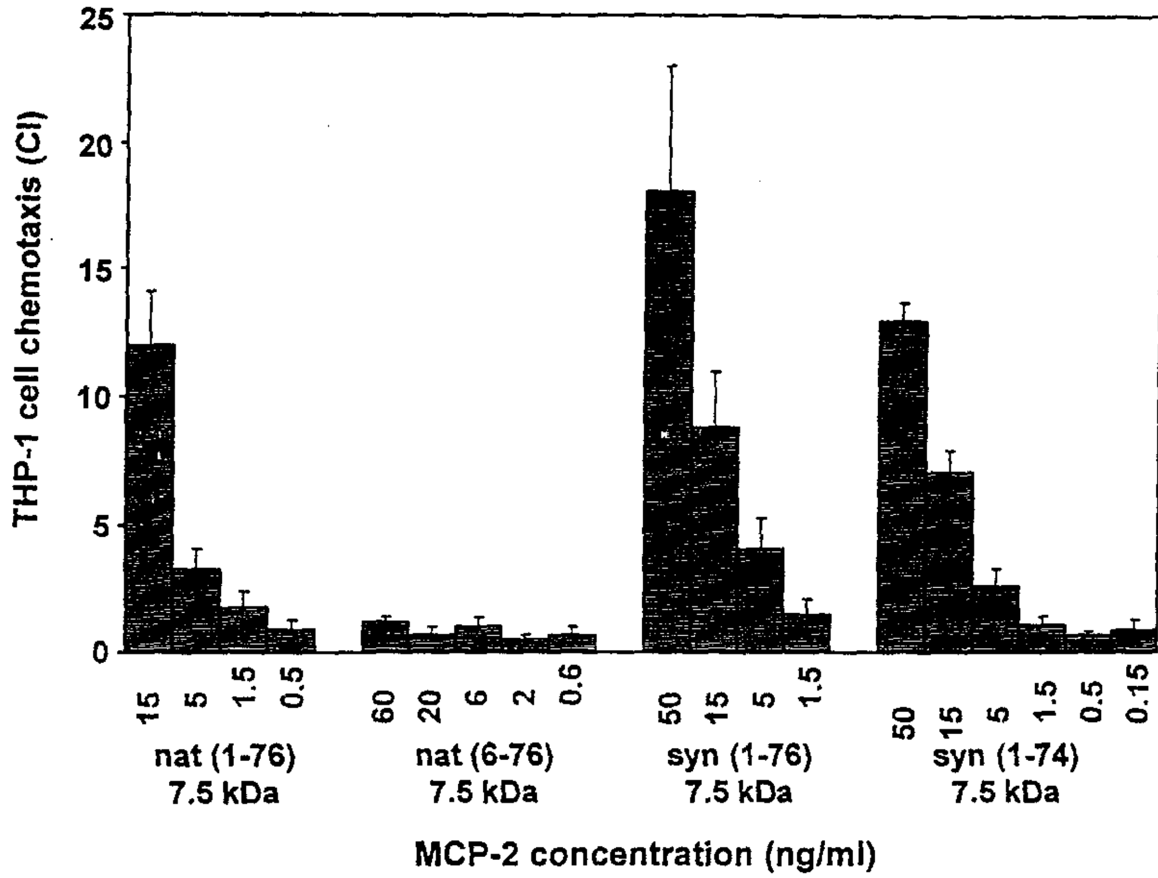
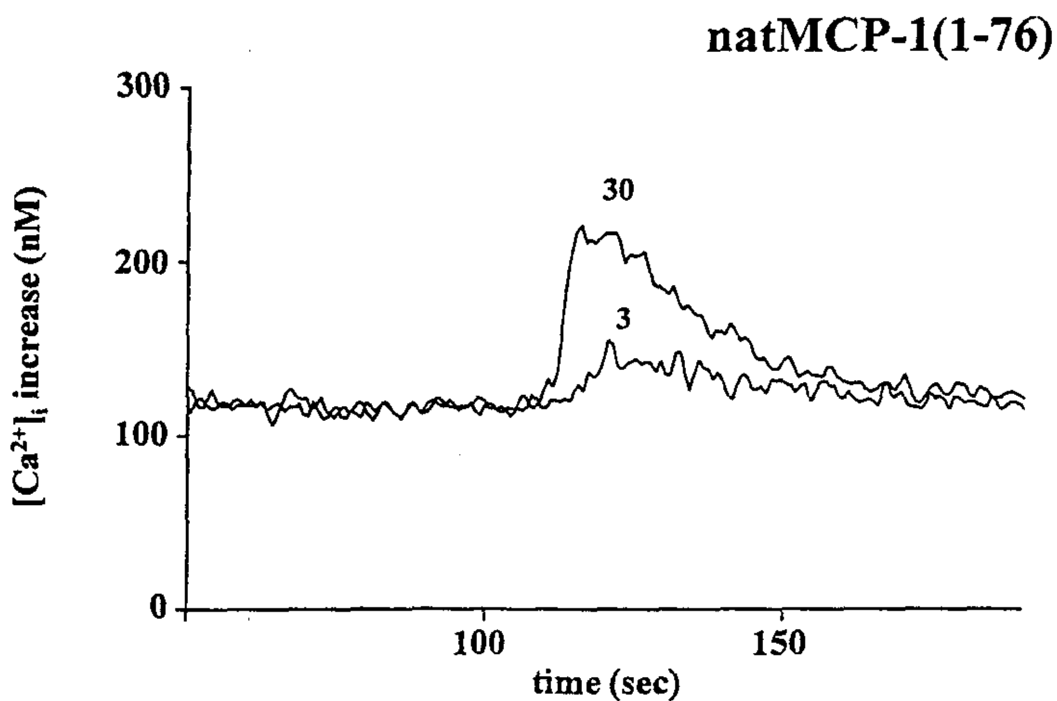


Figure 3



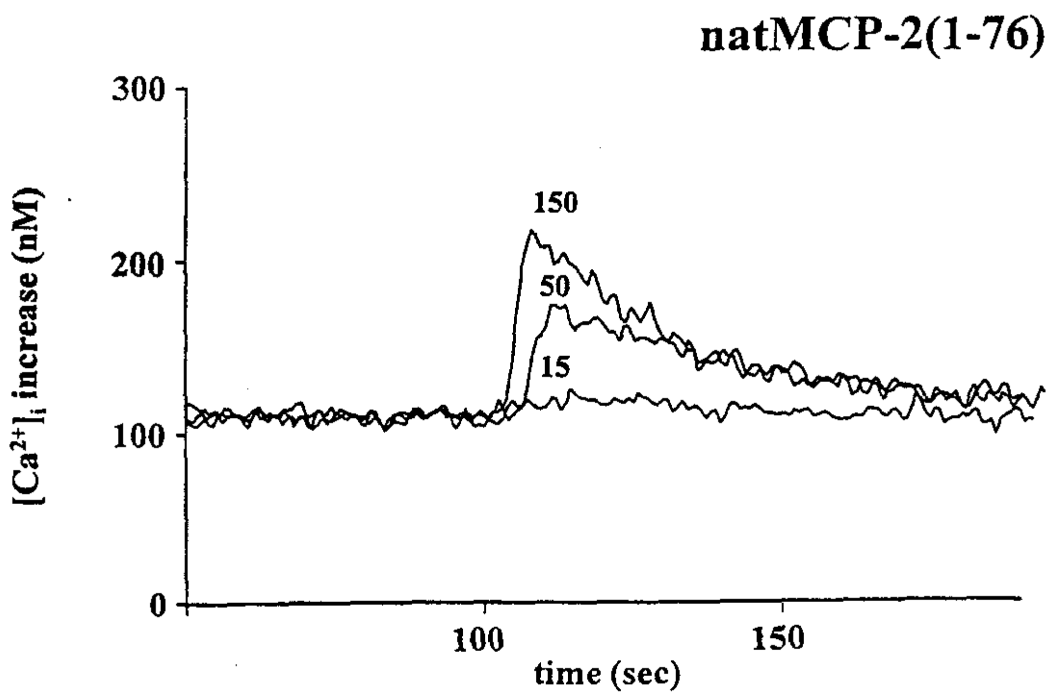


Figure 4

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: APPLIED RESEARCH SYSTEMS ARS HOLDING N.V.
(B) STREET: 14 JOHN B. GORSIRAWEG
(C) CITY: CURACAO
(E) COUNTRY: THE NETHERLANDS ANTILLES
(F) POSTAL CODE (ZIP): NONE
(G) TELEPHONE: 639300
(H) TELEFAX: 614129

(ii) TITLE OF INVENTION: AMINO-TERMINALLY TRUNCATED MCP-2 AS CHEMOKINE ANTAGONISTS

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION:1..76

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Met Lys Val Ser Ala Ala Leu Leu Cys Leu Leu Leu Met Ala Ala Thr
            -20                 -15                 -10

Phe Ser Pro Gln Gly Leu Ala Gln Pro Asp Ser Val Ser Ile Pro Ile
        -5                  1               5

Thr Cys Cys Phe Asn Val Ile Asn Arg Lys Ile Pro Ile Gln Arg Leu
10                  15                  20                  25

Glu Ser Tyr Thr Arg Ile Thr Asn Ile Gln Cys Pro Lys Glu Ala Val

                30                  35                  40
```

```
Ile Phe Lys Thr Lys Arg Gly Lys Glu Val Cys Ala Asp Pro Lys Glu
            45                  50                  55

Arg Trp Val Arg Asp Ser Met Lys His Leu Asp Gln Ile Phe Gln Asn
        60                  65                  70

Leu Lys Pro
    75
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION:1..76

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Lys Val Ser Ala Ala Leu Leu Cys Leu Leu Leu Met Ala Ala Thr
            -20                 -15                 -10

Phe Ser Pro Gln Gly Leu Ala Gln Pro Asp Ser Val Ser Ile Pro Ile
        -5                  1               5

Thr Cys Cys Phe Asn Val Ile Asn Arg Lys Ile Pro Ile Gln Arg Leu
10                  15                  20                  25

Glu Ser Tyr Thr Arg Ile Thr Asn Ile Gln Cys Pro Lys Glu Ala Val
                30                  35                  40

Ile Phe Lys Thr Gln Arg Gly Lys Glu Val Cys Ala Asp Pro Lys Glu
            45                  50                  55

Arg Trp Val Arg Asp Ser Met Lys His Leu Asp Gln Ile Phe Gln Asn
        60                  65                  70

Leu Lys Pro
    75
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
Ser Ile Pro Ile Thr Cys Cys Phe Asn Val Ile Asn Arg Lys Ile Pro
1               5                   10                  15

Ile Gln Arg Leu Glu Ser Tyr Thr Arg Ile Thr Asn Ile Gln Cys Pro
            20                  25                  30

Lys Glu Ala Val Ile Phe Lys Thr Lys Arg Gly Lys Glu Val Cys Ala
        35                  40                  45

Asp Pro Lys Glu Arg Trp Val Arg Asp Ser Met Lys His Leu Asp Gln
    50                  55                  60

Ile Phe Gln Asn Leu Lys Pro
65                  70
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Ser Ile Pro Ile Thr Cys Cys Phe Asn Val Ile Asn Arg Lys Ile Pro
1               5                   10                  15

Ile Gln Arg Leu Glu Ser Tyr Thr Arg Ile Thr Asn Ile Gln Cys Pro
            20                  25                  30

Lys Glu Ala Val Ile Phe Lys Thr Gln Arg Gly Lys Glu Val Cys Ala
        35                  40                  45

Asp Pro Lys Glu Arg Trp Val Arg Asp Ser Met Lys His Leu Asp Gln
    50                  55                  60

Ile Phe Gln Asn Leu Lys Pro
65                  70
```

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/06142

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/19 C07K14/52 A61K38/19 A61K38/48 G01N33/68

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)
IPC 6 C07K C12N A61K G01N

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)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	E. VAN COILLIE ET AL.: "The Human MCP-2 gene (SCYA8): Cloning, Sequence Analysis, Tissue Expression and Assignment to the CC Chemokine Gene Contig on Chromosome 17q11.2" GENOMICS, vol. 40, 1 March 1997, pages 323-331, XP000197686 see the whole document --- -/--	1-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in 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
28 January 1999	05/02/1999

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	Hix, R

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	E. VAN COILLIE ET AL.: "Human Monocyte Chemotactic Protein-2: cDNA cloning and regulated Expression of mRNA in Mesenchymal cells." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 231, 24 February 1997, pages 726-730, XP002044670 see the whole document	1-12
A	WO 97 25427 A (GENETICS INST) 17 July 1997 see the whole document	1-12
A	GONG J-H ET AL: "Antagonists of monocyte chemoattractant protein 1 identified by modification of functionally critical NH-2-terminal residues." JOURNAL OF EXPERIMENTAL MEDICINE 181 (2). 1995. 631-640. ISSN: 0022-1007, XP000644794 cited in the application see page 637, column 2	1-12
A	GONG J-H ET AL: "RANTES and MCP -3 antagonists bind multiple chemokine receptors." JOURNAL OF BIOLOGICAL CHEMISTRY 271 (18). 1996. 10521-10527. ISSN: 0021-9258, XP002047804 cited in the application see table 1	1-12
A	WEBER M ET AL: "Deletion of the NH-2-terminal residue converts monocyte chemotactic protein 1 from an activator of basophil mediator release to an eosinophil chemoattractant." JOURNAL OF EXPERIMENTAL MEDICINE 183 (2). 1996. 681-685. ISSN: 0022-1007, XP000609014 see the whole document	1-12
A	Y. ZHANG ET AL.: "A dominant negative inhibitor indicates that monocyte chemoattractant protein 1 functions as a dimer." MOLECULAR AND CELLULAR BIOLOGY, vol. 15, no. 9, September 1995, pages 4851-4855, XP000605358 see the whole document	1-12

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Y. ZHANG ET AL.: "MCP-1: Structure/activity analysis." METHODS: A COMPANION TO METHODS IN ENZYMOLOGY, vol. 10, 1996, pages 93-103, XP000609055 see the whole document	1-12
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." THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 22, 3 June 1994, pages 15918-15924, XP002025858 see the whole document	1-12
A	M. BAGGIOLINI ET AL.: "Human Chemokines: An update." ANNUAL REVIEW OF IMMUNOLOGY, vol. 15, 1997, pages 675-705, XP002055737 cited in the application see page 689 - page 691	1-12
A	J. VAN DAMME ET AL.: "Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family" JOURNAL OF EXPERIMENTAL MEDICINE, vol. 176, July 1992, pages 59-65, XP000673185 see the whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 98/06142

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9725427 A	17-07-1997	AU 1532697 A	01-08-1997

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[19]中华人民共和国国家知识产权局

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C12N 15/19
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A61K 38/48 G01N 33/68

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[11]公开号 CN 1271385A

[22]申请日 1998.9.28 [21]申请号 98809569.6

[30]优先权

[32]1997.9.29 [33]EP [31]97116863.8

[32]1997.12.19[33]EP [31]97122471.2

[32]1998.3.10 [33]EP [31]98104216.1

[86]国际申请 PCT/EP98/06142 1998.9.28

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[85]进入国家阶段日期 2000.3.27

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[74]专利代理机构 上海专利商标事务所

代理人 陈文青

权利要求书1页 说明书14页 附图页数4页

[54]发明名称 作为趋化因子拮抗剂的氨基端截短的MCP－2

[57]摘要

本发明涉及以氨基端截短的MCP－2，它缺少对应于天然存在的MCP－2的氨基酸残基1、1—2、1—3、1—4或1—5的NH_2端氨基酸并具有趋化因子拮抗活性，本发明还涉及编码这些MCP－2的cDNA序列，以及它们在需要有趋化因子作用拮抗活性的疾病治疗和/或诊断中的应用，以及包含它们的药物组合物。

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权利要求书

1. 一种氨基端截短的 MCP-2，它缺少对应于天然存在的 MCP-2 的氨基酸残基 1、1-2、1-3、1-4 或 1-5 的氨基端氨基酸，并具有趋化因子拮抗活性。

2. 根据权利要求 1 所述的氨基端截短的 MCP-2，它缺少对应于天然存在的 MCP-2 的氨基酸残基 1-5 的氨基端氨基酸，并具有趋化因子拮抗活性。

3. 根据权利要求 1 所述的氨基端截短的 MCP-2，它具有 SEQ ID NO: 3 或 SEQ ID NO: 4 所示的氨基酸序列。

4. 根据前述任一权利要求所述的氨基端截短的 MCP-2，它是糖基化的形式。

5. 一种 DNA 分子，它包含编码前述任一权利要求所述的本发明氨基端截短 MCP-2 的 DNA 序列，该 DNA 序列包括基本上相同的核苷酸序列。

6. 一种表达载体，它包含权利要求 5 所述的 DNA 分子。

7. 一种宿主细胞，它包含权利要求 5 所述的表达载体。

8. 一种制备权利要求 1 至 4 任一所述的蛋白质的重组方法，该方法包括将权利要求 6 所述的细胞培养在合适的培养基中。

9. 根据权利要求 1 至 4 任一所述的蛋白作为药物的应用。

10. 权利要求 1 至 4 任一所述的蛋白在生产用于治疗和/或诊断疾病的药物中的应用，其中该疾病的治疗和/或诊断需要有拮抗趋化因子作用的活性。

11. 根据权利要求 10 所述的应用，该应用用于生产用来治疗炎性疾病、HIV 感染、血管生成和血细胞生成相关疾病以及肿瘤的药物。

12. 一种药物组合物，它包含权利要求 1 至 4 任一所述的蛋白质以及一种或多种药学上可接受的载体和/或赋形剂。

作为趋化因子拮抗剂的氨基端截短的MCP-2

5

发明领域

本发明涉及氨基端截短的 MCP-2，它缺少对应于天然存在的 MCP-2 的氨基酸残基 1、1-2、1-3、1-4 或 1-5 的 NH_2 端氨基酸，并具有趋化因子拮抗活性，本发明还涉及编码这些 MCP-2 的 cDNA 序列，以及它们在需要有趋化因子作用拮抗活性的
10 疾病治疗和/或诊断中的应用，以及包含它们的药物组合物。

发明背景

趋化因子构成了具有白细胞趋化和活化性能的一个促炎性细胞因子小家族。根据第一个半胱氨酸的位置，趋化因子家族可以分为 C-C、C-X-C 和 $C-X_3-C$ 趋化因子
15 (Baggiolini M.等人, 1994; Baggiolini M.等人, 1997 和 Taub D.等人, 1996)。

许多 C-X-C 趋化因子，如白细胞介素-8(IL-8)，对于嗜中性粒细胞有趋化作用，而 C-C 趋化因子，如单核细胞趋化蛋白-3(MCP-3)，则对包括单核细胞、淋巴细胞、嗜酸性粒细胞、嗜碱性粒细胞、NK 细胞和树突细胞在内的各种白细胞有活性。

趋化因子的氨基端区域参与受体结合，氨基端的加工可以激活趋化因子或使趋
20 化因子完全没有活性。

C-X-C 趋化因子血小板碱性蛋白只有在除去氨基端的 24 个残基后才变成一种嗜中性粒细胞趋化肽(NAP-2)(Walz A.等人, 1989 和 Van Damme J.等人, 1990)。

IL-8 缺失 8 个氨基端残基导致趋化活性增强，但是位于所有嗜中性粒细胞趋化性 C-X-C 趋化因子中第一个 Cys 前的 Glu-Leu-Arg 基序进一步断裂却导致其完全失
25 去活性(Clark-Lewis I.等人, 1991)。

另一个 C-X-C 趋化因子粒细胞趋化蛋白-2(GCP-2)发生相似的氨基端蛋白水解(解离下 8 个氨基酸)却对嗜中性粒细胞趋化活性没有影响(Proost P.等人，1993a)。

缺少 8 到 9 个氨基端氨基酸的合成的 C-C 趋化因子 MCP-1、MCP-3 和 RANTES 对单核细胞没有活性，并被用作受体拮抗剂(Gong J.等人，1996；和 Gong J.等人，
30 1995)。

RANTES 延伸一个甲硫氨酸将导致该分子完全失去活性，Met-RANTES 表现为真正的 RANTES 拮抗剂(Proudfoot A. E.等人, 1996)。

已经用 cDNA 探针(从刺激的针对休眠的外周血淋巴细胞(PBL)衍生获得)通过差

示实验室筛选分离出人 MCP-2(单核细胞引诱蛋白-2)的克隆(它最初称为“HC14”，Chang H.C.等人，1989)。cDNA 衍生的蛋白序列与纯化的天然 MCP-2 相同；然而，还分离出推定的等位基因变体(其中 Gln46 代替 Lys46)(Van Coillie 等人, 1997)。

还用固相化学方法合成了 MCP-2(Proost P.等人, 1995)。

5

发明描述

本发明的主要目的是氨基端截短的 MCP-2，它缺少对应于天然存在的 MCP-2 的氨基酸残基 1、1-2、1-3、1-4 或 1-5 的氨基端氨基酸，并具有趋化因子拮抗剂活性。

10 更具体地说，本发明的一个目的是 MCP-2(6-76)，它是缺少氨基端氨基酸 1-5 的 MCP-2，如图 1 和 SEQ ID NO: 3 或 SEQ ID NO: 4 所示。

本发明的这种氨基端截短的 MCP-2 可以是糖基化形式或非糖基化形式。

术语“趋化因子拮抗剂”指“成熟的、天然存在的全长趋化因子的拮抗剂”。

本发明的另一个目的是包含编码本发明氨基端截短的 MCP-2 的 DNA 序列的

15 DNA 分子，其中所述 DNA 序列包括基本上相同的核苷酸序列。

“基本上相同的核苷酸序列”包括凭借遗传密码的简并性也能编码出给定氨基酸序列的所有其它核酸序列。

本发明还包括含有上述 DNA 的表达载体，转化了该载体的宿主细胞，以及通过将所述转化细胞培养在合适培养基中来制备本发明这些氨基端截短的 MCP-2 的方

20 法。

编码本发明蛋白的 DNA 序列可以插入合适的质粒并与其连接。一旦形成表达载体，就将其导入合适的宿主细胞，该宿主细胞进而表达载体，产生所需蛋白。

本文提到的任何本发明重组蛋白的表达均可用合适的表达载体在真核细胞(如酵母、昆虫或哺乳动物细胞)或原核细胞中进行。本领域中已知的任何方法均可采用。

25 例如，用本领域熟知的技术(参见 Sambrook 等人, 1989)将上述任一方法获得的编码蛋白的DNA分子插入适当构建的表达载体中。利用同聚物加尾、采用合成的DNA接头的限制性连接或平头连接技术，将双链 cDNA 连接到质粒载体中。用 DNA 连接酶连接 DNA 分子，通过碱性磷酸酶处理避免不希望发生的连接。

为了能表达所需的蛋白，表达载体还应包含特异的含有转录和翻译调控信息的

30 核苷酸序列，该核苷酸序列与编码所需蛋白的 DNA 连接，从而允许该基因表达和产生蛋白。首先，为了使基因被转录，它的前面必须有能被 RNA 聚合酶识别的启动子，该启动子与聚合酶结合，从而启动转录过程。可以采用具有不同工作效率的各种启

动子(强的和弱的启动子)。

对于真核宿主，可以采用不同的转录和翻译调控序列，这取决于宿主的性质。它们可以来自病毒，如腺病毒、牛乳头瘤病毒、猿猴病毒等，其中调控信号与具有高表达水平的特定基因相关联。例子是疱疹病毒的 TK 启动子、SV40 早期启动子、酵母 gal4 基因启动子等。可以选择转录起始调控信号，使其具有阻遏和激活作用，从而能够调节基因的表达。

将包含编码本发明蛋白的核苷酸序列的 DNA 分子插入载体中与转录和翻译调控信号操作性相连，该载体能将所需基因序列整合入宿主细胞中。

还可以通过导入一个或多个允许选择含有表达载体的宿主细胞的标记物，来选择被导入的 DNA 稳定转化的细胞。标记物还可为营养缺陷型宿主提供光营养、杀微生物的抗性，例如抗生素，或重金属如铜等。可选择的标记基因可以和待表达的 DNA 基因序列直接连接，或通过共转染导入同一细胞中。为了使本发明蛋白的合成最优，可能还需要额外的元件。

选择特定质粒或病毒载体的重要因素包括：从不含载体的受体细胞中识别和选出含有载体的受体细胞的容易程度；特定宿主中所希望的载体拷贝数；以及是否希望载体能在不同种宿主细胞之间“穿梭”。

一旦制得了用于表达的载体或含 DNA 序列的构建物，就可用以下多种合适的方法将该 DNA 构建物导入合适的宿主细胞中：转化、转染、偶联、原生质体融合、电穿孔、磷酸钙沉淀、直接显微注射等。

宿主细胞可以是原核细胞或真核细胞。较佳的是真核宿主，例如哺乳动物细胞，如人、猴、小鼠和中国仓鼠卵巢(CHO)细胞，因为它们为蛋白质分子提供了翻译后的修饰，包括正确折叠或在正确位点的糖基化。酵母细胞也可进行包括糖基化的翻译后肽修饰。有许多重组 DNA 策略采用了强启动子序列和高拷贝数的质粒，它们可用来在酵母中产生所需的蛋白质。酵母识别克隆的哺乳动物基因产物上的前导序列，并分泌携带前导序列的肽(即前肽(pre-peptide))。

在导入载体后，使宿主细胞在选择性培养基中生长，该培养基使含有载体的细胞选择性地生长。克隆的基因序列的表达导致产生所需的蛋白质。

本发明的氨基端截短的 MCP-2 可用本领域中其它熟知的方法来制备，尤其是已建立的化学合成方法：用自动化固相肽合成仪，然后进行层析纯化。

例如，本发明的趋化因子可用 Fmoc(9-芴甲氧基羰基)、tBoc(叔丁氧羰基)或其它任何类似的化学合成在不同氨基酸上有或没有合适的侧链保护基团的条件下合成。有或没有合适的侧链保护基团的氨基酸被预先激活(例如用 HBTU/HOBt[2-(1H-

苯并三唑-1-基)-1,1,3,3-四甲基-脲六氟磷酸/1-羟基苯并三唑)，并偶联到生长的肽链上。在加入下一个残基之前，从α氨基上除去保护基团(例如 Fmoc)。合成后除去所有保护基团，纯化完整的全长肽并用化学方法或酶法将肽折叠(包括在半胱氨酸之间形成二硫键)成本发明的对应的趋化因子。

5 天然的、合成的或重组的蛋白可用已知用于纯化的任一种方法(即任何常规方法，包括抽提、沉淀、层析、电泳等(例如参见 Proost P.等人，1996))来进行纯化。用于纯化本发明蛋白的另一优选的纯化方法是亲和层析，该方法利用的是结合靶蛋白并产生和固定在柱中所含凝胶基质上的单克隆抗体或肝素的亲和力。使含有蛋白质的不纯制备物通过该柱。蛋白质将会通过肝素或特异性抗体结合在柱上，而杂质

10 则将通过柱。洗柱后，通过改变 pH 或离子强度将蛋白质从凝胶上洗脱下来。

本发明的氨基端截短的 MCP-2 可用于治疗和/或诊断需要有趋化因子拮抗作用的疾病。这些疾病的例子包括：炎性疾病、血管生成和血细胞生成相关疾病、肿瘤、传染病(包括 HIV)、自身免疫疾病、动脉粥样硬化、肺疾病和皮肤病。

因此，本发明另一方面提供了本发明的蛋白在生产用于治疗上述疾病的药物中

15 的应用。

药物宜为包含本发明蛋白以及一种或多种药学上可接受的载体和/或赋形剂的药物组合物形式。这些药物组合物形成了本发明的另一个方面。

本发明还有一个方面是一种治疗上述疾病的方法，该方法包括给予处于发生这些疾病危险的对象或已经表现出这些病理学现象的对象药学上活性量的本发明氨基

20 端截短的 MCP-2。

现在将通过下列实施例来描述本发明，这些实施例不应被理解成以任何方式限定了本发明。实施例将参考下文所述的附图。

附图简述

25 图 1：它显示了 MCP-2 以及其已知变体的氨基酸序列。信号序列用斜体字表示，而 C 端残基用粗体表示。箭头表示本发明的氨基端截短的 MCP-2(6-76)的第一个氨基酸。下划线是与 MCP-2 变体中不同的氨基酸。

图 2：氨基端截短的 MCP-2(6-76)的 SDS-PAGE：

泳道 1：天然 MCP-2(1-76，100ng/泳道)；

30 泳道 2：天然 MCP-2(1-76，30 ng/泳道)；

泳道 3：天然 MCP-2(6-76，30 ng/泳道)；和

泳道 4：合成的 MCP-2(1-76，60 ng/泳道)。

凝胶在还原性条件下进行电泳，蛋白质用银染色。

图 3：它显示了修饰型 MCP-2 的趋化效力的比较。

测试完整的天然(nat)和合成(syn)MCP-2(1-76)、氨基端截短的天然 MCP-2(6-76)以及羧基端截短的合成的 MCP-2(1-74)在 THP-1 细胞上的趋化活性。结果表示成四次或四次以上单独实验的 CI 平均值±SEM。

图 4：与 MCP-1 相比，天然的 MCP-2 是使单核细胞中钙流动的较弱的激动剂。完整的 MCP-2 随剂量的增加(15、50 和 150ng/ml)而增加 THP-1 细胞中的$[Ca^{2+}]_i$。图中显示了两个实验中一个典型实验的结果。

实施例

实施例 1：氨基端截短的 MCP-2

材料和方法

趋化因子和免疫试验

如前所述的那样(Proost P.等人, 1995)合成和纯化 MCP-2。

从小鼠获得特异性抗人 MCP-2 抗体，并在 Sepharose 柱(CNBr 活化的 Sepharose 4B, Pharmacia, Uppsala, Sweden)上亲和纯化，用生产商提供的条件使合成的 MCP-2 与柱偶联。

用亲和纯化的抗人 MCP-2 包被 ELISA 板，并用生物素化的抗-MCP-2 作为捕获性抗体。用过氧化物酶标记的链霉亲和素和 TMB 进行检测。MCP-2 ELISA 的检测极限约为 0.1ng/ml。

MCP-2 的生产和纯化

从 Antwerp 和 Leuven 输血中心所得 132 份献血员的外周血单核细胞衍生的条件培养基中纯化获得单核细胞趋化蛋白(Proost P.等人, 1996)。

通过在羟乙基淀粉(Fresenius AG, Bad Homburg, Germany)中沉淀和在 3-乙酰氨基-5-乙酰甲氨基-2,4,6-三碘苯甲酸钠溶液(sodium metrizoate, Lymphoprep; Nyegaard, Oslo Norway)梯度离心，除去红细胞和粒细胞。

用 10 微克/毫升 Con A 和 2 微克/毫升 LPS 培育单核细胞(60×10^9 细胞)。48 至 120 小时后，收集条件培养基并置于-20℃直至纯化。

如以前所描述的那样(Proost P.等人，1996)，用四步纯化程序纯化天然的 MCP-2。

简言之，在控制孔径的玻璃或硅酸上浓缩条件培养基，并在肝素-Sepharose 柱(Pharmacia)上亲和层析部分纯化。

含有 MCP-2 免疫反应性的级分用 Mono S(Pharmacia)阳离子交换层析进一步纯化，并用 pH4.0 的 NaCl 梯度洗脱。

在用 0.1%三氟乙酸(TFA)平衡的 C-8 Aquapore RP-300 柱(Perkin Elmer, Norwalk CT)上通过 RP-HPLC 将天然的 MCP-2 纯化至均一。蛋白质用乙腈梯度洗脱。

5

用 SDS-PAGE、氨基酸序列分析和质谱法对 MCP-形式进行生物化学特性分析

在还原性条件下、Tris/麦黄酮(tricine)凝胶上用 SDS-PAGE 检测柱级分的纯度(Proost P.等人, 1996)。银染蛋白质，并用下列相对分子量(*Mr*)标记：OVA(*Mr* 45000), 碳酸酐酶(*Mr* 31000)、大豆胰蛋白酶抑制剂(*Mr* 21500)、β-乳球蛋白(*Mr* 18400)、溶菌

10 酶(*Mr* 14400)以及抑蛋白酶肽(*Mr* 6500)。

在脉冲液体 477A/120A 蛋白质测序仪(Perkin Elmer)上，用 N-甲基哌啶作为偶联碱，用 Edman 降解法测定纯化的趋化因子的氨基端序列。将封闭的蛋白置于 75%甲酸中 50 小时，使其在 Asp 和 Pro 之间断裂。不作进一步纯化就对甲酸消化物测序。用基质辅助性激光解吸电离/飞行时间质谱法(MALDI/TOF-MS)(Micromass TofSpec,

15 Manchester, UK)测定 MCP-2 的 *Mr*。分别用α-氰基-4-羟基肉桂酸和细胞色素 C 作为基质和内部标准。

趋化活性的检测

在 Boyden 微室中用孔径 5 微米的聚乙烯吡咯烷酮处理的聚碳酸膜测试 MCP-2

20 在新鲜纯化的单核细胞(2×10^6 细胞/毫升)或单核细胞性 THP-1 细胞(0.5×10^6 细胞/毫升；转种后 2 天)上的趋化活性。

将样品和细胞稀释在添加了 1 毫克/毫升人血清白蛋白(Red Cross Belgium)的 HBSS(Life technologies/Gibco BRL, Paisley, Scotland)中。37℃培育 2 小时后，固定细胞，用 Diff-Quick 染色液(Harleco, Gibbstown. NJ)染色，然后用显微镜在 10 个放大 500

25 倍的油浸视野中计数迁移通过膜的细胞。

根据迁移到样品中的细胞数与迁移至对照培养基中的细胞数之比(Van Damme J. 等人, 1992)，计算样品(每个室中一式三份)的趋化指数(CI)。

对于脱敏实验，在加入 Boyden 微室的上排孔之前，用无生物活性的趋化因子变体 37℃培育细胞 10 分钟。用 HBSS 处理的细胞的 CI 对样品的 CI 作为参照值，

30 计算 CI 的抑制%。

胞内 Ca^{2+}浓度的检测

如以前所述的那样(Wuyts A.等人, 1997)，测定胞内钙浓度([Ca^{2+}]$_i$)。在含荧光指示剂 fura-2(fura-2/AM 2.5μM; Molecular Probes Europe BV, Leiden, The Netherlands)和0.01% Pluronic F-127(Sigma, St Louis MO)的 Eagle 极限必需培养基(EMEM，Gibco)+0.05%FCS 中，培育纯化的单核细胞或 THP-1 细胞(10^7 细胞/毫升)。

37℃放置30分钟后，洗涤细胞两次，并以10^6细胞/毫升的浓度重悬于含 1mM Ca^{2+}和 0.1%FCS 的 HBSS(用 pH 7.4 的 10mM Hepes/NaOH 作为缓冲液)中。37℃平衡细胞 10 分钟，然后在 LS50B 发光分光光度计(Perkin Elmer)中测定 fura-2 荧光。

在 340 和 380nm 处激发后，在 510nm 检测荧光。用 Grynkiewicz 方程式(Grynkiewicz 等人, 1985)计算[Ca^{2+}]$_i$。为了测定 R_{max}，用 50μM 毛地黄皂苷裂解细胞。随后，用 20mM Tris 调节 pH 至 8.5，在裂解的细胞中加入 10mM EGTA，获得 R_{min}。所用 K_d 为 224nM。

对于脱敏实验，首先用缓冲液、不同浓度的趋化因子或趋化因子拮抗剂刺激单核细胞或 THP-1 细胞。作为第二刺激，MCP-2 所用浓度为缓冲液预先刺激后诱导[Ca^{2+}]$_i$显著增加的浓度。第二刺激在加入第一刺激后 2 分钟施加。比较用趋化因子或趋化因子拮抗剂预先刺激后的信号与加入缓冲液后的信号，计算对第二刺激反应中[Ca^{2+}]$_i$增加的抑制百分数。

结果

翻译后修饰型 MCP-2 的分离

用特异的、灵敏的 ELISA 追踪检测促分裂素和内毒素刺激的外周血单核细胞所产生的不同形式的 MCP-2。

根据标准的分离方法(Proost P.等人，1996)纯化条件培养基，这些方法包括吸附到孔径控制的玻璃上和肝素 Sepharose 层析。

随后，进行 FPLC mono S 阳离子交换层析纯化，然后采用 C-8 RP HPLC 作进一步纯化。用 SDS-PAGE 和 MALDI/TOF-MS 测定分子量。

分离获得不同形式的 MCP-2：除了真实的 7.5kDa 的 MCP-2(1-76)外，用 RP-HPLC 将缺少 5 个残基的 7kDa 氨基端截短型 MCP-2[MCP-2(6-76)]纯化至均一，并用氨基酸序列分析鉴定(图 2)。MALDI/TOF-MS(表 I)测得完整 MCP-2 的分子量为 8881Da(理论上的相对分子量为 8893Da)，而测得 MCP-2(6-76)的分子量为 8365Da，这确认了有 5 个氨基端氨基酸缺失(理论上的相对分子量为 8384Da)。这些天然形式的 MCP-2 在 THP-1 趋化试验中的功能比较表明，完整的 MCP 2 在 5ng/ml 下仍有活性，而截短的 MCP-2(6-76)在 0.6-60ng/ml 的浓度范围内测试时仍没有趋化活性(图 3)。还比较

了完整的天然 MCP-2 与合成的 MCP-2(1-76)以及缺少 2 个残基的羧基端截短的合成型 MCP-2[MCP-2(1-74)](Proost P.等人, 1995)的效力。

还发现这些形式的最低有效趋化浓度为 5ng/ml(图 3)。尽管在趋化试验中，天然的完整 MCP-1 和 MCP-2 的比活是相当的(Van Damme J，等人，1992)，但是 MCP-2 使钙迁移的性能仍有待争论。

然而，在 Ca^{2+}迁移实验中，天然的或合成的 MCP-2(1-76)的最低有效剂量比天然的完整的 MCP-1(1-76)高 10 倍(图 4)，而 MCP-2(6-76)却仍没有活性。

尽管如此，完整的 MCP-2(50ng/ml)却能使 MCP-2(15ng/ml)和 MCP-3(10ng/ml)脱敏，使两者的趋化作用分别受到 52%和 45%的抑制。

由于 MCP-2 在 Ca^{2+}试验中的比活较低，因此在 Boyden 微室中进行了 MCP-2(6-76)对趋化作用的脱敏。据报道，完整的 MCP-2 在单核细胞趋化试验中与活性 MCP-1、MCP2 和 MCP 3 交叉脱敏(Sozzani S.等人, 1994)，所以，我们研究天然的无活性的 MCP-2(6-76)是否也能使 MCP-1、MCP-2、MCP-3 和 RANTES 脱敏(表 II)。用 100ng/ml 无活性 MCP-2(6-76)预先培育 THP-1 细胞已经能明显抑制 10ng/ml MCP-1(63%)、5ng/ml MCP-2(75%)、30ng/ml MCP-3(62%)和 100ng/ml RANTES(75%)诱导的趋化作用。另外，浓度低 3 倍的各 MCP 所产生的趋化作用被 100ng/ml MCP-2(6-76)完全(91-100%)抑制。而且，在低达 10ng/ml 的浓度下，MCP-2(6-76)仍然能明显抑制 MCP-1(3ng/ml)、MCP-2(1.5ng/ml)或 MCP 3(10ng/ml)以及 RANTES(30ng/ml)诱导的趋化活性。总之，MCP-2(6-76)是天然产生的、无活性的化学引诱剂，并拮抗几种 C-C 趋化因子，对 MCP-3 的效果最突出。

表 I

天然型 MCP-2 的生物化学特性分析。氨基端氨基酸分析以及 C-8 RP-HPLC 纯化的天然 MCP-同种型的实验(SDS-PAGE 和 MALDI/TOF-MS)和理论的相对分子量的比较

MCP 形式	氨基端序列	相对分子量(Da)		
		未糖基化的理论值	SDS-PAGE	MALDI/TOF-MS
MCP-2(1-76)	封闭	8893	7500	8881
MCP-2(2-76)	SIPITCC	8384	7500	8365

表 II

MCP-2(6-76)使微室中的 MCP-1、MCP-2、MCP-3 和 RANTES 的单核细胞趋化反应脱敏。

趋化因子[a]	浓度	趋化反应的拮抗作用[b,c]		趋化作用的抑制%
		缓冲液	100ng/ml MCP-2(6-76)	
MCP-1	10	22.3±7.9	8.3±3.8	63±21
	3	15.0±8.0	1.3±0.3	99±1.0
MCP-2	5	36.0±15.6	10.8±6.1	75±8.0
	1.5	6.7±1.4	1.5±0.3	91±7.0
MCP-3	30	13.2±0.4	6.0±4.0	62±31
	10	3.0±1.5	<1	100±0.0
RANTES	100	6.3±0.8	2.6±1.3	75±19
	30	4.0±0.8	1.5±0.3	77±16
		缓冲液	100ng/ml MCP-2(6-76)	
MCP-1	10	12.7±2.3	10.5±3.8	24±1.8
	3	7.5±0.0	3.0±0.3	69±4.0
MCP-2	5	38.0±5.3	27.2±4.9	30±6.0
	1.5	18.3±4.6	9.2±1.4	45±23
MCP-3	30	13.2±1.9	8.0±1.0	37±19
	10	7.7±1.4	1.7±0.3	90±6.0
RANTES	100	5.5±0.6	5.8±0.9	17±7.0
	30	3.2±0.7	2.5±0.5	39±18

[a] 将 MCP-1、MCP-2、MCP-3 或 RANTES 作为化学吸引剂加入下排孔中。

5 [b] 微室的上排孔中加入预先用 MCP-2(6-76)或缓冲液培育的 THP-1 细胞

[c] 3 个独立实验的 CI 平均值±SEM

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序列表

(1) 一般信息:

(i) 申请人:

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(C) 城市: CURACAO

(E) 国家: THE NETHERLANDS ANTILLES

(F) 邮政编码: 没有

(G) 电话: 639300

(H) 电传: 614129

(I) 电报:

5

(ii) 发明名称: 作为趋化因子拮抗剂的氨基端截短的 MCP-2

(iii) 序列数目: 4

10 (v) 计算机可读形式:

(A) 记录介质类型: 软盘

(B) 计算机: IBM PC 兼容型

(C) 操作系统: PC-DOS/MS-DOS

(D) 软件: PatentIn Release #1.0, Version #1.30(EPO)

15

(2) SEQ ID NO:1 的信息:

(i) 序列特征:

(A) 长度: 99 氨基酸

20 (B) 类型: 氨基酸

(C) 链型:

(D) 拓扑结构: 线性

(ii) 分子类型: 蛋白质

25

(iii) 假设: 没有

(ix) 特征:

(A) 名称/关键: 蛋白质

30 (B) 位置: 1..76

(xi) 序列描述：SEQ ID NO:1:

```
Met Lys Val Ser Ala Ala Leu Leu Cys Leu Leu Leu Met Ala Ala Thr
            -20                 -15                 -10

Phe Ser Pro Gln Gly Leu Ala Gln Pro Asp Ser Val Ser Ile Pro Ile
        -5                  1               5

Thr Cys Cys Phe Asn Val Ile Asn Arg Lys Ile Pro Ile Gln Arg Leu
10                  15                  20                  25

Glu Ser Tyr Thr Arg Ile Thr Asn Ile Gln Cys Pro Lys Glu Ala Val

                30                  35                  40

Ile Phe Lys Thr Lys Arg Gly Lys Glu Val Cys Ala Asp Pro Lys Glu
            45                  50                  55

Arg Trp Val Arg Asp Ser Met Lys His Leu Asp Gln Ile Phe Gln Asn
        60                  65                  70

Leu Lys Pro
    75
```

(2) SEQ ID NO:2 的信息：

5 (i) 序列特征：

(A) 长度：99 氨基酸

(B) 类型：氨基酸

(C) 链型：

(D) 拓扑结构：线性

10

(ii) 分子类型：蛋白质

(iii) 假设：没有

15 (ix) 特征：

(A) 名称/关键：蛋白质

(B) 位置：1..76

(xi) 序列描述：SEQ ID NO:2:

Met Lys Val Ser Ala Ala Leu Leu Cys Leu Leu Leu Met Ala Ala Thr
-20 -15 -10

Phe Ser Pro Gln Gly Leu Ala Gln Pro Asp Ser Val Ser Ile Pro Ile
-5 1 5

Thr Cys Cys Phe Asn Val Ile Asn Arg Lys Ile Pro Ile Gln Arg Leu
10 15 20 25

Glu Ser Tyr Thr Arg Ile Thr Asn Ile Gln Cys Pro Lys Glu Ala Val
30 35 40

Ile Phe Lys Thr Gln Arg Gly Lys Glu Val Cys Ala Asp Pro Lys Glu
45 50 55

Arg Trp Val Arg Asp Ser Met Lys His Leu Asp Gln Ile Phe Gln Asn
60 65 70

Leu Lys Pro
75

(2) SEQ ID NO:3 的信息:

(i) 序列特征:

5 (A) 长度: 71 氨基酸

(B) 类型: 氨基酸

(C) 链型:

(D) 拓扑结构: 线性

10 (ii) 分子类型: 蛋白质

(iii) 假设: 没有

(xi) 序列描述: SEQ ID NO:3:

Ser Ile Pro Ile Thr Cys Cys Phe Asn Val Ile Asn Arg Lys Ile Pro
1 5 10 15

Ile Gln Arg Leu Glu Ser Tyr Thr Arg Ile Thr Asn Ile Gln Cys Pro
20 25 30

Lys Glu Ala Val Ile Phe Lys Thr Lys Arg Gly Lys Glu Val Cys Ala
35 40 45

Asp Pro Lys Glu Arg Trp Val Arg Asp Ser Met Lys His Leu Asp Gln
50 55 60

Ile Phe Gln Asn Leu Lys Pro
65 70

15

(2) SEQ ID NO:4 的信息:

(i) 序列特征:

(A) 长度: 71 氨基酸

(B) 类型: 氨基酸

(C) 链型:

5 (D) 拓扑结构: 线性

(ii) 分子类型: 蛋白质

(iii) 假设: 没有

10

(xi) 序列描述:SEQ ID NO: 4:

```
Ser Ile Pro Ile Thr Cys Cys Phe Asn Val Ile Asn Arg Lys Ile Pro
1               5                   10                  15

Ile Gln Arg Leu Glu Ser Tyr Thr Arg Ile Thr Asn Ile Gln Cys Pro
            20                  25                  30

Lys Glu Ala Val Ile Phe Lys Thr Gln Arg Gly Lys Glu Val Cys Ala
        35                  40                  45

Asp Pro Lys Glu Arg Trp Val Arg Asp Ser Met Lys His Leu Asp Gln
    50                  55                  60

Ile Phe Gln Asn Leu Lys Pro
65                  70
```

15

说 明 书 附 图

MCP-2

```
-23                           1   ↓
MKVSAALLCL LLMAATFSPQ GLAQPDSVSI PITCCFNVIN RKIPIQRLES YTRITNIQCP
KEAVIFKTKR GKEVCADPKE RWVRDSMKHL DQIFQNLKP
                                          76
```

MCP-2 变体

```
-23                           1   ↓
MKVSAALLCL LLMAATFSPQ GLAQPDSVSI PITCCFNVIN RKIPIQRLES YTRITNIQCP
KEAVIFQTKR GKEVCADPKE RWVRDSMKHL DQIFQNLKP
                                          76
```

图 1

图 2

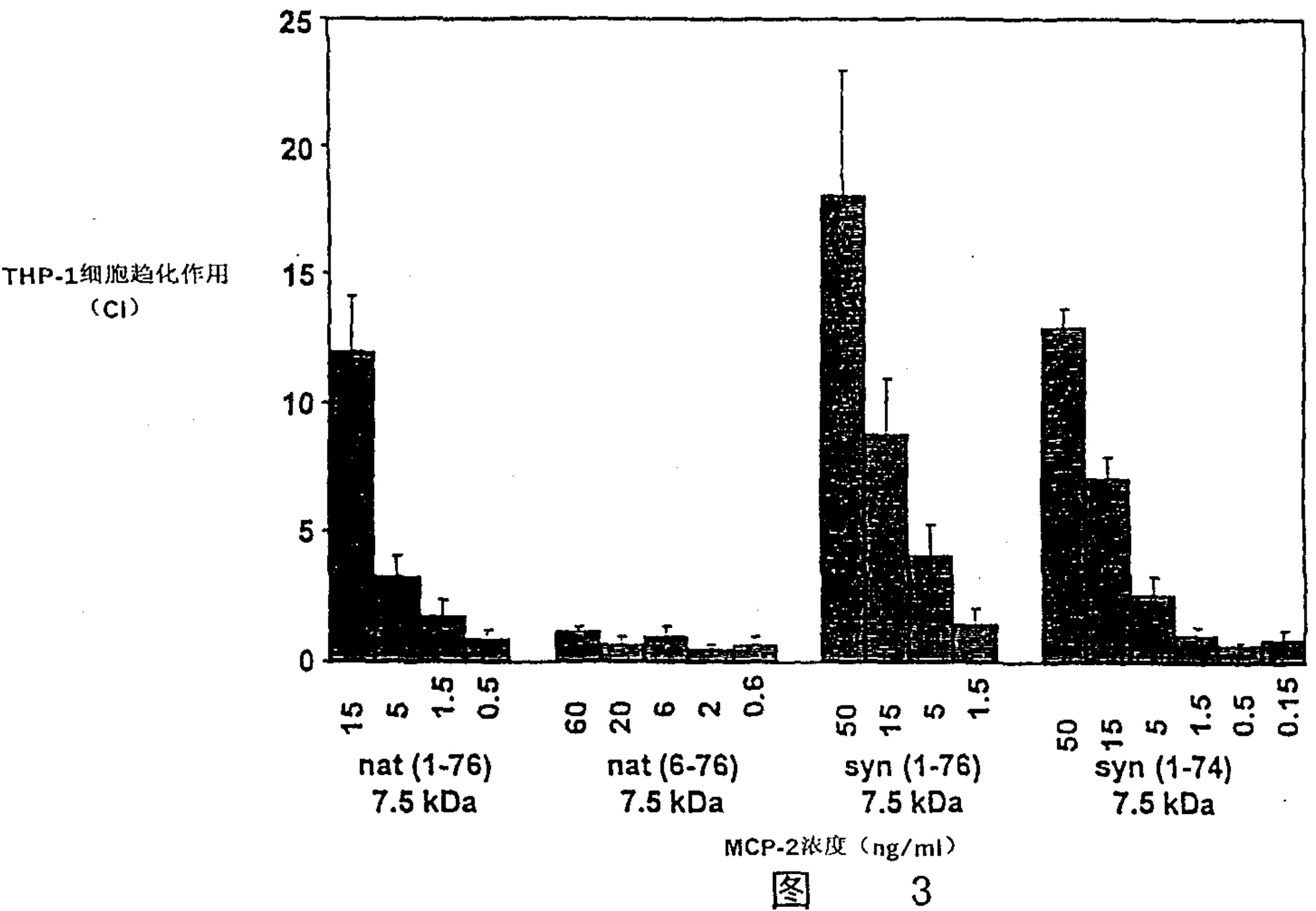
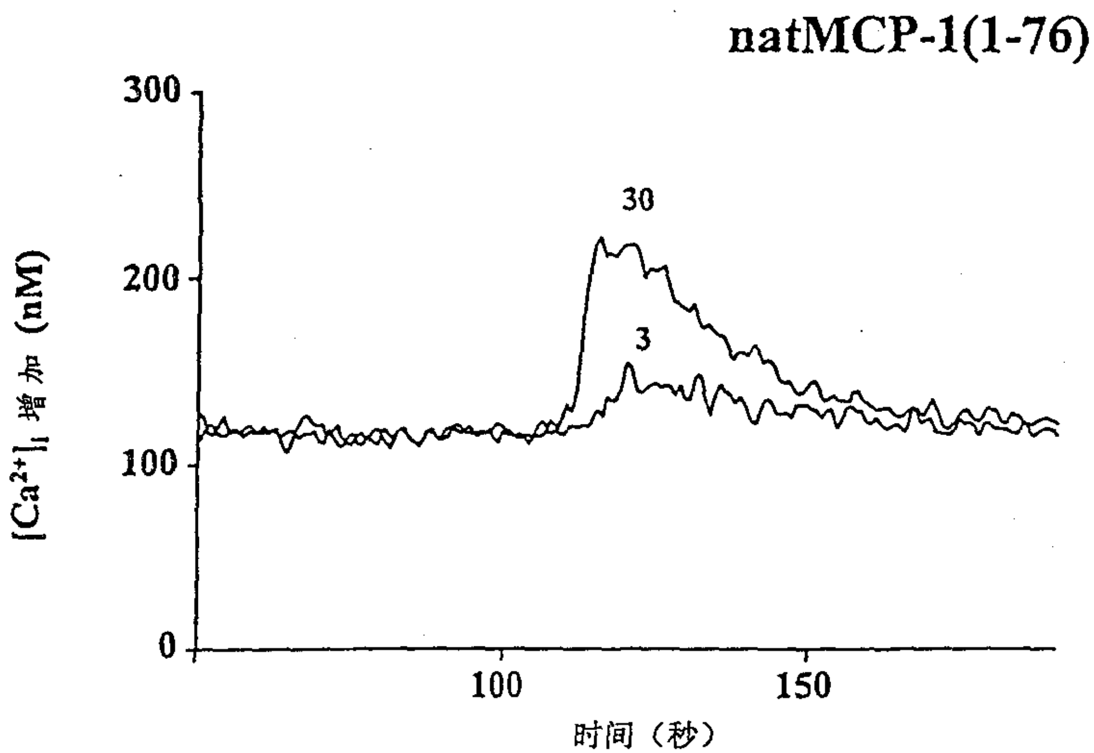


图 3



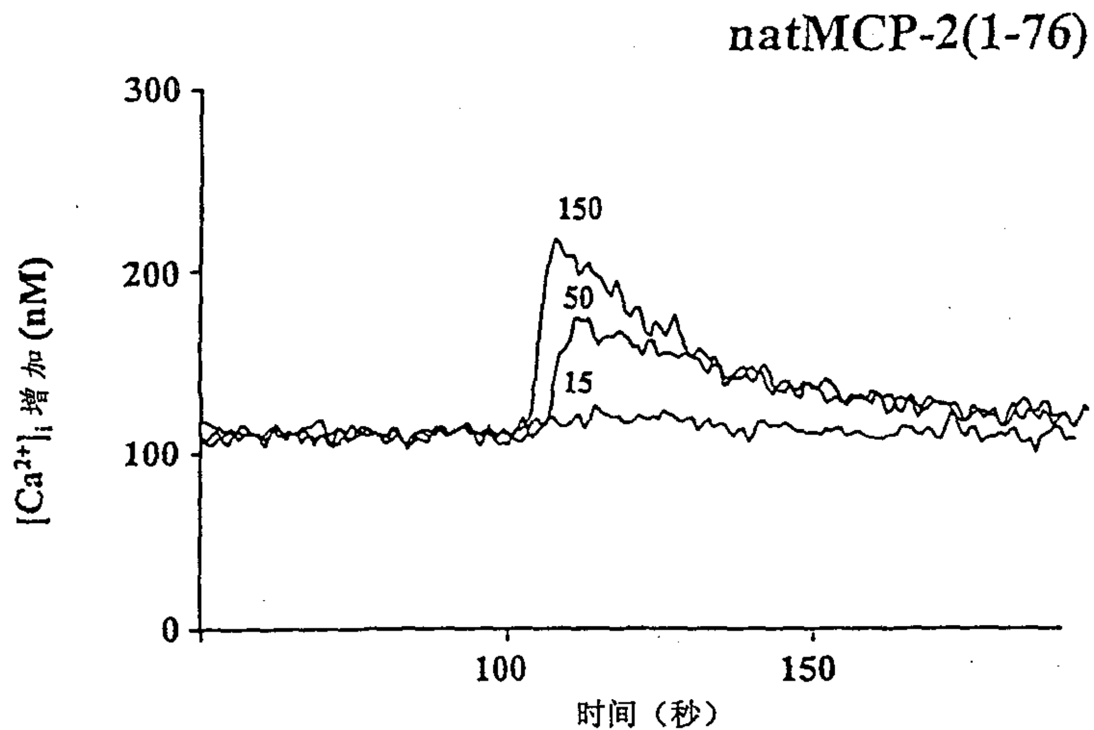


图 4