Title: COMBINATION OF CHIPS (CHEMOTAXIS INHIBITING PROTEIN FROM STAPHYLOCOCCUS AU-REUS)-BASED COMPOUNDS

Abstract: The invention relates to a combination of compounds, wherein one compound has CHIPS activity and consists of at least 6 building blocks the sequence of which is based on the N-terminal part of the CHIPS molecule, and which sequence may comprise one or more substitutions as compared to the original CHIPS amino acid sequence, whereas the one or more other compounds are derived from the amino acids 4-121 of CHIPS and supports CHIPS activity. One or more of the amino acids in the amino acid sequence of the one or more supportive compounds may be replaced by building blocks selected from the group consisting of proteinogenic amino acids, non-proteinogenic amino acids, D-amino acids and peptidomimetic building blocks.
COMBINATION OF CHIPS (CHEMOTAXIS INHIBITING PROTEIN FROM STAPHYLOCOCCUS AUREUS)-BASED COMPOUNDS

The present invention relates to combinations of peptides derived from the Chemotaxis Inhibitory Protein from Staphylococcus aureus (CHIPS) having CHIPS activity. The invention in addition relates to the use of non-peptide molecules having peptide structural features and a similar function as the peptides in such combinations. The combination of compounds (peptide and non-peptide molecules) can be used in the prophylaxis and treatment of various indications, such as inflammation reactions. The peptides and non-peptides can in addition be used for inhibiting activation of leukocytes and endothelial cells.

Leukocytes are mainly involved in protecting the body against foreign invaders (e.g. bacteria, viruses, fungi, and cancer cells). The most important cells are lymphocytes, monocytes and neutrophils. Lymphocytes form the specific immune system and cause immune reactions against invaders. Their most important task is to build up specific memory against the invader, so that the next time the invader enters the body it is recognized, killed and removed rapidly. Sometimes these lymphocytes not only attack invaders, but also react against certain structures and/or molecules (so-called auto-antigens) of the own body, causing auto-immune diseases (e.g. rheumatoid arthritis). Killing and removal of invaders is mostly done by monocytes and neutrophils, cells of the innate immune system, by direct recognition of the invaders or with the help of specific lymphocytes.

In contrast to the delicate network of the fine-tuned and controlled reactions of lymphocytes, cells of the innate system react in a relatively non-specific and aggressive way. Since they are part of the body's first line of defense, their most important task is to kill and remove the invading agent as quickly as possible. This is accomplished through very aggressive substances (e.g. free radicals and enzymes) that are not
only lethal to the invader, but also cause damage to host cells in the vicinity. Substances from these damaged cells and the locally activated cells from the innate system itself will further attract increasing numbers of neutrophils and monocytes, causing local inflammation. In most cases, such an aggressive and damaging inflammatory reaction, caused by overactivated neutrophils, is unnecessary. In some cases this inflammatory response is responsible for serious, sometimes lethal disorders and includes conditions like Adult Respiratory Distress Syndrome (ARDS), severe tissue damage following thrombotic events such as heart attacks and stroke, inflammatory bowel diseases and rheumatoid arthritis. The inflammation will subside once all the invaders have been killed and removed, together with the various cells killed in the process. Healing of the wound, caused by the inflammatory response, can then begin. Although there is some overlap in function, the main task of neutrophils is to attack the invaders and the main task of monocytes is to remove the debris resulting from this attack. In addition, neutrophils have another peaceful task in assisting the wound healing process.

When bacteria have invaded the body and, for example, infected the central nervous system (as in meningitis) they start to produce microbial substances, including the formylated polypeptides (like the fMLP peptide). Other substances of microbial origin activate the complement factor 5 (C5) convertase enzyme-complex, that converts C5 of the complement system into its activated C5a form. Both C5a and fMLP are chemo-attractants: substances that can activate and attract cells from the blood vessels (the migration process). Neutrophils are responsive to these two substances and also to interleukin-8 (IL-8). This "chemokine" (the name given to chemo-attractants that are produced by cells of the immune system) is produced mainly by activated monocytes (but also in minute amounts
by the activated neutrophils themselves). Neutrophils interact with these substances, because they have receptors for these substances on the outside of their cell membrane.

Activated neutrophils can easily migrate from blood vessels. This is because the chemo-attractants, microbial products and substances from activated monocytes will have increased the permeability of the vessels and stimulated the endothelial cells of the vessel walls to express certain adhesion molecules. Neutrophils express selectins and integrins (e.g. CD11b/CD18) that bind to these adhesion molecules. Once the neutrophil has adhered to the endothelial cells, it is able to migrate through the cells, under the guidance of chemo-attractants/chemokines, towards the site of infection, where the concentration of these substances is at its highest. These substances also activate neutrophils to produce a range of other molecules, some of which attract more neutrophils (and subsequently monocytes), but, mostly, they are responsible for destroying the invading bacteria. Some of these substances (e.g. free radicals, enzymes that break down proteins (proteases) and cell membranes (lipases)) are so reactive and non-specific that cells from the surrounding tissue (and the neutrophils themselves) are destroyed, causing tissue damage. This damage is exacerbated by the presence of blood derived fluid which has transgressed the leaky vessel wall and is responsible for the swelling that always accompanies inflammation (called oedema). The pressure build up caused by this excess fluid results in further cell damage and death.

Later in the inflammatory process, monocytes migrate to the scene and become activated. Besides their role in removing bacteria and cell debris, they also produce substances such as tumour necrosis factor (TNF) and IL-8, which in turn attract more activated neutrophils, causing further local damage. TNF also has a direct stimulatory effect on neutrophils. Once all the
invaders have been removed, the inflammatory response will subside and the area will be cleared of the remaining 'casualties'. Then the process of wound healing starts. Although it is known that neutrophils play a pivotal role in wound healing, it is not clear which neutrophil-derived substances are involved and how the neutrophils are active in healing without being aggressive to the surrounding tissue. In general, damaged tissue will be replaced by scar tissue formed mainly of fibroblasts and collagen. When inflammation occurs in areas of the body with an important function, like tissues formed from heart muscle cells, brain cells or lung alveolar cells, normal function will be compromised by the resulting scar formation, causing serious conditions like heart failure, paralysis and emphysema. To minimize the debilitating consequences of these conditions, it is important to 'dampen' the inflammatory reaction as quickly as possible.

Intervention to control the acute early phase inflammatory response presents an opportunity to improve the prognosis for a wide range of patients whose symptoms can be traced back to such an event. Such an approach has been advocated for many acute and chronic inflammation-based diseases and shown to have potential based on findings from relevant disease models. Classical anti-inflammatory drugs such as steroids and Non Steroid Anti-Inflammatory Drugs (NSAIDS) do not have the ideal profile of action, either in terms of efficacy or safety. Steroids affect the 'wrong' cell type (monocytes) and their dampening effects are easily bypassed. NSAIDS generally show a relatively mild effect partly because they intervene at a late stage in the inflammatory process. Both classes of drugs produce a range of undesirable side effects resulting from other aspects of their pharmacological activity. Drugs acting directly and specifically to prevent migration and activation of neutrophils may have a number of advantages. Several drugs under early development only interfere with one
individual aspect of neutrophil activation (e.g. C5 convertase inhibitors, antibodies against C5a, C5a-receptor blocking drugs) and migration (antibodies against integrins (like CD11b/CD18) and L-selectin on neutrophils and antibodies against adhesion molecules (like ICAM-1 and E-selectin) on endothelial cells). Antibodies against TNF and IL-8 have effects in chronic inflammation, but only marginal effects in acute inflammation, because of the minimal role monocytes (which are mainly responsible for these substances' production) play in the acute phase.

Sometimes, the cause of the acute inflammation cannot be removed and the inflammation becomes chronic. With the exception of tuberculosis, chronic hepatitis and certain other conditions, this is seldom the case with infections. However, chronic inflammation can also be caused by stimuli other than bacteria, such as auto-immune reactions. Research has shown that in chronic inflammation the role of monocytes is much more prominent, and that neutrophil migration and activation, monocyte migration and activation, tissue damage, removal of dead cells and wound healing are all going on at the same time. This complex cascade of interactions between cells and many different cytokines and chemokines has been the subject of intensive research for many years. It was believed that monocytes and their products were the most important elements that needed to be inhibited to dampen chronic inflammation. This explains why steroids, which specifically interact with monocytes, are generally more effective in chronic as opposed to acute inflammation. Long-term treatment with steroids however, is not a desirable option, because severe and unacceptable side effects can occur at the doses required to produce a clinical effect. Newer treatments using antibodies against TNF or IL-8 have shown good results, and were initially seen as proof of the major role monocytes were thought to play in chronic inflammation. Recent research casts doubts on an exclusive role for
monocytes in inflammation and points to a critical role for neutrophils, which are now seen to represent better targets for therapeutic intervention.

The underlying cause of a chronic inflammatory condition is not always clear, and the original cause may not always be responsible for future recurrence. Some scientists believe that in certain chronic inflammatory diseases there is a continuous cycle of events. Their idea is that existing activated neutrophils and monocytes continuously attract and activate new groups of cells, thus perpetuating the inflammatory response even when the initial stimulus is no longer present. This would suggest that an acute or periodic treatment with an effective inhibitor of the neutrophil and monocyte activation would stop the cycle of new cell recruitment, leading in due course to modification of disease progression, or even a complete cure, and not just symptomatic relief.

In the research that led to the present invention a new agent with inflammation-inhibiting properties was found in the extracellular medium of growing Staphylococcus aureus (S. aureus). This agent is the subject of application PCT/NL99/00442. The agent was found to be capable of directly or indirectly blocking different chemokine receptors. Incubation of different cells with the medium resulted in a greatly reduced expression of a number of the chemokine receptors, both of the expression of receptors of classical chemotactic agents such as fMLP and C5a on neutrophils, monocytes and endothelial cells and of the expression of CXCR4 and CCR5 receptors on lymphocytes, monocytes and macrophages. The reduced receptor expression was related to greatly reduced chemotaxis relative to the chemokines, as well as a reduced infection with HIV. The active protein could be further purified, as described in application PCT/NL99/00442. Because this protein is isolated from the supernatant of the Staphylococcus aureus and gives inhibition of chemotaxis, this protein was named "CHIPS": CHEmotaxis Inhibitory Protein from Staphylococcus aureus.
Isolation of the CHIPS protein out of the supernatant of *S. aureus* is not very cost-effective. The co-pending application PCT/EP01/00270 describes a nucleic acid molecule comprising a nucleotide sequence encoding a (poly)peptide having CHIPS activity. **Figure 1** gives the nucleotide sequence of this nucleic acid molecule. This sequence provides the means for producing the original CHIPS protein or other corresponding (poly)peptides that have CHIPS activity, as well as functional fragments, derivatives or analogues thereof by means of genetic engineering.

"CHIPS activity" is herein defined as the ability to specifically impair at least the responses induced by both fMLP and C5a, including at least impairment of ligand-(C5a or fMLP) binding, and optionally impairment of chemotaxis and cell-activation (e.g. calcium mobilization). However, the compounds of this invention may in addition have other biological activities, such as an inhibitory effect on the activation of leukocytes and endothelial cells.

In addition, it is desirable for the practical use of CHIPS in therapy that the active part of the protein is isolated. Smaller protein or peptide molecules have a reduced risk of inducing an immunological response in a subject receiving the protein or peptide for therapy. Furthermore, it is desirable to be able to modify the protein or peptide to further increase the biological activity and/or lower the immunogenicity thereof.

It is therefore the object of the present invention to provide the smallest as possible active site within the CHIPS molecule conferring CHIPS activity in order to be able to design individual peptides or non-peptides based on this active site for use in diagnosis, prophylaxis and therapy.

It was found according to the invention that this smallest as possible active site within the naturally occurring CHIPS molecule is the N-terminal
sequence FTFEPF. This peptide has however an activity that is 10,000 times lower (on molar basis) than the native CHIPS protein. This led the inventors to conclude that one or more further sites are present in the CHIPS molecule that support the CHIPS activity of the peptide.

The present invention therefore relates to combinations of compounds, wherein one compound has CHIPS activity (further identified herein as "the compound having CHIPS activity") and consists of at least 6 amino acids the sequence of which is based on the N-terminal part of the naturally occurring CHIPS molecule, and which sequence optionally comprises one or more substitutions as compared to the naturally occurring sequence, or peptidomimetic derivatives thereof, whereas the one or more other compounds are derived from the amino acids 4-121 of the naturally occurring CHIPS molecule and support CHIPS activity (further identified herein as the "supporting compound").

More in particular the invention relates to combinations of compounds, wherein the compound having CHIPS activity, has the sequence:

\[ X_1-X_2-X_3-X_4-X_5-X_6-X_7 \]

wherein the building blocks \( X_1, X_2, X_3, X_4, X_5, X_6 \) and \( X_7 \) are selected from the group consisting of proteinogenic amino acids, non-proteinogenic amino acids, D-amino acids and peptidomimetic building blocks and wherein \( X_7 \) may or may not be present and may represent either one or more building blocks. Optionally \( X_1 \) may be modified (cf. Theodora W. Greene, Peter G.M. Wuts; Protective groups in organic synthesis, Third edition 1999, John Wiley & Sons Inc, New York; W.C. Chan, P.D. White (eds); Fmoc Solid Phase Peptide Synthesis, a practical approach, 2000, Oxford University Press, New York; and Gunther Jung (ed); Combinatorial Peptide and Nonpeptide Libraries, 1996, VCH Verlagsgesellschaft mbH, Weinheim) with a group that is smaller than an amino acid and which is selected from
acyl (Greene & Wuts, 1999, supra) moieties, such as acetyl and formyl, and ureum moieties such as carbamoyl, or by reductive alkylation (Chan & White, 2000, supra; and Jung, 1996, supra).

In case the building blocks of the compound having CHIPS activity are proteinogenic amino acids then X₁ may be selected from phenylalanine (F), cysteine (C), asparagine (N), tryptophan (W); X₂ may be selected from alanine (A), cysteine (C), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), leucine (L), methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), valine (V), tryptophan (W), tyrosine (Y); X₃ may be selected from phenylalanine (F), tryptophan (W); X₄ may be selected from alanine (A), cysteine (C), aspartic acid (D), glutamic acid (E), leucine (L), asparagine (N), methionine (M), serine (S), tryptophan (W); X₅ may be selected from phenylalanine (F), isoleucine (I), leucine (L), proline (P), valine (V) tryptophan (W), tyrosine (Y); X₆ is selected from cysteine (C), phenylalanine (F), histidine (H), isoleucine (I), tryptophan (W), tyrosine (Y).

More in particular X₁ may be selected from phenylalanine (F), tryptophan (W); X₂ may be selected from phenylalanine (F), isoleucine (I), serine (S), threonine (T); X₃ is phenylalanine (F); X₄ may be selected from alanine (A), cysteine (C), aspartic acid (D), glutamic acid (E), asparagine (N), serine (S), tryptophan (W); X₅ may be selected from phenylalanine (F), isoleucine (I), leucine (L), proline (P), valine (V) tryptophan (W), tyrosine (Y); X₆ may be selected from cysteine (C), phenylalanine (F), histidine (H), isoleucine (I), tryptophan (W), tyrosine (Y).

Particular compounds having CHIPS activity of the invention have the sequence:

F-T-F-E-P-F-X₇,

wherein one or more of the amino acids on positions 1 to 6 are substituted with the building blocks as defined above, and wherein X₇ is a stretch of one or more
building blocks selected from the group consisting of non-proteinogenic amino acids, D-amino acids and peptidemimetic building blocks and may or may not be present. X7 can be present to further increase the potency (CHIPS activity) of the compound and/or to improve upon its pharmacological and pharmaceutical characteristics (e.g. increase potency, stability, bioavailability, in vivo half-life, decrease side effects, etc.).

Compounds having CHIPS activity for use in the combinations of the invention in which only one of the original amino acids has been substituted are the following:

X1-T-F-E-P-F-X7, F-X2-F-E-P-F-X7, F-T-X3-E-P-F-X7, F-T-F-X4-P-F-X7, F-T-F-E-X5-F-X7, F-T-F-E-P-X6-X7. In these X1 to X7 are as defined above. A preferred compound having CHIPS activity for use in the combinations of the invention is F-T-F-E-P-F-X7, wherein X7 may or may not be present.

The peptides described in Table 6 are part of the present invention as the compound that has CHIPS activity provided they have CHIPS activity as defined above and as tested in one or more of the CHIPS activity tests as given in the Examples.

Table 6

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The N-terminal of these peptides may be modified as described above for X1, whereas an X7 group may be present C-terminally. In addition, all of these peptides may be used as a starting point for further modification, for example by substitution of one or more of the amino acids with other building blocks.

Particularly preferred for use in combinations of the invention is a peptide of the naturally occurring N-terminal sequence of CHIPS, i.e. FTFEPF.

In this application a "combination of compounds" is to be understood as a mixture of at least one peptide or peptidomimetic that is based on the first six amino acids of the CHIPS protein and has CHIPS activity and one or more other peptides derived from the amino acid sequence of positions 4-121 of CHIPS, wherein the two or more peptides are either present as separate entities (that either stay as separate entities after administration into the body or become attached to each
other in the body) or physically attached (either directly or indirectly, such as via a spacer). The amino acid sequence of the one or more other peptides may be altered as compared to the amino acid sequence occurring in CHIPS in the same manner as described herein for the compound having CHIPS activity.

Both types of compounds that are used in the combinations of the invention can be produced by known chemical synthesis. Methods for constructing peptides by synthetic means are known to those skilled in the art. These synthetic peptides, by virtue of sharing primary, secondary and/or tertiary structural and/or conformational characteristics with CHIPS will possess an activity in common therewith, meaning CHIPS properties or supportive properties. Thus, such synthetically produced peptides can be employed as biologically active or immunological substitute for natural purified (poly)peptides having CHIPS activity or supportive properties.

The compounds having CHIPS activity provided herein also include peptides characterized by amino acid sequences into which modifications are naturally provided or deliberately engineered. Modifications in the peptide can be made by those skilled in the art using known conventional techniques. Modifications of interest in the CHIPS active peptide sequences may include replacement, insertion or deletion of selected amino acid residues in the coding sequence. The Examples describe such modified synthetic peptides.

In some cases the potential for use of peptides in drugs may be limited for several reasons. Peptides may for example be too hydrophilic to pass membranes like the cell-membrane and the blood-brain barrier, and may be rapidly excreted from the body by the kidneys and the liver, resulting in a low bioavailability. Furthermore, peptides may suffer from a poor biostability and chemical stability since they may be quickly degraded by proteases, e.g. in the gastro-intestinal tract. Also,
peptides generally are flexible compounds which can assume thousands of conformations. The bioactive conformation usually is only one of these possibilities, which sometimes might lead to a poor selectivity and affinity for the target receptor. Finally, the potency of the peptides may not be sufficient for therapeutic purposes.

As a result of the above described drawbacks, peptides are sometimes mainly used as sources for designing other drugs, and not as actual drugs themselves. In such case it is desirable to develop compounds in which these drawbacks have been reduced. Alternatives for peptides are the so-called peptidomimetics. Peptidomimetics based on the peptides of the present invention are also part of this application. In that case, one or more of X1 to X7 are substituted with peptidomimetic building blocks.

Various definitions for peptidomimetics have been formulated in literature. Among others, peptidomimetics have been described as "chemical structures designed to convert the information contained in peptides into small non-peptide structures", "molecules that mimic the biological activity of peptides but no longer contain any peptide bonds", "structures which serve as appropriate substitutes for peptides in interactions with receptors and enzymes" and as "chemical Trojan horses".

In general, peptidomimetics can be classified into two categories. The first consists of compounds with non-peptidelike structures, often scaffolds onto which pharmacophoric groups have been attached. Thus, they are low molecular-weight compounds and bear no structural resemblance to the native peptides, resulting in an increased stability towards proteolytic enzymes.

The second main class of peptidomimetics consists of compounds of a modular construction comparable to that of peptides, i.e. oligomeric peptidomimetics. These compounds can be obtained by
modification of either the peptide side chains or the peptide backbone. Peptidomimetics of the latter category can be considered to be derived of peptides by replacement of the amide bond with other moieties. As a result, the compounds are expected to be less sensitive to degradation by proteases. Modification of the amide bond also influences other characteristics such as lipophilicity, hydrogen bonding capacity and conformational flexibility, which in favourable cases may result in an overall improved pharmacological and/or pharmaceutical profile of the compound.

Oligomeric peptidomimetics can in principle be prepared starting from monomeric building blocks in repeating cycles of reaction steps. Therefore, these compounds may be suitable for automated synthesis analogous to the well-established preparation of peptides in peptide synthesizers. Another application of the monomeric building blocks lies in the preparation of peptide/peptidomimetic hybrids, combining natural amino acids and peptidomimetic building blocks to give products in which only some of the amide bonds have been replaced. This may result in compounds which differ sufficiently from the native peptide to obtain an increased biostability, but still possess enough resemblance to the original structure to retain the biological activity.


The vinylogous peptides and oligopyrrolinones have been developed in order to be able to form secondary structures (β-strand conformations) similar to those of peptides, or mimic secondary structures of peptides. All these oligomeric peptidomimetics are expected to be resistant to proteases and can be assembled in high-yielding coupling reactions from optically active monomers (except the peptoids).

Peptidosulfonamides are composed of α- or β-substituted amino ethane sulfonamides containing one or more sulfonamide transition-state isosteres, as an analog of the hydrolysis of the amide bond. Peptide analogs containing a transition-state analog of the hydrolysis of the amide bond have found a widespread use in the
development of protease inhibitor e.g. HIV-protease inhibitors.

Another approach to develop oligomeric peptidomimetics is to completely modify the peptide backbone by replacement of all amide bonds by nonhydrolyzable surrogates e.g. carbamate, sulfone, urea and sulfonamide groups. Such oligomeric peptidomimetics may have an increased metabolic stability. Recently, an amide-based alternative oligomeric peptidomimetics has been designed viz. N-substituted Glycine-oligopeptides, the so-called peptoids. Peptoids are characterized by the presence of the amino acid side chain on the amide nitrogen as opposed to being present on the α-C-atom in a peptide, which leads to an increased metabolic stability, as well as removal of the backbone chirality. The absence of the chiral α-C atom can be considered as an advantage because spatial restrictions which are present in peptides do not exist when dealing with peptoids. Furthermore, the space between the side chain and the carbonyl group in a peptoid is identical to that in a peptide. Despite the differences between peptides and peptoids, they have been shown to give rise to biologically active compounds.

Translation of a peptide chain into a peptoid peptidomimetic may result in either a peptoid (direct-translation) or a retropetid (retro-sequence). In the latter category the relative orientation of the carbonyl groups to the side chains is maintained leading to a better resemblance to the parent peptide.

Review articles about peptidomimetics that are incorporated herein by reference are:

The invention thus furthermore relates to combinations of molecules that are not peptides themselves but have a structure and function similar to those of the peptides described herein. Examples of such molecules are the above described peptidomimetics, but also compounds in which one or more of X1 to X7 are replaced by non-proteinogenic amino acids or D-amino acids. When reference is made in this application to peptides, it is intended to include also such other compounds that have a similar or the same structure and function and as a consequence a similar or the same biological activity as the peptides.

More in particular substitutions can be made with non-proteinogenic amino acids selected from the group consisting of 2-naphthylalanine (Nal(2)), B-cyclohexylalanine (Cha), p-amino-phenylalanine ((Phe(p-NH$_2$)), p-benzoyl-phenylalanine (Bpa), ornithine (Orn), norleucine (Nle), 4-fluoro-phenylalanine (Phe(p-F)), 4-chloro-phenylalanine (Phe(p-Cl)), 4-bromo-phenylalanine (Phe(p-Br)), 4-iodo-phenylalanine (Phe(p-I)), 4-methyl-phenylalanine (Phe(p-Me)), 4-methoxy-phenylalanine (Tyr(Me)), 4-nitro-phenylalanine (Phe(p-NO$_2$)).

Suitable D-amino acids for substituting the amino acids in the peptides of the invention are for example those that are selected from the group consisting of D-phenylalanine, D-alanine, D-arginine, D-asparagine, D-aspartic acid, D-cysteine, D-glutamic acid, D-glutamine, D-histidine, D-isoleucine, D-leucine, D-lysine, D-methionine, D-proline, D-serine, D-threonine, D-tryptophan, D-tyrosine, D-valine, D-2-naphthylalanine (D-Nal(2)), B-cyclohexyl-D-alanine (D-Cha), 4-amino-D-phenylalanine (D-Phe(p-NH$_2$)), p-benzoyl-D-phenylalanine (D-Bpa), D-ornithine (D-Orn), D-norleucine (D-Nle), 4-fluoro-D-phenylalanine (D-Phe(p-F)), 4-chloro-D-
phenylalanine (D-Phe(p-CI)), 4-bromo-D-phenylalanine (D-Phe(p-Br)), 4-iodo-D-phenylalanine (D-Phe(p-I)), 4-methyl-D-phenylalanine (D-Phe(p-Me)), 4-methoxy-D-phenylalanine (D-Tyr(Me)), 4-nitro-D-phenylalanine (D-Phe(p-NO2)).

One or more of the amino acids in the peptides can be replaced by peptoid building blocks selected from the group consisting of N-substituted glycines, such as N-benzylglycine (NPhe), N-methylglycine (NAla), N-(3-guanidinopropyl)glycine (NArg), N-(Carboxymethyl)glycine (NAsp), N-(carbamylmethyl)glycine (NAsn), N-(thioethyl)-glycine (NhCys), N-(2-carboxyethyl)glycine (NGlu), N-(2-carbamylethyl)glycine (NCl), N-(imidazolylethyl)glycine (NhHis), N-(1-methylpropyl)glycine (N1le), N-(2-methyl-propyl)glycine (NLeu), N-(4-aminobutyl)glycine (N1ys), N-(2-methylthioethyl)glycine (NMet), N-(hydroxyethyl)-glycine (NhSer), N-(2-hydroxypropyl)glycine (NhThr), N-(3-indolylmethyl)glycine (NTrp), N-(p-hydroxyphenethyl)-glycine (NTyr), N-(1-methylethyl)glycine (NVal).

All compounds of the invention may also be in cyclic form. A cyclic compound may have improved potency, stability, rigidity and/or other pharmaceutical and/or pharmacological characteristics.

In the combinations of the invention peptides having CHIPS activity may be combined with supportive non-peptides or with supportive peptides, whereas non-peptides having CHIPS activity may be combined with supportive peptides or supportive non-peptides or combinations thereof. In addition, both categories may be hybrid compounds (i.e. compounds in which not all proteinogenic amino acids are replaced by non-proteinogenic amino acids, D-amino acids or peptidomimetic building blocks).

The functional activity of compounds of the invention can be assayed by various methods. This CHIPS activity of a compound can be measured by its ability to prevent the binding of fluorescent-fMLP (such as Bodipy-fMLP) or fluorescent-C5a (such as FITC-C5a) to
neutrophils as determined by flow cytometry. CHIPS activity is also measured by its ability to prevent migration of neutrophils towards fMLP or C5a as determined by chemotaxis assays, such as the Transwell system. Furthermore, an assay based on the ability of chemokines, including fMLP and C5a, to initiate a rapid and transient rise in intracellular calcium concentration can be employed to screen for CHIPS activity, as described in Materials and Methods. Alternatively, an assay based on the ability of chemokines, including fMLP and C5a, to initiate an excretion of e.g. elastase in cytochalasin B-stimulated neutrophils, can be used to screen for CHIPS activity, as described in Materials and Methods. Various other assays known in the art can be used, including but not limited to the use of various calcium specific fluorescent probes in combination with flow cytometry or fluorometry, or microphysiometry. As cells for the screening of CHIPS activity by either method, e.g. freshly isolated neutrophils can be used or cells transfected with either FPR or C5aR, wild type or mutated forms of those receptors.

The supportive capacity of the other compound can be tested in the same manner as described above by comparing the CHIPS activity of the first compound having CHIPS activity alone with the CHIPS activity of the combination of the two compound. A compound has supportive capacity in case the CHIPS activity of the combination is higher than the CHIPS activity of the first compound alone.

Combinations of compounds of the invention may be useful in treating, preventing or ameliorating inflammatory conditions that are involved in many diseases and disorders.

The combination of compounds of the invention may for example be used in the treatment or prophylaxis of indications involving the C5a-receptor (C5aR) and/or formylated peptide receptor (FPR) on neutrophils, monocytes and endothelial cells. Such indications often
involves acute or chronic inflammation reactions, such as found in cardiovascular diseases, in particular arteriosclerosis, atherosclerosis, vasculitis, ischaemia reperfusion episodes, ischaemic shock, cardiopulmonary bypass, (large) vessel surgery, transplantation, myocardial infarction, diseases of the central nervous system, in particular bacterial meningitis, viral meningitis, multiple sclerosis, stroke, Alzheimer's disease, brain tumour, (traumatic) brain injury, gastrointestinal diseases, in particular pancreatitis, ulcerative colitis, Crohn's disease, alcoholic hepatitis, viral hepatitis, Helicobacter pylori gastritis, gastric carcinoma, peritonitis, skin diseases, in particular psoriasis, contact dermatitis, atopic dermatitis, and other dermatoses, cutaneous T-cell lymphoma, burns, genitourinary diseases, in particular urinary tract infection, glomerulonephritis, Trichomonas vaginalis infection, endometriosis, joint diseases, in particular rheumatoid arthritis, acute reactive arthritis, gout, respiratory diseases, in particular adult respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis, cystic fibrosis, asthma, pleural emphema, metal fume fever, bacterial pneumonia, chronic bronchitis, hypersensitivity pneumonia, Mycobacterium tuberculosis infection, viral respiratory tract infection, allergic rhinitis, sinusitis, bronchogenic carcinoma, and other diseases and indications, in particular periodontitis, chronic lymph leukemia, acute transplant rejection, glomerulonephritis, frost bite, repetitive strain injury (RSI), sepsis.


Furthermore, the combination of compounds of the invention may be used in the prophylaxis or treatment of indications involving CXCR4 and/or CCR5 receptors on leukocytes, in particular HIV infection.

The combinations of compounds of the invention are also useful in the treatment of indications involving
C5aR and/or FPR on cells other than neutrophils, monocytes and endothelial cells, in particular lymphocytes, dendritic cells, eosinophils, basophils, macrophages, microglia cells, astrocytes, Kupffer cells, hepatocytes and epithelial cells. An additional application is the use in prophylactic or therapeutic vaccines (to induce the production of anti-CHIPS antibodies to decrease the in vivo CHIPS activity, thereby stimulating the natural inflammation response against CHIPS producing bacteria) for infections with CHIPS-producing bacteria, such as Staphylococcus aureus.

Combinations of compounds of the invention may be used themselves as inhibitors of fMLP and C5a binding to their respective receptors FPR and C5aR, or to design inhibitors of CHIPS binding, by screening for competitive inhibition. This can be performed by various approaches, some of which are described hereunder. Inhibitors of CHIPS binding (to the putative CHIPS receptor or receptor domains) having CHIPS activity (CHIPS agonists) are also useful for treating inflammation conditions and HIV.

The invention furthermore relates to the use of the combination of compounds having CHIPS activity for the manufacture of a composition or preparation for prophylaxis or therapy, in particular for the prophylaxis or treatment of acute and chronic inflammation reactions and HIV infection, more in particular for the treatment of the indications listed above.

Also part of the present invention are therapeutic compositions comprising a suitable excipient and one or more of the combinations of compounds having CHIPS activity of the invention. Such composition can be used for the treatments as specified above.

The invention further relates to use of the combinations of compounds, wherein the separate compounds, such as peptides, are optionally incorporated in or fused to a larger protein, for various purposes, such as raising antibodies thereto, modulating the CHIPS activity or in a therapeutic preparation.
An antibody or biologically active fragment thereof specifically directed to one or both of the compounds in the combinations as claimed is also part of this invention. Such antibodies or biologically active fragments thereof like Fab, scFv fragments etc. can be used in the diagnosis, treatment or prophylaxis of infections of CHIPS producing bacteria, such as Staphylococcus aureus.

The information in the compounds of the combinations of the invention and the combinations of compounds themselves can be used to screen for other agents which are capable of interfering (e.g. through binding to CHIPS, thereby blocking the sites that CHIPS uses to bind to and/or activate its receptor or changing the CHIPS conformation such that the binding and/or activating capacity to, respectively of its receptor is impaired) with peptides having CHIPS activity. Such agents thus may act as inhibitors of CHIPS binding to and/or activation of its putative receptor, thereby improving for instance the neutrophil's activation and chemotactic capacities in the presence of combinations of compounds having CHIPS activity. Appropriate screening assays may for example use the labeled (e.g. fluorescent) purified CHIPS protein that binds to C5aR/FPR-bearing cells as analyzed by flow cytometry or fluorometry. A suitable binding assay may alternatively employ purified CHIPS receptor or receptor domain on a carrier with a form of CHIPS protein as ligand. Alternatively, an assay can be employed that screens for the ability to bind or compete with CHIPS for binding to a specific anti-CHIPS antibody (monoclonal, polyclonal, or single chain antibody) by various immunoassays known in the art, including but not limited to competitive and non-competitive ELISA techniques or Biosensor technology employing a sensor chip coated with either ligand (CHIPS), antibody or putative CHIPS receptor (Surface Plasma Resonance (SPR) technique like the BioCore). Any (poly)peptide having CHIPS activity other than CHIPS may
also be used in the screening assays described. All these methods can be adapted for High Throughput Screening (HTS).

Combinations of compounds interfering with CHIPS or with combinations of compounds of the invention are also part of this application. Such interfering compounds can be used in the diagnosis, treatment or prophylaxis of infections of CHIPS producing bacteria, such as *Staphylococcus aureus*.

The information in the combination of compounds and the combinations of compounds themselves can be used for other agents (including antibodies or fragments thereof) which are capable of interfering (e.g. through binding on the CHIPS receptor or changing the conformation of the CHIPS receptor) with the CHIPS receptor, without having (or only partial) CHIPS activity themselves. Such (partial) CHIPS antagonists may be found using appropriate screening assays. For example using fluorescent labeled purified CHIPS protein or peptide in various competitive CHIPS receptor binding and/or functional inhibiting screening assays, some of which have been mentioned above.

Combinations of compounds are claimed that interfere with the CHIPS receptor. Such antagonistic compounds can also be used in the treatment or prophylaxis of infections of CHIPS producing bacteria.

The CHIPS interfering compounds, anti-CHIPS antibodies or biologically active fragments thereof and chimerics, single chains, and expression libraries may be used to neutralize the activity of the CHIPS protein or related (poly)peptides in prophylaxis or therapy. Some of these may also be used for diagnostic purposes to bind CHIPS or related (poly)peptides. CHIPS receptor interfering compounds and such antibodies and such CHIPS interfering compounds that are all based on the combination of compounds of the invention, are for example useful for the prophylaxis or treatment of CHIPS
producing bacteria, such as *Staphylococcus aureus* infection.

Both the antibodies and their biologically active fragments and/or the CHIPS interfering compounds and/or the CHIPS receptor interfering compounds are useful for the preparation of a coating composition for use on surfaces of medical devices that are introduced into the human body through the skin or open surgical procedures, such as the surface of a catheter tip. In such cases the composition comprising the compounds that inhibit CHIPS activity or combinations thereof is a slow-release composition.

The invention further relate to therapeutic, diagnostic or prophylactic compositions comprising a suitable excipient and one or more antibodies and/or biologically active fragments thereof and/or one or more interfering compounds.

Also part of the invention are methods for prophylaxis or treatment of a subject suffering from indications involving C5aR and/or FPR on neutrophils, monocytes and endothelial cells comprising administering a therapeutically effective amount of the combinations of compounds of the invention. A further embodiment of the invention relates to a method for the prophylaxis or treatment of a subject suffering from indications involving CXCR4 and/or CCR5 receptors on lymphocytes comprising administering a therapeutically effective amount of the combinations of compounds of the invention. The indication to be treated is for example HIV infection.

Furthermore, the invention relates to methods for prophylaxis and treatment of a subject suffering from indications involving C5aR and/or FPR on leukocytes other than neutrophils, monocytes and endothelial cells comprising administering a therapeutically effective amount of the combinations of compounds of the invention. The other leukocytes are for example lymphocytes, dendritic cells, eosinophils, basophils or macrophages.
The invention further relates to amino acid sequences that can be identified by so-called "computer cloning". More specifically, this technique comprises of using the amino acid sequences of peptides used in the combinations of the invention, derivatives, and analogues thereof, as a query for screening protein sequences or protein sequence databases, using search algorithms that can identify regions with homology. Such algorithms are known to the person skilled in the art and include, but are not limited to, BLAST searches (Altschul et al., J. Mol. Biol. 215, 403-410 (1990)). The amino acid sequence database that may be searched include, but are not limited to, the Swissprot™ database. When using a BLAST search or modifications thereof, generally subjects that display homology can be identified. Identification is based on the value of the Score or the Smallest Sum Probability P(N).

The combination of compounds as claimed can also be used in a diagnostic kit for determining the presence of CHIPS in the body. The invention thus also relates to a diagnostic kit for determining the presence of CHIPS in the body, comprising a combination of compounds of the invention.

All compounds that are used in the combinations of the invention, i.e. peptides and non-peptides, may find various other applications. Such applications include, but are not limited to:
- Isolation of factors that can bind the above mentioned molecules. Examples of such factors being receptors and proteins. Such isolation can for instance be performed using the yeast two hybrid system or using tagged molecules of the invention as bait for fishing.
- Design of further peptidomimetics or small chemical compounds.
- Making phage display libraries, which can in turn be used for determining active domains, functional equivalents etc.
- Assay for determination of the biological CHIPS activity (chemotaxis inhibition or chemokine receptor expression or calcium flux)

All compounds that are used in the combinations of the invention can be labeled in any way. Examples of labeling include but are not limited to fluorescence, biotin, radioactive labeling etc. Such labeled molecules can be used for screening of compounds that resemble or overlap with the biological activity of CHIPS, as well as identification of binding sites, both in vivo and in vitro, and for tracing CHIPS protein in an organism.

The present invention will be further illustrated in the examples that follows and that is in no way intended to be limiting to this invention. In this description of the example, reference is made to the following figures and tables:

**Figure 1** shows the sequence of the chp gene from *S. aureus* Newman. The chp open reading frame (ORF) is underlined.

**Figure 2** shows the amino acid sequence deduced from the *S. aureus* Newman chp gene.

**Figure 3** shows the effect of the 5 amino acid peptide scan on the fMLP- (A) and PAF-induced (B) calcium mobilization in neutrophils.

**Figure 4** shows the concentration-dependent inhibition of the fMLP- and PAF-induced calcium mobilization in neutrophils by pep1-15.

**Figure 5** shows the specific effect of pep1-15 on the fMLP- C5a- and PAF-induced calcium mobilization in neutrophils.

**Figure 6** shows the effect of different lengths of CHIPS-derived peptides, ranging from pep1-5 to pep1-16 as depicted in Table 2, on the fMLP-induced elastase release in neutrophils.

**Figure 7** shows the effect of a one amino acid peptide scan, spanning the amino acids 1 to 22 of CHIPS as depicted in Table 2, on the fMLP-induced calcium mobilization in neutrophils.
Figure 8 shows the effect of the addition of small amino acids to the N-terminal phenylalanine of CHIPS-derived pep1-13 on the fMLP-induced calcium mobilization in neutrophils.

Figure 9 shows the effect of exchanging the first phenylalanine of pep 1-8 with non-proteinogenic amino acids, D-amino acids, peptidomimetic building blocks, and acetylation of the N-terminus on the fMLP-induced elastase release in neutrophils.

Figure 10A shows the effect of rCHIPS1-121 and rCHIPS4-121 on fMLP Bodipy binding.

Figure 10B shows the competition between rCHIPS1-121 and rCHIPS4-121 for CHIPS-FITC binding.

Figure 11 shows the structure of the various types of peptidomimetic building blocks.

Table 1 shows the sequences of 15 mer synthetic peptides derived from CHIPS that span the entire CHIPS protein.

Table 2 shows the sequences of synthetic peptides derived from CHIPS that vary in length from 1 to 40 (pep1-5 till pep1-40), that vary in length from 1 to 16 by one amino acid (pep1-5 till pep1-16), and a one amino acid peptide scan spanning amino acids 1 to 22 of CHIPS, and their effect on the fMLP-induced response in neutrophils (+).

Table 3 (page 1, 2, and 3) shows the sequences of CHIPS-derived peptide 1-10, containing all 20 available natural occurring amino acid exchanges at each position. Substitutions are in gray boxes and the substitutions generating the original pep1-10 are in bold.

Table 4A shows the summary of the pep1-10 single amino acids exchanges as determined by their effect on the fMLP-induced elastase release in neutrophils in a 100 μM concentration.

Table 4B shows the summary of the pep1-10 single amino acids exchanges as determined by their
effect on the fMLP-induced elastase release in neutrophils in a 10 µM concentration.

Table 5 shows the sequences of cyclic synthetic peptides derived from CHIPS that have two cysteine (C) substitutions in pep1-20 or pep1-10, and their effect on the fMLP-induced elastase release in neutrophils (+).

MATERIALS AND METHODS
1. Generating recombinant CHIPS
10 1.1 Sequence of the CHIPS-encoding gene (chp) of Staphylococcus aureus
DNA was sequenced by cycle sequencing on a DNA sequencer 4000 L (LI-COR Inc., Lincoln, Neb., USA) using the Thermo Sequenase™ fluorescent-labeled prime cycle sequencing kit (Amersham, Little Chalfont, UK). Suitable primers were used to directly sequence genomic DNA which was isolated according to J. Mamur (J. Mol. Biol., 3:208-218 (1961)). The sequencing method has been described briefly in Peschel et al. (J. Biol. Chem., 274:8405-8410 (1999)). To perform sequence similarity searches, the program BLAST 2.0 with the non-redundant protein database of the NCBI (Bethesda, Md., USA) was used. Sequence alignments were accomplished using the Higgins-Sharp algorithm of the program MacDNASIS Pro (Hitachi Software Engineering, San Bruno, Calif., USA).

Previously, the first 35 amino acids of CHIPS have been determined by N-terminal sequencing of the purified protein (PCT/NL99/00442). The S. aureus DNA is very rich in A and T nucleotides while G and C nucleotides are rare (only about 30% of total bases). Thus, for most amino acids, the most A- and T-rich codons are preferred. According to this rule, a primer sequence was derived from amino acids 15-24 (GAAAAAAGAAAAGCATATAAAG-AA (SEQ ID NO 1)). The primer was used to directly sequence genomic DNA from S. aureus Newman (a commonly used laboratory strain) yielding a sequence of several hundred base pairs. A new primer was
derived from this sequence to read toward the binding site of the first primer.

The combined DNA sequence contained the binding site of the first primer with two differences (G instead of A in position 3 and T instead of A in position 15). It encoded an open reading frame of 450 bp preceded by a reasonable Shine Dalgarno sequence for initiation of translation (J. Shine and L. Dalgarno, Proc. Natl. Acad. Sci. USA, 71:1342-1346 (1974)) and followed by three stop codons (Figure 1 with the gene underlined). The gene was named chp; it encodes a putative protein of 149 amino acids with no similarities to any protein in the databases. The N-terminal 28 amino acids seem to form a signal peptide for secretion across the cytoplasmic membrane (3 positively charged residues followed by a non-charged region of 22 amino acids and an ALA-X-ALA consensus motive for cleavage by the signal peptidase 1; Figure 5) (G. von Heijne, Nucl. Acids Res. 14:4683-4690 (1986)). The signal peptide is followed by a region that matches almost perfectly the N-terminal 35 amino acids of CHIPS. The only exception is a serine in position 33 of the deduced mature protein instead of an asparagine residue predicted by N-terminal sequencing. The deduced mature protein has a size of 121 amino acids and 14.1 kDa and an isoelectric point of 9.32. It thus fulfills all requirements for the CHIPS protein. The derived amino acid sequence is shown in Figure 2.

1.2. Generating recombinant CHIPS in E.coli

The DNA sequence for CHIPS from S.aureus is cloned into a suitable vector that enables efficient expression of CHIPS in competent E.coli host cells using conventional molecular biology techniques. The strategy used enables expression of the complete CHIPS protein linked to a removable HIS-tag at the N-terminus in the cytoplasm of E.coli. The trc Expression System (pTrcHIS B vector; Invitrogen) was used that enables expression of non-toxic proteins in E.coli. This system uses the trc
promotor for high-level, regulated expression in any
Escherichia coli strain with a multicloning vector. The vector
contains an N-terminal polyhistidine (6xHis) tag for
rapid purification, a Xpress epitope for easy detection
with an anti-Xpress antibody and an Enterokinase cleavage
site for removal of fusion tag. This leaves the purified
CHIPS protein with an N-terminal phenylalanine (F).

Staphylococcus aureus Newman chromosomal DNA was used as
template for the PCR reaction using Pwo-DNA polymerase
that results in a blunt ended PCR product. The primers
used are CHIPS-TTT (starts exactly with the first amino
acid of CHIPS (F) and CHIPS-TAA (containing a stop codon
and an EcoRI-site).

The PCR product is digested with EcoRI and the
pTrcHIS B vector with BamHI. The 5' overhang is removed
with S1-nuclease to make the BamHI site blunt ended
exactly where the enterokinase (EK) will digest the
protein. Thereafter the vector is digested with EcoRI and
ligated with the digested PCR product.

For transformation of the vector, TOP-10 E.coli
is used (Invitrogen) using standard calcium precipitation
(F.M. Ausubel et al., 1990, Current Protocols in
Molecular Biology, John Wiley and Sons, Inc., New York,
N.Y.). Clones are screened on Ampicillin containing
plates and proper ligation of CHIPS gene is verified by
sequencing of the isolated plasmid (clone29).

Once expressed, the E.coli bacteria are lysed
and the protein mixture is applied onto a Nickel-ion
affinity column (ProBond). Therefore a culture of clone29
in LB medium + 50 µg/ml Ampicillin is initiated with 1 mM
IPTG for 4 h at 37°C. Bacteria are centrifuged and the
pellet resuspended in cold Phosphate buffer pH 7.8 and
stored at in -20°C. For cell lysis, lysozyme (100 µg/ml)
is added for 15 min on ice, tubes are sonicated, frozen
in liquid N₂ and thawed in a 37°C waterbath. This cycle of
sonication/freeze/thaw is repeated another 3 times.
Thereafter RNase and DNase (5 µg/ml) is added for 30' on
ice. The mixture is centrifuged for at 3000 g for 30' at
4°C and filtered through a 0.45 μm filter. The final lysate is diluted 1:1 with cold Phosphate buffer pH 7.8 and run through a charged Nickel column (InvitroGen). The column is washed with Phosphate buffer pH 7.8, with Phosphate buffer pH 6.0 and with Phosphate buffer pH 5.3. The bound CHIPS is eluted with 500 mM imidazole in pH 6.0 Phosphate buffer.

The HIS-tag is removed by enterokinase cleavage followed by removal of the protease with an EK-Away enterokinase affinity resin. Therefor the eluate is dialysed overnight in cold digestion buffer (50 mM Tris-HCl, 1 mM CaCl₂ and 0.1% Tween20, pH 8.0), filtered through a 0.45 μm filter and digested with 0.175 μl Enterokinase/ml HIS-CHIPS product. This amount of Enterokinase is batch-dependent and results in a partial digestion to avoid the generation of breakdown products. The digested product is dialyzed against Phosphate buffer pH 7.8 and passed over a fresh Nickel column to eliminate uncleaved HIS-CHIPS; the run through is pure rCHIPS.

Undigested HIS-CHIPS can be eluted again from Nickel column for a second digestion round. The Nickel column is finally washed with 50 mM EDTA, 0.5 M NaOH, water, 5 mg/ml NiCl₂, water and stored in 20% ethanol.

All steps in the isolation and digestion of HIS-CHIPS are checked by SDS-PAGE on a 16.5% Tris-Tricine Ready gel using the Mini-ProteanII system (BioRad).

1.3. Production of recombinant CHIPS₄⁻¹²¹

When several E.coli colonies containing the plasmid with recombinant CHIPS were analyzed for proper insertion of the chp gene by sequencing, several incomplete insertions were found. One of them that contains the complete HIS-tag, enterokinase cleavage site and the CHIPS protein minus the first three amino acids (CHIPS₄⁻¹²¹; clone 19) was further propagated and purified as described above for complete CHIPS.
2. Synthesis of CHIPS-derived peptides

2.1 Method one.

Peptides were prepared by automated simultaneous multiple peptide synthesis, set up by using a standard autosampler (Gilson 221). Standard 9-fluorenylmethoxycarbonyl chemistry with in situ PyBop/N-methylmorpholine (Novabiochem, Laufelfingen, Switzerland) activation of the amino acids in a fivefold molar excess with respect to 2 μmol/peptide PAL-PEG-PS resin (Perseptive Biosystems, Framingham, Mass.) was used. Peptides were obtained as C-terminal amides after cleavage with 90 to 95% trifluoroacetic acid-containing scavenger cocktails. Most peptides were dissolved in distilled H₂O to a concentration of 1 to 3 mM; others were dissolved in dimethyl sulfoxide. References describing similar methods are:


2.2 Method two

Synthesis of CHIPS derived peptides was performed on TGT resin (5 g, 0.3 mmol, NovaBiochem) and transferred to peptide synthesizer, were a solution of piperidine (12 ml) in dimethylformamide (DMF; 18 ml) was added to the resin. The solution was swirled for 1 hour and the resin washed with DMF (3 x 30 ml) followed by dichloromethane (DCM; 3 x 30 ml) and allowed to dry under vacuum for 5 minutes. The remainder of the peptides was sequentially assembled employing standard Fmoc chemistry. Cleavage of the peptides was accomplished by treating the protein resin with a solution of trifluoroacetic acid/triisopropylsilane/H₂O [90:8:2 v/v/v] for 2.5 hours. The crude product was isolated by ether precipitation followed by purification by using High Performance Liquid
Chromatography. References describing similar methods are:

2.3 Method three

Na-Fmoc-protected amino acids were purchased from Alexis (Switzerland). For the Na-Fmoc-protected amino acids, the following side chain protecting groups were used: Tyr(t-Bu), Lys(Boc), Thr(t-Bu), Ser(t-Bu), Asn(Trt), Glu(0t-Bu) and Phe(p-NHBoc). The oligomers were synthesized on ArgoGel™ Rink-NH-Fmoc resin, 0.32 mmol/g (Argonaut Technologies, Muttenz, Switzerland). Peptide grade NMP, DCE, and TFA and HPLC-grade CH₂CN, MTBE, and n-hexane were purchased from Biosolve B.V. (Valkenswaard, The Netherlands). All other solvents and reagents were acquired from commercial sources and used without further purification.


The oligomers were synthesized on a MultiSynTech Syro II Robot Synthesizer (MultiSynTech, Witten, Germany). HPLC runs were performed on two HPLC systems: a Gilson automated HPLC workstation with UV detector system operating at 214 nm and 278 nm, or a Shimadzu automated HPLC system with a UV detector system operating at the same wavelengths. Analytical HPLC was conducted by using an Alltech Adsorbosphere XL C18 (300 Å, 5 μm, 250 x 4.6 mm) column at a flow rate of 1.0 mL
min-1. Elution was effected with a gradient from 0.1% TFA in water/acetonitrile (90/10) to 0.1% TFA in acetonitrile/water (90/10) over 40 minutes. Electrospray ionization mass spectrometry (ESI-MS) was measured on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode. For the MS full scan spectra, data were acquired in continuum mode over the range m/z 200 to 1500 in 1 second at unit mass resolution. A deflector voltage of 50 V was set, which produces predominantly (M+H)+ ions. Instrumental control, data acquisition and data processing were carried out using the CLASS 8000 software package (version 1.10). The observed average m/z was compared with the calculated (M+H)+ average mass.

The oligomers were synthesized on 0.05 mmol scale in a polypropylene reaction tube equipped with a polypropylene frit. The syntheses were carried out on ArgoGel™ Rink-NH-Fmoc resin (0.32 mmol/g) to obtain C-terminal amides. The synthesis started with 156 mg dry resin. The resin was swollen in DCE (two times with 2 mL for 4 min) followed by NMP (two times with 2 mL for 4 min). The Fmoc group was removed by a double treatment with 2 mL 20% piperidine in NMP during 8 min. Subsequently, the resin was extensively washed with NMP (five times with 2.5 mL for 2 min). To this deprotected resin, 1 mL of a 0.2 M Fmoc-protected N-substituted glycine monomer or Fmoc-protected amino acid solution in NMP was added followed by 0.75 mL of a 0.267 M solution of HBTU/HOBt in NMP and 0.5 mL of a 0.4 M solution of DiPEA in NMP. After 45 min, the reaction tube was drained and the resin was washed with NMP (five times with 2.5 mL for 2 min). During the resin swelling and washing steps, Fmoc deprotection and coupling reactions, the resin suspension was magnetically mixed by levitation stirring for 15 s per min. After the final Fmoc removal the resin was washed with NMP (five times with 2.5 mL for 2 min) and DCE (three times with 2.5 mL for 2 min). The anchored oligomers thus obtained were cleaved from the
resin and deprotected by treatment with 2 mL TFA/H₂O/TIS (95:2.5:2.5, v/v/v) solution for 2 h at room temperature. The oligomers were precipitated with MTBE/n-hexane (1:1, v/v, 50 mL). The precipitate was collected by centrifugation (3000 rpm, 5 min), the supernatant was decanted, and the pellet was resuspended in MTBE/n-hexane (1:1, v/v) and centrifuged again. Subsequently, the pellet was washed two times with MTBE (50 mL). After this, the pellet was dissolved in tert.-BuOH/water (1:1, 10 v/v, 5-10 mL) and lyophilized to obtain the oligomers as a white fluffy solid. The purity was analyzed by analytical HPLC and the hybrids were characterized by mass spectrometry.

3. Assays for CHIPS activity.

3.1 Calcium mobilization assay

The effect of CHIPS and the synthetic peptides on the intracellular free calcium release induced by fMLP, C5a and PAF (Platelet Activating Factor) in neutrophils was tested as follows. Cells were loaded with 1 μM of a calcium specific intracellular probe (Fluo-3, acetoxyethyl (AM) ester; Molecular Probes), washed and incubated with various concentrations of synthetic peptides (1 to 100 μM) or rCHIPS (1 μg/ml) for 15 min at room temperature. From each sample the initial fluorescence value was determined in the FACScan by measuring 2000 cells. Subsequently, stimulus was added (10⁻⁹ M fMLP (Sigma), 10⁻¹⁰ M rC5a (Sigma) or 10⁻¹⁰ M PAF (Calbiochem)) and the fluorescence intensity from the same sample was determined exactly 15 seconds after administration of the stimulus (the optimal time point for all agonists). Triggering neutrophils with fMLP, C5a or PAF initiates a rapid and transient increase in free intracellular calcium concentration that is measured by an increase Fluo-3 fluorescence signal. From each activated sample, the initial basal fluorescence value is subtracted. Results are expressed as a percentage of
buffer treated cells stimulated with either fMLP, C5a or PAF.

3.2 Elastase release assay.

Human neutrophils contain enzymes in their granules, among which elastase. Furthermore, the granules contain the fMLP receptors ready for quick recruitment to the cell surface. Cytochalasin B treatment of neutrophils stimulates the excretion of the granules, which results in an increase of the amount of fMLP receptors expressed on the cell surface of the neutrophils. Upon subsequent stimulation with fMLP, the neutrophils will effectively excrete their granule content into the medium, thereby releasing elastase. CHIPS and CHIPS peptides will inhibit the activation of the neutrophils with fMLP, which can be measured via a decrease in elastase release. The amount of elastase is determined via a specific enzymatic reaction using the fluorescent substrate methoxysuccinyl-L-Ala-L-Ala-L-Pro-L-Val-MAC (elastase substrate V; Calbiochem).

In a round bottom microtiterplate neutrophils (1x10^4 per well) were incubated for 15 min with 5 µg/ml cytochalasin B together with rCHIPS (1 µg/ml) or CHIPS-derived peptides (0.4 to 100 µM) at room temperature. Subsequently, fMLP (1x10^8M) was added. After 1 h incubation at 37°C, the microtiterplate was centrifuged and fluorescent substrate (250 µM) was added to each well. The elastase response was measured for 30 min at 37°C in a FluostarII microtiter fluorometer. Results are expressed as a percentage of cytochalasin B- and buffer-treated cells stimulated with fMLP after substraction of the values for non-stimulated cells.
EXAMPLES

EXAMPLE 1

Identification of the active side within the CHIPS protein

1.1 Scan of the CHIPS protein by overlapping peptides.

The sequence of CHIPS (amino acids 1 to 121) was divided into 22 different 15-mer peptides that progressed along the CHIPS sequence by initiating a new peptide every sixth amino acid as shown in Table 1. Peptides were prepared according to method one (MATERIALS AND METHODS section 2.1).

Figure 3 shows the effect of these peptides spanning the CHIPS on the fMLP- (A) and PAF-induced (B) calcium mobilization in neutrophils. It can be seen that pep1-15 and pep61-75 strongly inhibit the fMLP-induced calcium mobilization in neutrophils. The effect of pep61-75 seems to be non-specific for fMLP, as it also inhibits the PAF-induced response. This might be due to a non-specific toxicity of this peptide. Concluding, only pep1-15 shows CHIPS activity. Figure 4 shows the concentration-dependent inhibition of the fMLP-induced calcium mobilization by pep1-15. Figure 5 shows the effect of pep1-15, on the fMLP-, C5a- and PAF-induced calcium mobilization in neutrophils and confirms the specificity of pep1-15 and rCHIPS for the fMLP- and C5a-induced response.

1.2 Optimal length for the active site.

To verify and optimize the effective length for the active CHIPS peptide, peptides of increasing length spanning amino acid 1 to 40 were chosen all starting with the first phenylalanine (see Table 2), including newly synthesized pep1-15. Peptides were prepared according to method two (MATERIALS AND METHODS section 2.2).

Table 2 shows the effect of different lengths of CHIPS-derived peptides on the fMLP-induced calcium mobilization in neutrophils. This table shows that at least the first 8 amino acids are necessary for CHIPS
activity, as pep1-5 shows no CHIPS activity in contrast to pep1-8 and all other depicted longer peptides. No improved activity of longer peptides was observed using different concentrations.

To redefine the exact minimal length for the active CHIPS peptide, highly purified (>95% pure) peptides were synthesized starting with pep1-5 with a one amino acid elongation until pep1-12. Peptides were prepared according to method three (MATERIALS AND METHODS section 2.3).

Figure 6 shows the effect of different lengths of CHIPS-derived peptides, as depicted in Table 2, and their effect on the fMLP-induced elastase release in neutrophils. This Figure shows that at least the first 6 amino acids are necessary for CHIPS activity, as pep1-5 shows no CHIPS activity in contrast to pep1-6 and all other depicted longer peptides.

1.3 Crucial role for the first phenylalanine.

The first 22 amino acids of CHIPS were divided into 8 different 15-mer peptides that progressed along the CHIPS sequence by initiating a new peptide every next amino acid. Peptides were prepared according to method one (MATERIALS AND METHODS section 2.1).

Figure 7 shows the effect of the one amino acid peptide scan, as depicted in Table 2, spanning the amino acids 1 to 22 of CHIPS, on the fMLP-induced calcium mobilization in neutrophils. It can be seen that the first phenylalanine plays a crucial role in the CHIPS activity since only pep1-15 is active while all others are inactive.

1.4 Requirement for a non-blocked first phenylalanine.

To determine the essence for a free available N-terminal phenylalanine, peptides were constructed spanning the amino acids 1 to 13 of CHIPS with an additional amino acid before the first phenylalanine.
Peptides were prepared according to method one (MATERIALS AND METHODS section 2.1).

Figure 8 shows the effect of the peptides spanning CHIPS 1 to 13 with additional N-terminal amino acids attached on the fMLP-induced calcium mobilization in neutrophils. It can be seen that blocking the CHIPS N-terminal phenylalanine with the chosen small amino acids Ala (alanine), Gly (glycine), Pro (proline), Ser (serine), Thr (threonine) or Val (valine) completely abrogated effective inhibition of the fMLP-induced calcium mobilization by the non-blocked pepl-15. The PAF-induced response is not affected by any peptide nor rCHIPS.

EXAMPLE 2

Modifications of the active site

2.1 Proteinogenic amino acid substitutions.

Substitutions with all 20 natural occurring amino acids were done in the CHIPS-derived peptide comprising of the first 10 amino acids of CHIPS. Each of the 10 amino acids were consecutively replaced with all 20 natural occurring amino acids, being A (alanine), C (cysteine), D (aspartic acid), E (glutamic acid), F (phenylalanine), G (glycine), H (histidine), I (isoleucine), K (lysine), L (leucine), M (methionine), N (asparagine), P (proline), Q (glutamine), R (arginine), S (serine), T (threonine), V (valine), Y (tyrosine), W (tryptophan), as shown in Table 3. Peptides were prepared according to method two or three (MATERIALS AND METHODS section 2.2 and 2.3). The peptides were tested in the elastase release assay in a concentration of 100 μM (optimal) as well 10 μM (suboptimal) to find the most potent combinations. Individual substitutions also resulted in the original pepl-10 that were indeed positive for each newly synthesized peptide (in bold). Tables 4A (100 μM) and 4B (10 μM) summarizes the positive single amino acid substitutions in pepl-6 (the minimal active part of CHIPS).
The results from the single amino acid substitutions (Table 4B) lead to a new set of possible amino acid substitutions as listed in the description resulting in \(2 \times 4 \times 1 \times 7 \times 7 \times 6 = 2352\) new combinations. To synthesize and test all those new peptides for CHIPS activity, 200 individual combinations are synthesized chosen at random from the 2352 possibilities as examples for the total combinations.

2.2 Non-proteinogenic amino acid substitutions.

Non-proteinogenic, natural occurring (meaning non-human) amino acid substitutions were performed on the CHIPS-derived peptide comprising of the first 8 amino acids of CHIPS. Peptides were prepared according to method three (MATERIALS AND METHODS section 2.3).

The first phenylalanine (Phe) of pep1-8 was replaced by the structurally related, non-proteinogenic amino acids Nal(2) 2-naphthylalanine), Cha (cyclohexylalanine), Phe(p-NH\(_2\)) (p-amino-phenylalanine) and Bpa (p-phenylalanine). Figure 9 shows the effect of these exchanges on the fMLP-induced elastase release in neutrophils. These results show that pep1-8, in which the first phenylalanine is exchanged for Cha or Bpa still shows considerable CHIPS activity. Exchange for Nal(2) results in a somewhat less active pep1-8, whereas exchange for Phe(p-NH\(_2\)) strongly reduces the CHIPS activity of pep1-8.

2.3 D-amino acid substitutions.

D-amino acid (in contrast to the natural L-isoform) substitutions were performed on the CHIPS-derived peptide comprising of the first 8 amino acids of CHIPS. Peptides were prepared according to method three (MATERIALS AND METHODS section 2.3).

The first phenylalanine (Phe) of pep1-8 was replaced by D-Phe and tested in the fMLP-induced elastase release in neutrophils. Exchange for D-Phe results in a somewhat less active pep1-8 as shown in Figure 9.
2.4 Peptidomimetic substitutions.

Peptidomimetic substitutions were performed on the CHIPS-derived peptide comprising of the first 8 amino acids of CHIPS. Peptides were prepared according to method three (MATERIALS AND METHODS section 2.3).

The first phenylalanine (Phe) of pep1-8 was replaced by the peptidomimetic oligomer Nphe (N-Benzylglycine) and tested in the fMLP-induced elastase release in neutrophils. Exchange for NPhe results in an equally active pep1-8 as shown in Figure 9.

2.5 N-terminal modifications.

Chemical modifications (with a small molecular mass) on the N-terminal phenylalanine (Phe) were performed on the CHIPS-derived peptide comprising of the first 8 amino acids of CHIPS. Peptides were prepared according to method three (MATERIALS AND METHODS section 2.3).

The first phenylalanine (Phe) of pep1-8 was acylated by the introduction of an N-terminal acetyl group (Ac-Phe). This peptide was tested in the fMLP-induced calcium mobilization in neutrophils and showed diminished CHIPS activity of pep1-8 as shown in Figure 9.

EXAMPLE 3

Cyclisation of the peptides having CHIPS activity

3.1 Cysteine cyclisation.

Two cysteine residues were substituted into the peptide comprising of amino acids 1 to 20 (pep1-20) and 1 to 10 (pep1-10) of CHIPS, to enable these peptides to become cyclic. The position of the 2 cysteine residues varies as is depicted in Table 5. Peptides were prepared according to method two (MATERIALS AND METHODS section 2.2).

Table 5 shows the sequences of the CHIPS-derived peptides 1-20 and 1-10, each containing 2 cysteine residues to enable the formation of cyclic peptides, and
their effect on the fMLP-induced elastase release in neutrophils (+).

EXAMPLE 4

5 Competition for CHIPS binding to its putative receptor

Competition with CHIPS-FITC binding

In Falcon tubes 5 µl serial dilutions of combinations of compounds of the invention were prepared and mixed with 5 µl CHIPS-FITC (10 µg/ml). Thereafter 50 µl isolated neutrophils at 5 x 10⁶ cells/ml are added and incubated for 30 min on ice. Cells are washed and analyzed for CHIPS-FITC binding by flow cytometry.

EXAMPLE 5

15 Indication for an additional binding site within the CHIPS protein

5.1 Assay for CHIPS activity: fMLP-Bodipy binding.

Granulocytes are isolated from heparinized blood of healthy volunteers via a Histopaque-Ficoll gradient in accordance with the standard method (Troelstra et al., J. Leukocyte Biol. 61, 173-178 (1997)). The remaining erythrocytes in the granulocyte fraction are lysed with sterile water (for 30 sec.) and washed after recovery of the isotonicity. The cells are finally resuspended in PRMI (Gibco) with 0.05% Human Serum Albumin (RPMI/HSA). In Falcon tubes 50 µl cells (5 x 10⁶ cells/ml) are incubated with 5 µl CHIPS (10-fold concentrated) for 15 min at room temperature. The cells are placed on ice and washed once with RPMI/HSA (at 4°C) and resuspended in 50 µl fresh medium. 5 µl BODIPY-labeled fMLP (final concentration 0.1 µM; Molecular Probes) is then added and the sample is incubated for 60 minutes on ice. RPMI/HAS is added to a final volume of 200 µl and the fluorescent fMLP binding to the granulocytes is analysed with a flow cytometer (FACScan; Becton Dickinson). The average fluorescence value of 5000 granulocytes is calculated with LysisII software (Becton Dickinson).
RESULTS

Figure 10A shows the effect of CHIPS on the binding of fMLP-Bodipy to the cells. The complete rCHIPS\textsuperscript{1-121} abrogates concentration dependent the binding of fMLP-Bodipy. This is the characteristic feature of CHIPS activity. In contrast the mutant rCHIPS\textsuperscript{4-121} in the same concentration range tested did not affect the binding of fMLP-Bodipy to the cells, indicating this mutant has no CHIPS activity.

5.2 Competition for CHIPS-FITC binding.

In Falcon tubes 5 \( \mu l \) CHIPS-FITC (20 \( \mu g/ml \)) is incubated with various concentrations unlabeled rCHIPS or mutant rCHIPS\textsuperscript{4-121} for 15 min at room temperature. Subsequently, 50 \( \mu l \) granulocytes (5 x 10\textsuperscript{6} cells/ml) are added to the tubes and incubated for 60 min on ice. Cells are washed once with medium (RPMI containing 0.05% HSA) and resuspended in 200 \( \mu l \) fresh medium. Binding of CHIPS-FITC to the leukocyte is measured by flow cytometry (FACScan; Becton Dickinson). The average fluorescence value of the cells is calculated with LysisII software (Becton Dickinson).

RESULTS

Figure 10B shows the concentration dependent inhibition of CHIPS-FITC binding by both the complete rCHIPS\textsuperscript{1-121} as well as the mutant rCHIPS\textsuperscript{4-121}. Both preparations show a similar inhibition pattern with equal effective concentrations. This indicates that the mutant rCHIPS\textsuperscript{4-121} still can bind to the cells, but lacks the intrinsic CHIPS activity (Figure 10A). These results point to a multi-site model for CHIPS that involves one or more supportive sites (in the 4 to 121 part of CHIPS) and a activity site that comprises the first 6 amino acids as shown with the peptides.
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### Table 2

#### Varying lengths

| P 1-40 | + FTFEPFPPTNEEIESNKKMLEKEKAYKESFKNSGLPTTLGK |
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| P 1-30 | + FTFEPFPPTNEEIESNKKMLEKEKAYKESFK          |
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| P 1-23 | + FTFEPFPPTNEEIESNKKMLEK                   |
| P 1-20 | + FTFEPFPPTNEEIESNKKMLE                    |
| P 1-18 | + FTFEPFPPTNEEIESNKKM                     |
| P 1-15 | + FTFEPFPPTNEEIESN                   |
| P 1-13 | + FTFEPFPPTNEEIE            |
| P 1-10 | + FTFEPFPPTNE           |
| P 1-8  | + FTFEPFPFPT          |
| P 1-5  | - FTFEP                           |

#### Varying lengths

| P 1-5  | - FTFEP                           |
| P 1-6  | + FTFEPF                          |
| P 1-7  | + FTFEPFP                         |
| P 1-8  | + FTFEPFPPT                       |
| P 1-9  | + FTFEPFPPTN                      |
| P 1-10 | + FTFEPFPPTNE                      |
| P 1-11 | + FTFEPFPPTNEE                     |
| P 1-12 | + FTFEPFPPTNEEI                    |
| P 1-13 | + FTFEPFPPTNEEI                    |
| P 1-14 | + FTFEPFPPTNEIEES                  |
| P 1-15 | + FTFEPFPPTNEEIES                 |
| P 1-16 | + FTFEPFPPTNEIESN                 |

#### 1 aa scan

| P 1-15 | + FTFEPFPPTNEEIESN           |
| P 2-16 | - FTFEPFPPTNEEIESNK         |
| P 3-17 | - FEPFPPTNEEIESNKK          |
| P 4-18 | - EPFPPTNEEIESNKKKM         |
| P 5-19 | - PFPTNEEIESNKKML           |
| P 6-20 | - FPTNEEIESNKKMLE           |
| P 7-21 | - PNEEIESNKKMLEK            |
| P 8-22 | - TNEEIESNKKMLEKE           |
Table 3 page 1/3

| p 1-10 subst. | p 1-10 A10 | p 1-10 C10 | p 1-10 D10 | p 1-10 E10 | p 1-10 F10 | p 1-10 G10 | p 1-10 H10 | p 1-10 I10 | p 1-10 J10 | p 1-10 K10 | p 1-10 L10 | p 1-10 M10 | p 1-10 N10 | p 1-10 P10 | p 1-10 Q10 | p 1-10 R10 | p 1-10 S10 | p 1-10 T10 | p 1-10 U10 | p 1-10 V10 | p 1-10 W10 | p 1-10 X10 | p 1-10 Y10 | p 1-10 Z10 | p 1-10 D9 | p 1-10 E9 | p 1-10 F9 | p 1-10 G9 | p 1-10 H9 | p 1-10 I9 |
|---------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
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SINGLE amino acid substitutions showing CHIPS activity as tested at 100uM peptide
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SINGLE amino acid substitutions showing CHIPS activity as tested at 10 uM peptide
### Table 5

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**Cyclic peptides**

- **P1-20 C1C20**: + C T F E P F F P T N E E I E S N K K M L C
- **P1-20 C3C20**: - F T C E P F F P T N E E I E S N K K M L C
- **P1-20 C5C20**: - F T F E C F F P T N E E I E S N K K M L C
- **P1-20 C7C20**: + F T F E P F C T N E E I E S N K K M L C
- **P1-20 C9C20**: + F T F E P F P T C E E I E S N K K M L C
- **P1-20 C11C20**: + F T F E P F P T N E C I E S N K K M L C
- **P1-20 C13C20**: + F T F E P F P T N E E I E S N K K M L C
- **P1-20 C15C20**: + F T F E P F P T N E E I E S N K K M L C
- **P1-20 C17C20**: + F T F E P F P T N E E I E S N K K M L C
- **P1-20 C1C18**: - C T F E P F P T N E E I E S N K K C L E
- **P1-20 C1C16**: - C T F E P F P T N E E I E S N C K M L E
- **P1-20 C1C14**: - C T F E P F P T N E E I E C N K K M L E
- **P1-20 C1C12**: - C T F E P F P T N E E C E S N K K M L E
- **P1-20 C1C10**: - C T F E P F P T N C E I E S N K K M L E
- **P1-20 C1C8**: + C T F E P F P C N E E I E S N K K M L E
- **P1-20 C1C6**: - C T F E P C P T N E E I E S N K K M L E
- **P1-20 C1C4**: - C T F C P F P T N E E I E S N K K M L E
- **P1-10 C1C10**: + C T F E P F P T N C
- **P1-10 C3C10**: ND F T C E P F F P T N C
- **P1-10 C5C10**: + F T F E C F F P T N C
- **P1-10 C7C10**: ND F T F E P F C T N C
- **P1-10 C1C8**: ND C T F E P F P C N E
- **P1-10 C1C6**: + C T F E P C P T N E
- **P1-10 C1C4**: ND C T F C P F P T N E
CLAIMS

1. Combination of compounds, wherein one compound has CHIPS activity and consisting of at least 6 building blocks the sequence of which is based on the N-terminal part of the CHIPS molecule, and which sequence may comprise one or more substitutions as compared to the original CHIPS amino acid sequence, whereas the one or more other compounds are derived from the amino acids 4-121 of CHIPS and supports CHIPS activity.

2. Combination of compounds as claimed in claim 1, wherein one or more of the amino acids in the amino acid sequence of the one or more supportive compounds are replaced by building blocks selected from the group consisting of proteinogenic amino acids, non-proteinogenic amino acids, D-amino acids and peptidomimetic building blocks.

3. Combination of compounds as claimed in claim 1 or 2, wherein the compound having CHIPS activity has the sequence:

$$X_1-X_2-X_3-X_4-X_5-X_6-X_7$$

wherein the building blocks $X_1$, $X_2$, $X_3$, $X_4$, $X_5$, $X_6$ and $X_7$ are selected from the group consisting of proteinogenic amino acids, non-proteinogenic amino acids, D-amino acids and peptidomimetic building blocks and wherein $X_7$ may or may not be present and may represent either one or more building blocks.

4. Combination of compounds as claimed in claim 3, wherein in the compound having CHIPS activity $X_1$ may be modified with a group that is smaller than an amino acid and which is selected from acyl moieties, such as acetyl and formyl, and urea moieties such as carbamoyl, or by reductive alkylation.

5. Combination of compounds as claimed in claim 3 and 4, wherein the building blocks of the compound having CHIPS activity are proteinogenic amino acids and
X1 is selected from phenylalanine (F), cysteine (C), asparagine (N), tryptophan (W);
X2 is selected from alanine (A), cysteine (C), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), leucine (L), methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), valine (V), tryptophan (W), tyrosine (Y);
X3 is selected from phenylalanine (F), tryptophan (W);
X4 is selected from alanine (A), cysteine (C), aspartic acid (D), glutamic acid (E), leucine (L), asparagine (N), methionine (M), serine (S), tryptophan (W);
X5 is selected from phenylalanine (F), isoleucine (I), leucine (L), proline (P), valine (V), tryptophan (W), tyrosine (Y);
X6 is selected from cysteine (C), phenylalanine (F), histidine (H), isoleucine (I), tryptophan (W), tyrosine (Y).

6. Combination of compounds as claimed in claims 3-5, wherein the building blocks of the compound having CHIPS activity are proteinogenic amino acids and
X1 is selected from phenylalanine (F), tryptophan (W);
X2 is selected from phenylalanine (F), isoleucine (I), serine (S), threonine (T);
X3 is phenylalanine (F);
X4 is selected from alanine (A), cysteine (C), aspartic acid (D), glutamic acid (E), asparagine (N), serine (S), tryptophan (W);
X5 is selected from phenylalanine (F), isoleucine (I), leucine (L), proline (P), valine (V), tryptophan (W), tyrosine (Y);
X6 is selected from cysteine (C), phenylalanine (F), histidine (H), isoleucine (I), tryptophan (W), tyrosine (Y).
7. Combination of compounds as claimed in claims 1-6, wherein the compound having CHIPS activity has the sequence:

F-T-F-E-P-F-X7,

wherein one or more of the amino acids on positions 1 to 6 are substituted with the building blocks as defined in claim 3, 5 or 6, and wherein X7 is as defined in claim 3 and may or may not be present.

8. Combination of compounds as claimed in claims 1-7, wherein the compound having CHIPS activity has the sequence:

X1-T-F-E-P-F-X7,

wherein X1 is as defined in claim 3, 4, 5 or 6 and X7 is as defined in claim 3 and may or may not be present.

9. Combination of compounds as claimed in claims 1-7, wherein the compound having CHIPS activity has the sequence:

F-X2-F-E-P-F-X7,

wherein X2 is as defined in claim 3, 5 and 6 and X7 is as defined in claim 3 and may or may not be present.

10. Combination of compounds as claimed in claims 1-7, wherein the compound having CHIPS activity has the sequence:

F-T-X3-E-P-F-X7,

wherein X3 is as defined in claim 3, 5 or 6 and X7 is as defined in claim 3 and may or may not be present.

11. Combination of compounds as claimed in claims 1-7, wherein the compound having CHIPS activity has the sequence:

F-T-F-X4-P-F-X7,

wherein X4 is as defined in claim 3, 5 or 6 and X7 is as defined in claim 3 and may or may not be present.

12. Combination of compounds as claimed in claims 1-7, wherein the compound having CHIPS activity has the sequence:

F-T-F-E-X5-F-X7,

wherein X5 is as defined in claim 3, 5 or 6 and X7 is as defined in claim 3 and may or may not be present.
13. Combination of compounds as claimed in claims 1-7, wherein the compound having CHIPS activity has the sequence:

F-T-F-E-P-X6-X7,

wherein X6 is as defined in claim 3, 5 or 6 and X7 is as defined in claim 3 and may or may not be present.

14. Combination of compounds as claimed in claim 3, wherein the compound having CHIPS activity has the sequence:

F-T-F-E-P-F-X7.

wherein X7 is as defined in claim 3 and may or may not be present.

15. Combination of compounds as claimed in claims 1-7, wherein the compound having CHIPS activity belongs to the group listed in Table 6.

16. Combination of compounds as claimed in claim 15, wherein the compound having CHIPS activity further comprises a group X7 at the C-terminal end thereof, wherein X7 is as defined in claim 3.

17. Combination of compounds as claimed in claims 3-16, wherein in the compound having CHIPS activity and/or in the supportive compound one or more of the proteinogenic amino acids is replaced by a non-proteinogenic amino acid.

18. Combination of compounds as claimed in claim 17, wherein the non-proteinogenic amino acid is selected from the group consisting of 2-naphtylalanine (Nal(2)), β-cyclohexylalanine (Cha), p-amino-phenylalanine ((Phe(p-NH₃), p-benzoyl-phenylalanine (Bpa), ornithine (Orn), norleucine (Nle), 4-fluoro-phenylalanine (Phe(p-F)), 4-chloro-phenylalanine (Phe(p-Cl)), 4-bromo-phenylalanine (Phe(p-Br)), 4-iodo-phenylalanine (Phe(p-I)), 4-methyl-phenylalanine (Phe(p-Me)), 4-methoxy-phenylalanine (Tyr(Me)), 4-nitro-phenylalanine (Phe(p-NO₂)).

19. Combination of compounds as claimed in claims 3-18, wherein in the compound having CHIPS
activity and/or in the supportive compound one or more of
the amino acids is replaced by a D-amino acid.

20. Combination of compounds as claimed in
claim 19, wherein the D-amino acid is selected from the
group consisting of D-phenylalanine, D-alanine, D-
arginine, D-asparagine, D-aspartic acid, D-cysteine, D-
glutamic acid, D-glutamine, D-histidine, D-isoleucine, D-
leucine, D-lysine, D-methionine, D-proline, D-serine, D-
threonine, D-tryptophan, D-tyrosine, D-valine, D-2-
naphtylalanine (D-Nal(2)), β-cyclohexyl-D-alanine (D-
Cha), 4-amino-D-phenylalanine (D-Phe(p-NH₂)), p-benzoyl-D-
phenylalanine (D-Bpa), D-Ornithine (D-Orn), D-Norleucine
(D-Nle), 4-fluoro-D-phenylalanine (D-Phe(p-F)), 4-chloro-
D-phenylalanine (D-Phe(p-Cl)), 4-bromo-D-phenylalanine
(D-Phe(p-Br)), 4-iodo-D-phenylalanine (D-Phe(p-I)), 4-
methyl-D-phenylalanine (D-Phe(p-Me)), 4-methoxy-D-
phenylalanine (D-Tyr(Me)), 4-nitro-D-phenylalanine (D-
Phe(p-NO₂)).

21. Combination of compounds as claimed in
claims 3-20, wherein in the compound having CHIPS
activity and/or in the supportive compound one or more of
the amino acids is replaced by a peptidomimetic building
block selected from the group consisting of oligo-β-
peptides, oligosulfonamides, vinyllogous sulfonamides,
ydrazinepeptide/azatides, oligocarbamates, ureapeptoids,
oligoureia, phosphodiesters, peptoids, oligosulfones,
peptoid sulfonamides, vinyllogous peptides.

22. Combination of compounds as claimed in
claim 21, wherein the peptidomimetic building block is
selected from the group consisting of N-substituted
glycines, such as N-benzylglycine (NPh), N-methylglycine
(NAla), N-(3-guanidinopropyl)glycine (NArg), N-
(Carboxymethyl)glycine (NASp), N-(carbamylmethyl)glycine
(NAsn), N-(thioethyl)-glycine (NhCys), N-(2-
carboxyethyl)glycine (NGlu), N-(2-carbamylethyl)glycine
(NGln), N-(imidazolylethyl)glycine (NhHis), N-(1-
methylpropyl)glycine (Nle), N-(2-methylpropyl)glycine
(NLeu), N-(4-aminobutyl)glycine (NLys), N-(2-

35
methylthioethyl)glycine (NMet), N-(hydroxyethyl)glycine (NHSer), N-(2-hydroxypropyl)glycine (NHThr), N-(3-indolylmethyl)glycine (NTrp), N-(p-hydroxyphenethyl)glycine (NTyr), N-(1-methylethyl)glycine (NVal).

23. Combination of compounds as claimed in claims 1-22, wherein at least one of the compounds is in cyclic form.

24. Combination of compounds as claimed in claims 1-23 for use in prophylaxis or therapy.

25. Combination of compounds as claimed in claims 1-23 for use in the prophylaxis or treatment of indications involving the C5a-receptor (C5aR) and/or formylated peptide receptor (FPR) on neutrophils, monocytes and endothelial cells.

26. Combination of compounds as claimed in claim 25, wherein the indication involves acute or chronic inflammation reactions.

27. Combination of compounds as claimed in claim 26 wherein the indication is selected from the group consisting of cardiovascular diseases, in particular arteriosclerosis, atherosclerosis, vasculitis, ischaemia reperfusion episodes, ischaemic shock, cardiopulmonary bypass, (large) vessel surgery, transplantation, myocardial infarction, diseases of the central nervous system, in particular bacterial meningitis, viral meningitis, multiple sclerosis, stroke, Alzheimer's disease, brain tumour, (traumatic) brain injury, gastrointestinal diseases, in particular pancreatitis, ulcerative colitis, Crohn's disease, alcoholic hepatitis, viral hepatitis, Helicobacter pylori gastritis, gastric carcinoma, peritonitis, skin diseases, in particular psoriasis, contact dermatitis, atopic dermatitis, and other dermatoses, cutaneous T-cell lymphoma, burns, genitourinary diseases, in particular urinary tract infection, glomerulonephritis, Trichomonas vaginalis infection, endometriosis, joint diseases, in particular rheumatoid arthritis, acute reactive arthritis, gout, respiratory diseases, in particular
adult respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis, cystic fibrosis, asthma, pleural emphema, metal fume fever, bacterial pneumonia, chronic bronchitis, hypersensitivity pneumonia, *Mycobacterium tuberculosis* infection, viral respiratory tract infection, allergic rhinitis, sinusitis, bronchogenic carcinoma, and other diseases and indications, in particular periodontitis, chronic lymph leukemia, acute transplant rejection, glomerulonephritis, frost bite, repetitive strain injury (RSI), sepsis.

28. Combination of compounds as claimed in claims 1-23 for use in the prophylaxis or treatment of indications involving CXCR4 and/or CCR5 receptors on leukocytes.

29. Combination of compounds as claimed in claim 28, wherein the indication is HIV infection.

30. Combination of compounds as claimed in claims 1-23 for use in the prophylaxis or treatment of indications involving C5aR and/or FPR on cells other than neutrophils, monocytes and endothelial cells.

31. Combination of compounds as claimed in claim 30, wherein the other leukocytes are lymphocytes, dendritic cells, eosinophils, basophils, macrophages, microglia cells, astrocytes, Kupfer cells, hepatocytes and epithelial cells.

32. Combination of compounds as claimed in claims 1-23 for use in prophylactic or therapeutic vaccines for infections caused by CHIPS-producing bacteria.

33. Combination of compounds as claimed in claim 32, wherein the CHIPS-producing bacterium is *Staphylococcus aureus*.

34. Use of the combination of compounds as claimed in claims 1-23 for the manufacture of a therapeutic preparation for prophylaxis or therapy.

35. Use as claimed in claims 34, wherein the therapeutic preparation is for prophylaxis and treatment
of indications involving C5aR and/or FPR on neutrophils, monocytes and endothelial cells.

36. Use as claimed in claim 35, wherein the indication involves acute or chronic inflammation reactions.

37. Use as claimed in claim 36 wherein the indication is selected from the group consisting of cardiovascular diseases, in particular arteriosclerosis, atherosclerosis, vasculitis, ischaemia reperfusion episodes, ischaemic shock, cardiopulmonary bypass, (large) vessel surgery, transplantation, myocardial infarction, diseases of the central nervous system, in particular bacterial meningitis, viral meningitis, multiple sclerosis, stroke, Alzheimer's disease, brain tumour, (traumatic) brain injury, gastrointestinal diseases, in particular pancreatitis, ulcerative colitis, Crohn's disease, alcoholic hepatitis, viral hepatitis, Helicobacter pylori gastritis, gastric carcinoma, peritonitis, skin diseases, in particular psoriasis, contact dermatitis, atopic dermatitis, and other dermatoses, cutaneous T-cell lymphoma, burns, genitourinary diseases, in particular urinary tract infection, glomerulonephritis, Trichomonas vaginalis infection, endometriosis, joint diseases, in particular rheumatoid arthritis, acute reactive arthritis, gout, respiratory diseases, in particular adult respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis, cystic fibrosis, asthma, pleural emphema, metal fume fever, bacterial pneumonia, chronic bronchitis, hypersensitivity pneumonia, Mycobacterium tuberculosis infection, viral respiratory tract infection, allergic rhinitis, sinusitis, bronchogenic carcinoma, and other diseases and indications, in particular periodontitis, chronic lymph leukemia, acute transplant rejection, glomerulonephritis, frost bite, repetitive strain injury (RSI), sepsis.

38. Use as claimed in claim 34, wherein the therapeutic composition is for prophylaxis and treatment
of indications involving CXCR4 and/or CCR5 receptors on leukocytes.

39. Use as claimed in claim 38, wherein the indication is HIV infection.

40. Use as claimed in claim 34, wherein the therapeutic composition is for prophylaxis or treatment of indications involving C5aR and/or FPR on cells other than neutrophils, monocytes and endothelial cells.

41. Use as claimed in claim 40, wherein the other leukocytes are lymphocytes, dendritic cells, eosinophils, basophils, macrophages, microglia cells, astrocytes, Kupfer cells, hepatocytes and epithelial cells.

42. Use as claimed in claim 34, wherein the therapeutic preparation is a prophylactic or therapeutic vaccine that can be used in the prophylaxis or treatment of infections caused by CHIPS-producing bacteria.

43. Use as claimed in claim 42, wherein the CHIPS-producing bacterium is Staphylococcus aureus.

44. A therapeutic composition comprising a suitable excipient and one or more combinations of compounds as claimed in claims 1-23.

45. A prophylactic composition comprising a suitable excipient and one or more combinations of compounds as claimed in claims 1-23.

46. An antibody or biologically active fragment thereof specifically directed to one or more of the compounds of the combination as claimed in claims 1-23.

47. Antibody or biologically active fragment thereof as claimed in claim 46 for use in the diagnosis, treatment or prophylaxis of infections of CHIPS producing bacteria.

48. Antibody or biologically active fragment thereof as claimed in claim 47, wherein the CHIPS producing bacterium is Staphylococcus aureus.

49. A compound interfering with CHIPS or one or more of the compounds of the combination as claimed in claims 1-23.
50. Compound as claimed in claim 49 for use in the diagnosis, treatment or prophylaxis of infections of CHIPS producing bacteria.

51. Compound as claimed in claim 50, wherein the CHIPS producing bacterium is *Staphylococcus aureus*.

52. A compound interfering with the CHIPS receptor.

53. Compound as claimed in claim 52 for use in the treatment or prophylaxis of infections of CHIPS producing bacteria.

54. Compound as claimed in claim 52, wherein the CHIPS producing bacterium is *Staphylococcus aureus*.

55. Use of one or more antibodies and/or biologically active fragments thereof as claimed in claim 46 and/or one or more CHIPS interfering compounds as claimed in claim 49 and/or one or more CHIPS receptor interfering compounds as claimed in claim 52 for the preparation of a coating composition for use on surfaces of medical devices that are introduced into the human body through the skin, or placed in the body during surgical procedures.

56. Use as claimed in claim 55, wherein the surface is the surface of a catheter tip.

57. Use as claimed in claims 55 and 56, wherein the composition is a slow-release composition.

58. Therapeutic, diagnostic or prophylactic composition comprising a suitable excipient and one or more antibodies and/or biologically active fragments thereof as claimed in claim 46 and/or one or more interfering compounds as claimed in claim 49 or 52.

59. Method for prophylaxis or treatment of a subject suffering from indications involving C5aR and/or FPR on neutrophils, monocytes and endothelial cells comprising administering a prophylactically or therapeutically effective amount of one or more combinations of compounds as claimed in claims 1-23.
60. Method as claimed in claim 59, wherein the indication involves acute or chronic inflammation reactions.

61. Method as claimed in claim 60 wherein the indication is selected from the group consisting of cardiovascular diseases, in particular arteriosclerosis, atherosclerosis, vasculitis, ischaemia reperfusion episodes, ischaemic shock, cardiopulmonary bypass, (large) vessel surgery, transplantation, myocardial infarction, diseases of the central nervous system, in particular bacterial meningitis, viral meningitis, multiple sclerosis, stroke, Alzheimer's disease, brain tumour, (traumatic) brain injury, gastrointestinal diseases, in particular pancreatitis, ulcerative colitis, Crohn's disease, alcoholic hepatitis, viral hepatitis, *Heliobacter pylori* gastritis, gastric carcinoma, peritonitis, skin diseases, in particular psoriasis, contact dermatitis, atopic dermatitis, and other dermatoses, cutaneous T-cell lymphoma, burns, genitourinary diseases, in particular urinary tract infection, glomerulonephritis, *Trichomonas vaginalis* infection, endometriosis, joint diseases, in particular rheumatoid arthritis, acute reactive arthritis, gout, respiratory diseases, in particular adult respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis, cystic fibrosis, asthma, pleural emphema, metal fume fever, bacterial pneumonia, chronic bronchitis, hypersensitivity pneumonia, *Mycobacterium tuberculosis* infection, viral respiratory tract infection, allergic rhinitis, sinusitis, bronchogenic carcinoma, and other diseases and indications, in particular periodontitis, chronic lymph leukemia, acute transplant rejection, glomerulonephritis, frost bite, repetitive strain injury (RSI), sepsis.

62. Method for prophylaxis or treatment of a subject suffering from indications involving CXCR4 and/or CCR5 receptors on lymphocytes comprising administering a prophylactically or therapeutically effective amount of
one or more combinations of compounds as claimed in claims 1-23.

63. Method as claimed in claim 62, wherein the indication is HIV infection.

64. Method for prophylaxis or treating a subject suffering from indications involving C5aR and/or FPR on cells other than neutrophils, monocytes and endothelial cells comprising administering a prophylactically or therapeutically effective amount of one or more combinations of compounds as claimed in claims 1-23.

65. Method as claimed in claim 64, wherein the other cells are lymphocytes, dendritic cells, eosinophils, basophils, macrophages, microglia cells, astrocytes, Kupfer cells, hepatocytes and epithelial cells.

66. Method for the prophylactic or therapeutic treatment of a subject against infections with CHIPS-producing bacteria comprising the administration of a prophylactically or therapeutically effective amount of one or more combinations of compounds as claimed in claims 1-23.

67. Combination of compounds as claimed in claims 1-23 for use in the identification of competitors for CHIPS binding.

68. Combination of compounds as claimed in claim 67, wherein the competitor is a CHIPS agonist.

69. Combination of compounds as claimed in claim 67, wherein the competitor is a CHIPS antagonist.

70. Combination of compounds as claimed in claims 1-23 for use in computer modeling to design chemicals having the same function as the combination of compounds as claimed or having an antagonist function.

71. Combination of compounds as claimed in claims 1-23 for use in a diagnostic kit for determining the presence of CHIPS.

72. Diagnostic kit for determining the presence of CHIPS, comprising one or more combinations of
compounds as claimed in claim 1-23.
FIG. 1

1 ATAAATTTAAATATAAGGAGAAATTAACATCATTTATGAAAAAGAAATTTAGCAACACAGTTT

69 TAGCATTAAGTTTTTTACCGCAGGAATCGTACACACCATCATTCAGCGAAAGCTTTTACTTTTGAA

137 CGGTTCCTCATAATGAAGAAATAGATCAAAATAAGAAATATGTTAGAGAAGAAGAAAAGCTTATAAGA

205 ATCATTTAAAAATAGTGGTCTTCTTCTACAACGGTTAATTAAGATGAAACGTTTGAGAAAATTATTTAA

273 AGAAAGGCACAAAAATTTCTGCTCAATTTTGAAAATGGTTATTTTAACTGAAAAATAAAGCTTACTAT

341 ACAGTATATCTGAAATACACCCACTTGCTGAAGATAGAAAAATGTTGAGTTACTAGGTAATAGTATAA

409 AACATACCTTTTTAAAAAGGAGAATCTCAAATCATCTTTATGTAATTAATGGTCCTGGAAAAACTAATG

477 AATATGCATACTAAATAGTGTTACATAAAATTTAAAGGTAGATATTCTTTTTTTTATATAAAGGTTGGC

545 AGACATTTCATAACTTGGCAACAACTTTATATATATCTAAATTTTTATCAAACCTGCACTAAACTT
Derived amino acid sequence of CHIPS

FTFEPFPTNEEIESNKKMLEKEKAYKESFKNSGLPTTLGKL
DERLRLNYLKGTKNSAQFEKMVILTENKGYYTQYLYNLTP
AEKLEKNAVELLGKMYKYFFKKGESKSYVINGPGKTEYAY

FIG.2
5 amino acid peptide scan for CHIPS
Figure 7

1 amino acid peptide scan

Response

pep8-22
pep7-21
pep6-20
pep5-19
pep4-18
pep3-17
pep2-16
pep1-15
rCHIPs
RPMI
Substitutions of the first phenylalanine in pep1-8

Response

FIG. 9

Ac-Phe
NPhe
D-Phe
Bpa
Phe(p-NH2)
Cha
Nal(2)
Phe
rCHIPS
RPMI
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/08  A61P29/00  A61P35/00  A61P31/04  C07K16/12
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 7 A61K C07K

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, PAJ, BIOSIS, MEDLINE, SEQUENCE SEARCH, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>WO 00 02913 A (EIJKMAN WINKLER INSTITUUT; KESSEL CORNELIS PETRUS MARIA V (NL); ST) 20 January 2000 (2000-01-20) cited in the application the whole document</td>
<td>1-48,50, 51,55-72</td>
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Date of the actual completion of the international search 16 July 2002

Date of mailing of the international search report 05/08/2002

Name and mailing address of the ISA
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Authorized officer
Döpfer, K-P

* Special categories of cited documents:
**A** document defining the general state of the art which is not considered to be of particular relevance
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**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 novel or 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.

* Patent family members are listed in annex.

** Further documents are listed in the continuation of box C. **
**Box I  Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

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

1. ☒ Claims Nos.: - because they relate to subject matter not required to be searched by this Authority, namely:

   see FURTHER INFORMATION sheet PCT/ISA/210

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 II  Observations where unity of invention is lacking (Continuation of Item 2 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.
Continuation of Box I.1

Although claims 59-66 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.

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Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy
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<th>Patent document cited in search report</th>
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