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<p>(54) Title: INHIBITORS OF HEMATOPOIETINS INTERLEUKIN-3, INTERLEUKIN-5 AND GRANULOCYTE/MACROPHAGE COLONY STIMULATING FACTOR AND USES THEREOF</p>		
<p>(57) Abstract</p> <p>The present invention provides peptide inhibitors of the haematopoietins interleukin-3, interleukin-5 and granulocyte/macrophage colony-stimulating factor signalling, wherein said peptide inhibits the activation of Lyn tyrosine kinase and, thereby, blocks signal transduction via the haematopoietin βc receptor common to interleukin-3, interleukin-5 and granulocyte/macrophage colony-stimulating factor. Also provided is a method of treating a condition involving increased production and function of eosinophils and other granulocytes, comprising the step of administering a pharmacologically effective dose of the pharmaceutical composition disclosed herein.</p>		

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INHIBITORS OF HEMATOPOIETINS INTERLEUKIN-3, INTERLEUKIN-5
AND GRANULOCYTE/MACROPHAGE COLONY STIMULATING FACTOR
AND USES THEREOF

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The present invention relates to peptide inhibitors of the haematopoietins: interleukin-3 (IL-3), interleukin-5 (IL-5) and granulocyte/macrophage colony-stimulating factor (GM-CSF) and to the use of such inhibitors in medical therapy.

10

The mechanism of action of many modern pharmacotherapeutic agents involves stimulation or inhibition of cellular receptors (e.g., beta-adrenergic receptor agonists or antagonists). Such receptors are frequently coupled to multiple signalling pathways leading to different cellular functions. The disadvantage of receptor antagonists is that they
15 indiscriminately inhibit all receptor-associated functions. This type of complete receptor blockade is not always desirable since it frequently results in adverse side effects. Ideally, one would like to block only an unwanted signal from a receptor without interfering with other important signalling processes.

20 Interleukin-3 (IL-3), interleukin-5 (IL-5) and granulocyte/macrophage colony-stimulating factor (GM-CSF) are three important growth and differentiation factors for myeloid progenitors. IL-3 is the pluripotent stem cell growth factor. GM-CSF promotes the growth and differentiation of granulocytes and macrophages whereas IL-5 stimulates the differentiation of eosinophils and basophils.

25

All three cytokines have a specific α receptor but they share a common β receptor (β_c). The β_c receptor is the principal signalling receptor for these three cytokines and consequently all three haematopoietins have significant functional overlap, especially, with regard to their activity on eosinophils.

30

Asthma and allergic diseases are very common. Approximately 20% of the population has an allergic disease and 4% has asthma. In 1990, the cost of asthma medication alone was about \$1.1 billion. Currently, there are two classes of anti-asthma drugs. The symptomatic drugs such as beta-agonists, anti-cholinergics and methylxanthines do not inhibit eosinophilic inflammation. The anti-inflammatory drugs for asthma include cromolyn sodium and corticosteroids. Cromolyn sodium is effective in only about 30% of asthmatic patients. Corticosteroids are a highly effective medicine for asthma but have many side effects. For these reasons, there is an intense effort in the industry to develop novel drugs for asthma that block eosinophilic inflammation.

The prior art is deficient in the lack of effective means of inhibiting the haematopoietins IL-3, IL-5 and GM-CSF. The present invention fulfils this long-standing need and desire in the art.

In one embodiment of the present invention, there is provided a peptide inhibitor of the haematopoietins IL-3, IL-5 and GM-CSF signalling, wherein said peptide inhibits the activation Lyn tyrosine kinase and thereby, blocks signal transduction of the haematopoietin βc receptor common to IL-3, IL-5 and GM-CSF

In another embodiment of the present invention, there is provided a pharmaceutical composition, comprising a peptide inhibitor of the present invention and a pharmaceutically acceptable carrier.

In yet another embodiment of the present invention, there is provided a method of treating a condition involving increased production and function of eosinophils and other granulocytes, comprising the step of administering a pharmacologically effective dose of the pharmaceutical composition of present invention.

Thus, the present invention is directed to a peptide inhibitor of the haematopoietins IL-3, IL-5 and GM-CSF signalling, wherein said peptide inhibits the activation of the Lyn

tyrosine kinase and blocks signalling via the haematopoietin βc receptor common to IL-3, IL-5 and GM-CSF. Preferably, the peptide is an N-terminally modified peptide. More preferably, the peptide is modified by N-stearation.

5 Recent studies with various receptors suggest that specific regions within their cytosolic tail generate different signals leading to disparate cellular functions. The strategy described herein is to map the cytosolic region of the receptor for specific signal generating sites (e.g. tyrosine kinase binding sites) and design small peptides based upon the sequence information of the signal-generating site. The next step is to modify the peptides in order
10 to enable their cellular internalisation and to improve their *in vivo* stability. The modified peptides should function to block targeted cellular functions.

Peptide inhibitors of the present invention may inhibit signalling of the haematopoietins IL-3, IL-5 and GM-CSF in any of several ways, including inhibition of tyrosine
15 phosphorylation, blocking the eosinophil survival promoting effects of IL-5, inhibiting GM-CSF-stimulated proliferation of GM-CSF-dependent cells, inhibiting the growth and differentiation of eosinophils from bone marrow cells.

The peptide inhibitor of the present invention is useful in the treatment of conditions
20 involving increased production and function of eosinophils and other granulocytes. Representative examples of such conditions include asthma, allergic rhinitis, allergic conjunctivitis, idiopathic eosinophilic syndrome, eosinophilic pneumonia, allergic bronchopulmonary aspergillosis, Churg-Strauss syndrome, eosinophilic gastritis, Loeffler's syndrome and myelopietic abnormalities such as myelodysplastic syndromes.

25

In a preferred embodiment of the present invention the peptide inhibitor corresponds to amino acid residues 450-465 of the βc receptor. A particularly preferred peptide of this embodiment may have the sequence: stearate-YGYRLRRKWEKIPNP-NH₂.

In a further embodiment of the present invention, the peptide inhibitor corresponds to amino acid residues 462-481 of the βc receptor. A particularly preferred peptide of this embodiment may have the sequence: stearate-IPNPSKSHLFQNGSAELWPP-NH₂.

- 5 It further preferred embodiments of the present invention the peptide inhibitor may comprise a D-basic amino acid residue at the end of the peptide; this is preferably the amino acid D-Arg

Other and further aspects, features, and advantages of the present invention will be
10 apparent from the following description of the presently preferred embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

- 15 So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarised above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however,
20 that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows the mapping of the Lyn kinase binding site of βc receptor. A previous publication indicated that the membrane proximal residues 450-517 of the βc receptor is
25 critical for activation of Lyn, Fes and Jak2 kinases. Thus, four overlapping and/or sequential peptides (βc 450-465, βc 456-470, βc 462-481, βc 482-498) derived from this region were synthesised. The peptides were biotinylated. The βc 605-624 (pY612) peptide was used as a positive control because it binds to the SH2 domain of Lyn. Two control peptides derived from FceRI β (amino acids 29-48) and gp130 (amino acids 658-677) which
30 amino acid sequences were similar to βc 462-481 were also obtained. These receptor

subunits were known to bind Lyn. TF-1 cells were used as the source of Lyn kinase. The precleared lysates were incubated with or without one of above-described peptides (50 mM) for 2-4 hours followed by incubation with avidin-agarose for an additional 2 hours. The avidin-agarose bound proteins were separated, eluted and immunoblotted with anti-Lyn antibody. Lyn kinase exists in two molecular weight forms: p53 and p56. Both bands are typically visible in most cells and cell lines. The left lane containing the unprocessed cell lysate shows the position of the p53/p56 Lyn kinase. Three overlapping peptides (β c 450-465, β c 456-470, β bc 462-481), but not β c 482-498, FceRIb 29-48 and gp130 658-677 peptides, bound to Lyn kinase.

Figure 2 shows the specific binding of β c 462-481 peptide to Lyn kinase. TF-1 cell lysates were precleared with avidin-conjugated agarose and then incubated with or without the biotinylated β c 462-481 peptide or a mutated β c 462-481 peptide with Pro \rightarrow Ala substitutions. After incubation for 2 hours, the avidin-agarose conjugates were added and the bound proteins were separated. The proteins were electrophoresed and immunoblotted with the anti-Lyn antibody. The mutated β c 462-481 peptide did not bind to Lyn kinase.

Figure 3 shows the effect of the steared peptide on GM-CSF-induced 3 H-thymidine incorporation. TF-1 cells (a) or THP-1 cells (b) (105 cells/ml) were incubated with or without the N-steared bc 462-481 peptide for 3 hours. The biotinylated β c 462-481 and steared-IL-5a 316-335 peptides were used as controls. The cells were further incubated with 5% FCS and GM-CSF (1 ng/ml) for 96 hours. During the last 6 hour incubation, 5 mCi/ml of 3 H-thymidine was added to the culture. The cells were harvested and then their radioactivity was counted. (a) The thymidine uptake by GM-CSF-stimulated TF-1 cells was 1,132,702 \pm 331,855 cpm (n=3) considered 100%. Other results were expressed as percent of the GM-CSF-stimulated uptake. (b) Basal thymidine uptake by THP-1 cells was 458,187 \pm 145,848 cpm (n=3) and was considered 100%. The N-steared β c462-481 peptide inhibited GM-CSF-induced 3 H-thymidine incorporation in TF-1 cells, but not in GM-CSF-independent THP-1 cells. *: p(0.05 vs without steared- β c 462-481 peptide (ANOVA).

Figure 4 shows the effect of the N-stearated β c 462-481 peptide on GM-CSF-induced tyrosine phosphorylation of proteins in eosinophils. Eosinophils (2×10^6 cells/ml) were incubated in the presence or absence of the N-stearated β c 462-481 peptide for 3 hours and then stimulated with or without GM-CSF (10 ng/ml) for 5 minutes. The biotinylated β c 462-481 and steared-IL-5 α 316-335 peptides were used as controls. After lysing the cells, the lysates were subjected to electrophoresis and western blotting with anti-phosphotyrosine antibody. The N-stearated β c 462-481 peptide inhibited GM-CSF-induced tyrosine phosphorylation of cellular proteins in eosinophils in a dose-dependent manner.

Figure 5 shows the effect of the N-stearated β c 462-481 peptide on GM-CSF-induced tyrosine phosphorylation of Lyn in eosinophils. Eosinophils (5×10^6 cells/ml) were incubated in the presence or absence of the N-stearated β c 462-481, biotinylated β c 462-481 and steared-IL-5 α 316-335 peptides for 3 hours followed by the stimulation with or without GM-CSF (10 ng/ml) for 5 minutes. The lysates of esinophils were immunoprecipitated with anti-Lyn antibody. The immunocomplex was subjected to electrophoresis and western blotting with anti-phosphotyrosine antibody. Lyn activation after GM-CSF stimulation was significantly inhibited in the presence of the N-stearated β c 462-481 peptide.

Figure 6 shows the results of eosinophil survival assay. Eosinophils (10^6 cells/ml) were incubated with or without the N-stearated β c 462-481 peptide for 3 hours. The biotinylated β c 462-481 and steared-IL-5 α 316-335 peptides were used as controls. The cells were further cultured with or without GM-CSF (1 ng/ml) or (b) IL-5 (1 ng/ml) for 72 hours, and then the viability of eosinophils was counted. The N-stearated β c 462-481 peptide inhibited GM-CSF- and IL-5-induced eosinophil survival in a dose-dependent manner. Data are expressed as means \pm SD (n=4). *: $p < 0.05$ vs without steared- peptides (ANOVA).

Figure 7 shows the effect of the steared peptide on ECP release from eosinophils.

Eosinophils (106 cells/ml) were incubated with or without the N-steared β c 462-481

peptide for 3 hours. The biotinylated β c 462-481 and steared-IL-5 α 316-335 peptides

were used as controls. The cells were primed with or without GM-CSF (1 ng/ml) for 2

5 hours, and then stimulated with PAF (5×10^{-8} M) for 20 minutes. ECP concentration in the supernatant of eosinophils was measured by RIA. Data are expressed as means \pm SEM (n=4). The ECP release from eosinophils was not affected by the N-steared β c 462-481 peptide.

10 Figure 8 shows the binding of SHPTP2 (SHP-2) to a phosphotyrosine based ITIM motif-containing peptide (peptide 1) derived from IL-5 β cR (residues 605-624). The controls were a nonphosphorylated F612 peptide (peptide 2) and a β c450-465 peptide (peptide 3).

The peptides were biotinylated and used in a peptide binding assay using the HL-60 cell lysate. The bound proteins were precipitated with streptavidin-agarose followed by

15 immunoblotting with anti-SHPTP2. The first lane, (L: cell lysate) shows the position of the 70 kD SHPTP2. The identity of the upper band is unknown. The buffer control is shown in the last lane (0). SHPTP2 binds to the phosphorylated β c 605-624 but not to the β c 450-465 or mutated β c 605-624 peptides.

20 Figure 9 shows the effect of the β c 462-481 peptide on Jak2 kinase activation. TF-1 cells (5×10^6 cells/ml) were incubated in the presence or absence of the N-steared β c 462-481, biotinylated β c 462-481 and steared-IL-5 α 316-335 peptides for 3 hours followed by the stimulation with or without GM-CSF (10 ng/ml) for 5 minutes. The cell lysates were immunoprecipitated with an anti-Jak2 antibody. The immune-complex was subjected to

25 electrophoresis and western blotting with an anti-phosphotyrosine antibody. The Jak2 activation after GM-CSF stimulation was not inhibited by the N-steared β c 462-481 peptide.

Figure 10 shows the effect of the β c 462-481 peptide on activation of ERK2 MAP kinase

30 by GM-CSF. TF-1 cells were incubated with or without the N-steared β c 462-481

peptide for 3 hours. The cells were then stimulated with GM-CSF (10 ng/ml) for 5 minutes. The cell lysate was immunoprecipitated with an anti-ERK2 antibody. The immune complex was separated, electrophoresed and western blotted with anti-phosphotyrosine antibody (left panel). GM-CSF stimulated tyrosine phosphorylation of ERK2 MAP kinase which was not affected by pre-treatment with the N-stearated peptide. The membrane was stripped and reprobod with the anti-ERK2 antibody which shows equal amount of the ERK protein in each lane (right panel).

Figure 11 shows the effect of the β c 462-481 peptide on anti-IgE-induced histamine release from basophils. Peripheral blood leukocytes were preincubated with buffer or increasing concentrations of the steared β c 462-481 peptide. Thirty minutes later, a predetermined concentration of anti-IgE was added. Histamine was analysed by an automated spectrofluorometer. The control histamine release was $18 \pm 5\%$ (n=3). The histamine release from basophils was not affected by the N-steared β c 462-481.

Figure 12 shows the effect of the steared peptide on ^3H -thymidine incorporation of T cells (a) and B cells (b). Purified T cells and B cells (5×10^6 cells/ml) were incubated with or without the N-steared β c 450-465 peptide for 3 hours in the presence of 10% FCS. The T cells were stimulated with Con A (5 mg/ml), whereas the B cells were stimulated with immobilised anti-IgM antibody (10 (g/ml). The cells were further incubated for 96 hours with the addition of 5 mCi/ml of ^3H -thymidine during the last 6 hours. After harvesting the cells, their radioactivity was counted. The thymidine uptakes by Con A-stimulated T cells and anti-IgM-stimulated B cells were $192,133 \pm 82,772$ and $7,950 \pm 1865$ cpm (n=3), respectively, and were considered 100%. The N-steared β c 450-465 peptide has no inhibitory effect on Con A-induced T cell proliferation nor anti-IgM-induced B cell proliferation.

Definitions

As used herein, the term "ECP or eosinophil cationic protein" shall refer to a granular protein of eosinophilic leukocytes.

5

As used herein, the term "GM-CSF or granulocyte-macrophage colony-stimulating factor" shall refer to a growth factor for stem cells.

10

As used herein, the term "haematopoietins" shall refer to cytokines that stimulate the growth of blood cells from bone marrow cells.

15

As used herein, the term "IL or interleukin" shall refer to specific cytokines that are proteins or glycoproteins made by leukocytes and other cells and modulate the function of a variety of cells.

As used herein, the term "Lyn" and "Jak2" shall refer to tyrosine kinases that catalyse the phosphorylation of tyrosine residues on target proteins.

20

As used herein, the term "MAP kinase (mitogen-activated protein kinase" shall refer to an enzyme that phosphorylates serine and threonine residues of target proteins.

As used herein, the term "Ras" shall refer to a small GTP binding protein that activates the MAP kinase pathway.

25

As used herein, the term "SH2 domain" or "src homology domain" shall refer to a structural motif that binds to proline-rich residues.

As used herein, the term "SH3" or "src homology" shall refer to domain or structural motif that binds to phosphorylated tyrosine residues.

30

As used herein, the term "SHP-2/Syp/SHPTP2" shall refer to a tyrosine phosphatase that dephosphorylates tyrosine residues of target proteins.

As used herein, the term "TF-1" shall refer to a cell line that is dependent upon GM-CSF
5 for its continuous growth.

As used herein, the term 'variant' shall mean a peptide having a deletion, substitution
addition or chemical modification of one or more amino acids such that it has an ability to
inhibit the activity of IL-3, IL-5 or GM-CSF of not less than 50% of that of the
10 corresponding non-variant peptide which has the same amino acid sequence as the variant
peptide but for that deletion, substitution, addition or chemical modification. More
preferably the variant has 60%,70%,80%,90%,100%,110%, of the inhibitory activity of the
non-variant and most preferably, greater than 120%.

15 It is specifically contemplated that pharmaceutical compositions may be prepared using
the peptide inhibitors of the present invention. In such a case, the pharmaceutical
composition comprises the peptide inhibitors of the present invention and a
pharmaceutically acceptable carrier. A person having ordinary skill in this art would
readily be able to determine, without undue experimentation, the appropriate dosages and
20 routes of administration of the novel peptide inhibitors of the present invention. When
used *in vivo* for therapy, the peptide inhibitors of the present invention are administered to
the human patient or other mammal in therapeutically effective amounts, i.e., amounts that
effectively inhibit function of the haematopoietins. They will normally be administered
parenterally, preferably intravenously, but other routes of administration will be used as
25 appropriate. The dose and dosage regimen will depend upon the nature of the disease, the
characteristics of the particular inhibitor, e.g., its therapeutic index, the patient, the patient's
history and other factors. The amount of the peptide inhibitor administered will typically
be in the range of about 1 to about 100 mg/kg of patient weight. The schedule will be
continued to optimise effectiveness while balanced against negative effects of treatment.
30 See Remington's Pharmaceutical Science, 17th Ed. (1990) Mark Publishing Co., Easton,

Penn.; and Goodman and Gilman's: The Pharmacological Basis of Therapeutics 8th Ed (1990) Pergamon Press; which are incorporated herein by reference. For parenteral administration, the peptide inhibitors will most typically be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are preferably non-toxic and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Non-aqueous vehicles such as fixed oils and ethyl oleate may also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives.

The hallmark of allergic diseases is eosinophilic inflammation. IL-5 is the single most important cytokine that regulates growth, differentiation and function of eosinophils. The present invention develops inhibitors of IL-5 as well as other haematopoietins (IL-3 and GM-CSF). Critical signalling molecules were identified that are involved in eosinophil growth and differentiation. Lyn tyrosine kinase is an important signalling molecule. The Lyn-binding site was mapped on βc receptor which is common to IL-3, IL-5 and GM-CSF. Peptides encompassing the Lyn-binding site were used to interfere with IL-5 and GM-CSF signalling. Two N-terminally modified peptides blocked Lyn kinase activation by the haematopoietins. Further, they inhibited tyrosine phosphorylation of many other signalling molecules. The peptides blocked the eosinophil survival promoting effects of IL-5 and inhibited GM-CSF-stimulated proliferation of factor-dependent cell lines but not autonomous tumour cell lines. Finally, the peptides inhibited the growth and differentiation of eosinophils from bone marrow cells. Based upon these observations, these peptides are useful in the treatment of diseases wherein eosinophils play an important role.

The present invention is the first mapping of the Lyn-binding site of βc receptor. The Lyn-binding peptide was modified by N-stearamation. The modified peptides inhibit eosinophil

growth, differentiation and survival in a specific manner. Thus, the peptides may be used to treat allergic and other eosinophilic inflammatory conditions.

The inhibitors described herein are useful for treatment of diseases involving increased production and function of eosinophils and other granulocytes. Diseases with increased production and function of eosinophils: asthma, allergic rhinitis, allergic conjunctivitis, idiopathic eosinophilic syndrome, eosinophilic pneumonia, allergic bronchopulmonary aspergillosis, Churg-Strauss syndrome, eosinophilic gastritis, Loeffler's syndrome. Diseases with myelopoietic abnormalities include myelodysplastic syndromes.

Two overlapping βc peptides that bind to Lyn kinase *in vitro* were designed. The peptides was modified by N-stearation and used to inhibit IL-5 and GM-CSF activity. The structure of the peptides are as follows:

peptide βc 450-465: Stearate-YGYRLRRKWEKIPNP-NH₂
peptide βc 462-481: Stearate-IPNPSKSHLFQNGSAELWPP-NH₂

These two peptides have been found to block βc receptor signalling and inhibit the growth and survival promoting effects of haematopietins *in vitro*. The degradation of small peptides can be reduced by adding D-basic amino acid residues such as D-Arg at the end of the peptides or by substituting L-amino acid residues with D-amino acid residues. The half-life can be increased by making a homobifunctional linker according to the method of Fein et al (JAMA, 1997;277:482-7).

In particular embodiments the invention provides the following

A peptide capable of blocking signal transduction via the haematopietin βc receptor common to IL-3,IL-5 and GM-CSF.

A peptide of the present invention is preferably one which has been derived from the sequence of the haematopoietin βc receptor common to IL-3,IL-5 and GM-CSF, or is a variant thereof.

5 A peptide capable of inhibiting the binding of Lyn kinase to the haematopoietin βc receptor common to IL-3,IL-5 and GM-CSF.

A peptide capable of inhibiting IL-3,IL-5 or GM-CSF which preferably acts by binding to Lyn tyrosine kinase.

10

A peptide comprising or more preferably consisting of a variant of the amino acid sequence of residues 450-465 or 462-481, of the haematopoietin βc receptor and having not less than 50% of the inhibitor activity of a peptide comprising or consisting of the non-variant amino acid sequence.

15

The peptides: stearate- YGYRLRRK WEEKIPNP-NH₂ or
stearate- IPNPSKSHLFQNGSAELWPP-NH₂.

A peptide according to the present invention, for use in medical therapy.

20

The use of a peptide of the present invention, in the preparation of a medicament for use in the therapy of conditions displaying increased production or function of granulocytes or in the therapy of conditions requiring any one of inhibition of tyrosine phosphorylation; blocking of the eosinophil survival promoting effects of interleukin-5 and granulocyte/macrophage colony-stimulating factor; or
25 inhibition of the growth and differentiation of eosinophils from bone marrow cells.

30

The use of a peptide according of the present invention, in the preparation of a medicament for use in the therapy of asthma, allergic rhinitis, allergic conjunctivitis,

idiopathic eosinophilic syndrome, eosinophilic pneumonia, allergic bronchopulmonary aspergillosis, Churg-Strauss syndrome, eosinophilic gastritis, Loeffler's syndrome or myelodysplastic syndromes.

5

A pharmaceutical formulation comprising a peptide of the present invention, and a pharmaceutically acceptable carrier therefor.

10

A method of treating a condition involving increased production or function of eosinophils or other granulocytes, comprising the step of administering a pharmacologically effective dose of a peptide of the present invention.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

15

20 **EXAMPLE 1**

Mapping the Lyn-binding site on βc receptor of IL-3/GM-CSF/IL-5.

25

Lyn is a member of the src family of tyrosine kinases and physically associates with the IL-5 βc receptor [Pazdrak et al., J Exp Med 1995;181:1827-34]. IL-5 activates Lyn kinase and transduces signals via the Ras-MAP kinase pathway. Lyn kinase plays a critical role in IL-5-stimulated eosinophil survival. The present invention maps the Lyn-binding site on βc receptor of IL-3/GM-CSF/IL-5.

The membrane-proximal β c 450-517 region is important for Lyn binding. In order to identify the exact binding site of Lyn, four biotinylated peptides were designed and synthesised:

- 5 β c 450-465 YGYRLRRKWEKIPNP,
 β c 456-470 KWEEKIPNPSKSHLF,
 β c 462-481 IPNPSKSHLFQNGSAELWPP,
 β c 482-498 GSMSAFTSGSPPHQGPW derived sequentially from this region.

10 The peptides were biotinylated. The β c 605-624 (pY612) peptide was used as a positive control because it binds to the SH2 domain of Lyn. Control peptides were derived from FceRIb (amino acids 29-48) and gp130 (amino acids 658-677) and their sequences were similar to that of β c 462-481.

15 The binding of the biotinylated peptides to Lyn was studied in a solution binding assay using TF-1 cell lysate as a source of Lyn. The detection of the peptide-bound Lyn kinase was performed by western blotting. Proline residues have been implicated in hydrophobic interactions (e.g. SH3 binding) among various proteins. These peptides had two to four Pro residues in their sequences. In order to determine the role of the Pro residues,
20 experiments were performed with a Pro \rightarrow Ala substituted peptide.

Three overlapping peptides (β c 450-465, β c 456-470, β c 462-481) bound to Lyn kinase in the binding studies (Figure 1). The mutated β c 462-481 peptide with Pro \rightarrow Ala substitutions did not bind to Lyn (Figure 2). These results suggest that Lyn binds to the
25 overlapping region of β c 450-465 and β c 462-481, and that the Pro residues from this region have a critical role for Lyn binding. This is the first identification of a binding site for Lyn kinase.

EXAMPLE 2

Effect of the N-stearated Lyn-binding peptides on GM-CSF signalling and proliferative responses in factor-dependent myeloid cell lines

To study the effect of the peptides on cytokine signalling, the peptides were modified by N-acylation which has been shown to induce internalisation of small peptides [Eichholz et al., J Biol Chem 1993;268:1982-6, Ward et al., Biochemistry 1993;32:11903-9]. To study whether an N-acylation of the Lyn-binding peptides helps internalisation and thereby, interferes with Lyn-coupled receptor signalling, two β c peptides were N-stearated.

To examine the effect of N-stearated Lyn-binding peptides on GM-CSF signalling, tyrosine phosphorylation of cellular proteins and cell proliferation in GM-CSF-dependent cell lines was studied. There are no human IL-5-dependent cell lines; therefore, GM-CSF-dependent cell lines were used since GM-CSF also utilises the β c receptor. TF-1 cells (2×10^6 cells/ml) were incubated in the presence or absence of the N-stearated β c 462-481 peptide or the control peptides for 3 hours and then stimulated with or without GM-CSF (10 ng/ml) for 5 minutes. After lysing TF-1 cells, the lysates were subjected to electrophoresis and western blotting with anti-phosphotyrosine antibody. For thymidine incorporation study, TF-1 cells were incubated with 5% FCS and GM-CSF (1 ng/ml) for 96 hours and their radioactivity was counted.

20

The N-stearated β c 462-481 peptide, but not the biotinylated β c 462-481 peptide nor a control N-stearated peptide derived from IL-5 α receptor (IL-5 α 316-335), inhibited GM-CSF-induced tyrosine phosphorylation of cellular proteins in eosinophils (Figure 4) and 3 H-thymidine incorporation in TF-1 cells (Figure 3). The cell proliferation of TF-1, but not THP-1 cells which was a GM-CSF-independent tumour cell line, was affected by the N-stearated β c 462-481 peptide (Figure 3). These results are suggestive of β c-specific signalling interference.

25

EXAMPLE 3

Effect of the N-stearated Lyn-binding peptide on GM-CSF/IL-5 signalling in eosinophils.

30

To show the effect of N-stearated Lyn-binding peptides on GM-CSF/IL-5 signalling in eosinophils, the capability of the peptide to interfere with the signal transduction of GM-CSF/IL-5 was examined by the two parameters. First, GM-CSF-induced activation of Lyn kinase in eosinophils in the presence of the peptide was determined. The activation was investigated by immunoprecipitation with anti-Lyn antibody followed by anti-phosphotyrosine immunoblotting. Secondly, an assessment of eosinophil stimulation by GM-CSF or IL-5 using an eosinophil survival assay and the ECP release method was performed.

10

The N-stearated β c 462-481 peptide inhibited GM-CSF-induced tyrosine phosphorylation of cellular proteins in eosinophils in a dose-dependent manner (Figure 4). In the immunoprecipitation study, Lyn activation after GM-CSF stimulation was significantly inhibited in the presence of the N-stearated β c 462-481 peptide (Figure 5). Eosinophil survival induced by GM-CSF was also inhibited by this steared peptide (Figure 6). ECP release from eosinophils was not affected by the N-stearated β c 462-481 peptide (Figure 7).

15

EXAMPLE 4

20

Effect of the N-stearated Lyn-binding peptide on the differentiation of murine bone marrow cells.

25

To investigate the role of Lyn kinase in myeloid cell differentiation, the effect of the N-stearated Lyn-binding peptide on murine bone marrow cells was studied. BALB/c mice were sacrificed and the femurs were removed. The bone marrow cavity was washed with saline to obtain cells. The bone marrow cells (2×10^5 cells/ml) were incubated for 1 week with 10 ng/ml of murine IL-3 and IL-5 and then harvested. The total cell count was obtained, and the rest of the cells were used for cytopsin preparations. The cytopsin preparations were stained with Wright's stain for a differential count of bone marrow cells.

30

IL-3 and IL-5 stimulated the proliferation of bone marrow cells and the cell count increased nearly four-fold. Further, 56% of the cells differentiated with eosinophilic lineage (eosinophilic myelocytes and mature eosinophils). The effect of N-stearated β c 462-481 peptide on the bone marrow cell differentiation was investigated. When the cells were pretreated with the peptide, the total cell number and the percentage of cells in eosinophilic lineage were reduced by nearly 50%. TABLE I shows the effect of the N-stearated β c 462-481 peptide on the proliferation and differentiation of murine bone marrow stem cells. The cells (2×10^5 cells) were incubated with or without 20 mM of the peptide for 3 hours, and then 10 ng/ml of IL-3 and IL-5 were added. One week later the cells were harvested and subjected to cytopspin to examine cell differentiation. The combination of IL-3 and IL-5 stimulated the differentiation of stem cells predominantly into eosinophils and basophils. The N-stearated β c 462-481 significantly inhibited the differentiation of eosinophils from stem cells without affecting the basophilic lineage. Data are expressed as means (SD (n=3)).

TABLE I

Murine IL-3 (ng/ml)	10	10
Murine IL-5 (ng/ml)	10	10
N-stearated β c 462-481 peptide (mM)	0	20
Total cell count (x 10 ⁵ cells)	7.1 \pm 0.4	3.7 \pm 0.3
Cell differentiation (%)		
Blast cell	5.0 \pm 1.7	5.7 \pm 2.5
Promyelocyte & Myelocyte	23.7 \pm 8.0	44.7 \pm 2.1

	Eosinophilic myelocyte	29.7± 4	17±3
	Basophilic myelocyte	10.3±3.1	10.3±3.2
	Eosinophil	18±11.7	9.7±3.1
	Basophil	5 ±1.0	5.3±0.6
5	Megakaryocyte	9.3±2.1	7.7±3.8

EXAMPLE 5

Specificity of Lyn inhibition

10

The effect of the β c 462-481 peptide was shown on the activation of other signalling molecules including Jak2 kinase, a member of the Janus family and SHP-2, (previously known as Syp and SHPTP2, and contains two SH2 domains) and ERK2 kinase, a member of the MAP kinase family. The Lyn-binding peptides do not bind or activate these

15 molecules (Figures 8-10). Lyn is predominantly expressed in myeloid cells and B cells. One study has demonstrated that the Lyn knockout mice have decreased myelopoiesis and dysregulated immunoglobulin production. Further, the IgE-dependent mast cell activation is impaired (Hibbs et al. Cell 83: 301-311,1995). Interestingly, another study failed to find any abnormalities of haematopoiesis or mast cell degranulation in Lyn knockout mice

20 (Nishizumi et al, J. Immunol. 158: 2350-2355, 1997). The effect of the β c peptide on anti-IgE-stimulated histamine release from basophils was tested. There was no inhibition of histamine release (Figure 11). Further, the peptide does not inhibit the differentiation of basophils from stem cells while it significantly blocks the differentiation eosinophils.

25

This selective action of the β c 462-481 peptide can be explained by the molecular interaction of Lyn with various receptors. Lyn has an N-terminal unique domain, followed by an SH3 domain, SH2 domain and the tyrosine kinase domain. Lyn can interact with receptors via three different binding sites: unique domain, SH3 and SH2 domains. The

30 lack of binding of the β c 462-481 peptide to SHP-2, which has two SH2 domains, suggest

that the SH2 domain is not involved in Lyn binding to βc . The molecular interaction of the βc 462-481 peptide with recombinant GST fusion proteins of the full length Lyn (without the kinase domain), the Lyn-unique domain, and the Lyn-SH3 domain. The peptide bound to the Lyn-unique domain and the full-length Lyn (without the kinase domain) but not to Lyn-SH3 (data not shown). On the basis of these results, the βc 462-481 peptide compete only with the receptors that bind Lyn via the unique domain but not via the SH2 or SH3 domains. Thus, this selective binding property further increases the specificity of the βc peptide.

10 EXAMPLE 6

Susceptibility to peptidases

Small peptides are generally susceptible to amino- and carboxypeptidases. The lipopeptide of the present invention is N-terminally acylated which will protect it from aminopeptidases. Further, the peptide has C-terminal amide which may make it partially resistant to carboxypeptidases. Formation of another ester C-terminal modification can be used to improve lipophilicity. One may also progressively replace L-amino acid residues with D-amino acids and perform routine binding and cellular studies. The D-amino acid residues are known to be resistant to peptidases. A number of peptide drugs have been designed using this principle as is well known in the art (e.g., bradykinin antagonists, neuropeptide antagonists, etc.). Additional modifications include introduction of pseudopeptide bonds (resistant to peptidases). Cyclization of peptides is also known to increase their stability.

25

The present invention involves the concept that signal specific inhibitors such as the βc 462-481 peptide will inhibit only the unwanted signal(s) of a receptor keeping other signalling processes intact. This is in contrast to receptor antagonists which block all receptor-associated signalling processes indiscriminately. Thus, using the teachings of the present invention, one can create inhibitors that will block only targeted functions of cells.

30

Consequently, signal specific inhibitors would have less toxicity than receptor antagonists. The therapeutic concentration of many currently used drugs is within 10-20 mg/ml, e.g., theophylline. Others require much higher concentrations, e.g., β -lactam antibiotics, aspirin. Thus, usage of 10-20 mM concentrations does not necessarily indicate that the peptide will be toxic. Some predictions about the toxicity of the β c peptide can be made. The knockout mice for β c receptor have eosinopenia but no other haematological or immunological abnormalities. Unlike humans, mice have an additional receptor for IL-3. Thus, double knockout mice were created by crossing β c knockout mice with IL-3 knockout mice. Haematopoiesis and immunologic studies were performed. The results were similar. Eosinopenia was the only haematological abnormality noted in these mice (Nishinakamura et al., Blood 88: 2458-2464, 1996). This study suggests the β c receptor is critical only for eosinopoiesis but not for other myeloid lineages. It has been postulated that cytokines such as G-CSF, M-CSF, IL-8 may regulate important aspects of myeloid cell haematopoiesis. From these studies one can conclude that a blockade of the β c receptor by a signal specific inhibitor may not have toxic effects on the growth and differentiation of neutrophils.

EXAMPLE 7

Delivery of the peptide to the target site.

The delivery of peptide of the present invention can be accomplished in a variety of conventional ways as well as using other technologies. In the fields of allergy and asthma, it is preferable to deliver the drugs topically, i.e., intranasally or intrabronchially via inhalation. Technology in the field of nasal and inhalation delivery of drugs is rapidly changing. The delivery of proteins with large porous particles such as poly(lactic acid-co-glycolic acid) (PLGA) or poly(lactic acid-co-lysine-graft-lysine) (PLAL-Lys) may prolong their half-life and provides steady delivery. The inhalation of insulin (serum half-life of 11 minutes) with PLGA caused sustained release of insulin for about 96 hours (Edwards, et

al., Science 276:1868-1871, 1997). These compounds may be useful in enhancing peptide delivery.

Recent studies with various receptors suggest that specific regions within their cytosolic
5 tail generate different signals leading to disparate cellular functions. The strategy described
herein is to map the cytosolic region of the receptor for specific signal generating sites (e.g.
tyrosine kinase binding sites) and design small peptides based upon the sequence
information of the signal-generating site. The next step is to modify the peptides in order
to enable their cellular internalisation and to improve their in vivo stability. The modified
10 peptides should function to block targeted cellular functions.

IL-5 activates multiple signalling pathways in eosinophils including the activation of Lyn
kinase, the Jak2-Stat and the Raf-MAP kinase pathways. Lyn and Jak2 kinases are
important for eosinophil growth, differentiation, and survival whereas the Raf-MAP kinase
15 pathway is critical for eosinophil degranulation. The Lyn-binding site of IL-5 β c receptor
was mapped and small peptides which bind Lyn in vitro were designed. Modified Lyn
binding peptides enter the cells and act as competitive inhibitors, and block Lyn-specific
eosinophil functions without interfering with other functions of IL-5 β c receptor.

20 Using the teaching herein, one with ordinary skill in this art could develop Lyn signal
specific inhibitors. By truncation mutations, various regions of IL-5R β c have been found
responsible for distinct signalling processes. For example, the membrane proximal 450-
517 residues are important for activation of Lyn, Jak2, c-myc, and pim-1, whereas the
distal 626-763 residues are critical for the activation of Ras-MAP kinase pathway as well
25 as c-fos and c-jun. Based upon this observation, overlapping peptides encompassing
residues 450-498 were synthesised. Three overlapping peptides β c 450-465 β c 456-470
and bc462-481 bind to Lyn kinase in vitro indicating that the binding site lies within
residues β c 450-481. Proline residues are critical for this binding since Pro(Ala mutation
of bc462-481 abrogates this binding. Based upon the Lyn binding residues of β c, signal
30 specific inhibitors may be designed that block Lyn specific IL-5 function.

EXAMPLE 8

IL-5 signal specific inhibitors (SSI) based upon the Lyn-binding site of the IL-5R β c.

5

The general strategy for developing signal specific inhibitors involves 5 steps. The first step is to identify a critical signalling molecule that associates with the receptor. The second step is to map the signalling molecule-binding site of the receptor. The third step is to design small peptide(s) based upon the binding site and study the in vitro binding activity. The fourth step is to N-acylate the peptide for cellular internalisation and examine the specificity of signalling inhibition and biologic effects in vitro. Finally, the fifth step is to modify the peptide for increased stability.

The peptides bc450-465 and bc462-481 are N-stearated to enhance cellular internalisation. All peptides and their modification can be obtained from Quality Controlled Biochemicals, Hopkinton, MA. The internalisation of N-stearated peptides and their effect on cytosolic signalling (protein tyrosine phosphorylation) are studied using eosinophils and a GM-CSF-dependent cell line TF-1. Further, the peptides are tested for inhibition of Lyn and other kinases/signalling molecules (e.g. Jak2, ERK MAP kinase, SHP-2) using established methods such as immunoprecipitation followed by western blotting with anti-phosphotyrosine antibodies and immune complex kinase assay. Controls include peptides without N-stearation and N-stearated peptides of similar length obtained from the IL-5 receptor b chain, the IgE receptor b chain, and the IL-6/LIF receptor common chain gp130. Once the specificity of Lyn inhibition is established, one can determine the minimal length of the peptide for biologic activity. The strategy is to progressively reduce the size by two residues and determine its Lyn-binding capability.

The effect of the N-stearated peptides on haematopoietin function is studied in the following assays: 1) eosinophil survival; 2) proliferation (thymidine incorporation) of GM-CSF-dependent cell lines; 3) IL-3 and IL-5-stimulated differentiation of eosinophils

30

from stem cells; and 4) GM-CSF-stimulated degranulation (release of eosinophil cationic protein ECP as measured by a commercially available RIA) of eosinophils. Further the effects of the N-stearated peptides on the following Lyn kinase-dependent cell functions are shown; 5) IgE-mediated histamine release from basophils; 6) Anti-IgM-stimulated B
5 cell proliferation.

A rationale for choosing Lyn as the target for signal specific inhibitors is that Lyn physically associates IL5R β c. Lyn is important for eosinophil growth and survival but not necessary for eosinophil degranulation (ECP release) and upregulation of adhesion
10 molecule CD11b. There is concern about the importance of secondary and tertiary structures of the receptor in Lyn binding which may be lost in a small peptide. However, recent experience with SH2 and SH3 domain-binding small peptides suggests that the tertiary structure is not essential. N-acylation of small peptides causes their internalisation through the lipid membranes as demonstrated by spin label electron spin resonance and 2H
15 NMR. The N-myristoylation of a protein kinase C substrate analogue causes its internalisation and specific inhibition of the kinase. Because of the bigger size of these peptides, they are N-stearated. Peptides from receptors (Fc(RI(and gp130) that are known to activate Lyn can be used as controls. The control peptides have equal numbers of similarly spaced proline residues.

20

EXAMPLE 9

Effect of IL-5 signal specific inhibitors on eosinophilia and eosinophilic inflammation *in vivo*.

25

To maximise the *in vivo* half-life of the peptides and study the biologic effects *in vivo*, a number of strategies have been shown to prevent enzymatic cleavage of peptides by amino- and carboxypeptidases. They include N-terminal modification, C-terminal modification, internal modification, cyclization and amide bond replacement. The N-terminus of the
30 peptide is modified by N-acylation and the C-terminus is modified to an amide. Both of

these modifications should stabilise the peptide. Alternatively, the C-terminus can be modified to an ester which increases lipophilicity.

D-amino acids are relatively resistant to digestion by peptidases. For this reason, L-amino acid residues are replaced systematically with D-amino acid residues except the critical
5 proline residues. Additional ways to improve stability include C(-alkyl amino acid or cyclopropyl amino acid substitutions. The modified peptides are biotinylated and tested for Lyn binding by western blotting according to the method described in Figure 2. Then their serum half-life is assessed by serial measurement (ELISA) of the serum concentration of
10 biotin following injection into the mice. Simultaneously, the kinetics and "half-life" of Lyn inhibition in blood granulocytes are monitored as a measure of in vivo effects of signal specific inhibitors.

The effect of optimised signal specific inhibitors are studied on the following models of IL-
15 5 action in vivo. IL-5-induced peripheral blood eosinophilia in mice. Mice are pre-treated with increasing concentrations of signal specific inhibitors or a steared control peptide. At the time of maximal serum concentration, the mice are injected with IL-5. Peripheral blood eosinophilia are monitored every 30 minutes for 4 hours. Next, an allergen-induced airway eosinophilia in a mouse model of asthma is used. Eosinophils constitute up to 60%
20 of the bronchoalveolar lavage cells upon allergen inhalation challenge. The immunised mice are pre-treated intrabronchially with increasing concentrations of signal specific inhibitors or a steared control peptide. Thirty minutes later the mice undergo allergen inhalation change. Bronchoalveolar lavage are performed 12, 24 or 48 hours later. The total and differential cell count are obtained. Further, the lung tissue are analysed
25 histologically for inflammatory cell influx. The bronchoalveolar lavage cells and inflammatory influx into the airways in signal specific inhibitors- and control peptide-treated mice are compared.

Any patents or publications mentioned in this specification are indicative of the levels of
30 those skilled in the art to which the invention pertains. These patents and publications are

herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to
5 carry out the objects and obtain the ends and advantages mentioned, as well as those
inherent therein. The present examples along with the methods, procedures, treatments,
molecules, and specific compounds described herein are presently representative of
preferred embodiments, are exemplary, and are not intended as limitations on the scope of
the invention. Changes therein and other uses will occur to those skilled in the art which
10 are encompassed within the spirit of the invention as defined by the scope of the claims.

CLAIMS

1. A peptide capable of blocking binding of Lyn tyrosine kinase to the
5 haematopoietin β c receptor.
2. A peptide according to claim 1 comprising the amino acid sequence of residues 450-465 or amino acid residues 462-481, of the haematopoietin β c receptor .
- 10 3. A peptide according to claim 1 comprising a variant of the amino acid sequence of residues 450-465 or 462-481, of the haematopoietin β c receptor and having not less than 50% of the inhibitor activity of a peptide comprising the non-variant amino acid sequence.
- 15 4. A peptide according to any of claims 1 to 3, wherein the peptide comprises the amino acid sequence:

YG YRLRRK WEEKIPNP-NH₂, or

20 IPNPSKSHLFQNGSAELWPP-NH₂.

5. A peptide according to any of claims 1 to 4, wherein the peptide is an N-terminally modified peptide.
- 25 6. A peptide according to claim 5, wherein the N-terminal modification is stearamide.
7. The peptides:
- 30 stearamide- YG YRLRRK WEEKIPNP-NH₂ or
stearamide- IPNPSKSHLFQNGSAELWPP-NH₂

8. A peptide according to any one of claims 1 to 7 additionally comprising a D-basic terminal amino acid.
- 5 9. A peptide according to claim 8 wherein the D-basic amino acid is D-Arg.
10. A peptide according to any one of claims 7 to 9, wherein one of the amino acids has been substituted by a D- amino acid.
- 10 11. A peptide according to any one of claims 1 to 10, for use in medical therapy.
12. The use of a peptide according to any of claims 1 to 10, in the preparation of a medicament for use in the therapy of conditions displaying increased production or function of granulocytes or in the therapy of conditions requiring any one of
15 inhibition of tyrosine phosphorylation; blocking of the eosinophil survival promoting effects of interleukin-5 and granulocyte/macrophage colony-stimulating factor; or inhibition of the growth and differentiation of eosinophils from bone marrow cells.
13. The use of a peptide according to any of claims 1 to 10, in the preparation of a
20 medicament for use in the therapy of asthma, allergic rhinitis, allergic conjunctivitis, idiopathic eosinophilic syndrome, eosinophilic pneumonia, allergic bronchopulmonary aspergillosis, Churg-Strauss syndrome, eosinophilic gastritis, Loeffler's syndrome or myelodysplastic syndromes.
- 25 14. A pharmaceutical formulation comprising a peptide according to any one of claims 1 to 10, and a pharmaceutically acceptable carrier therefor.

15. A method of treating a condition involving increased production or function of eosinophils or other granulocytes, comprising the step of administering a pharmacologically effective dose of a peptide as claimed in any one of claims 1 to 10.
- 5 16. The method of claim 15, wherein the conditions is one of: asthma, allergic rhinitis, allergic conjunctivitis, idiopathic eosinophilic syndrome, eosinophilic pneumonia, allergic bronchopulmonary aspergillosis, Churg-Strauss syndrome, eosinophilic gastritis, Loeffler's syndrome or is a myelodysplastic syndrome.

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IP: Biotinylated Peptide
WB: Anti-Lyn

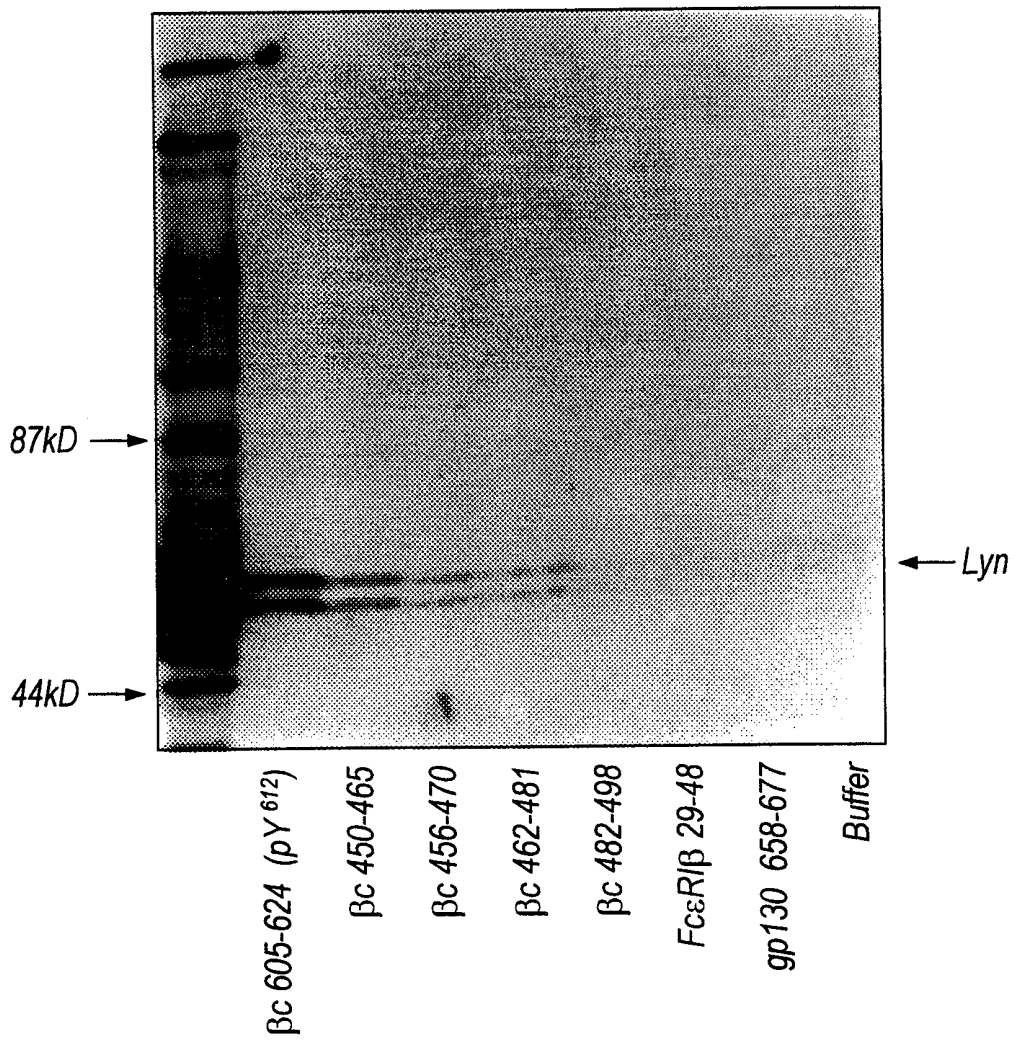
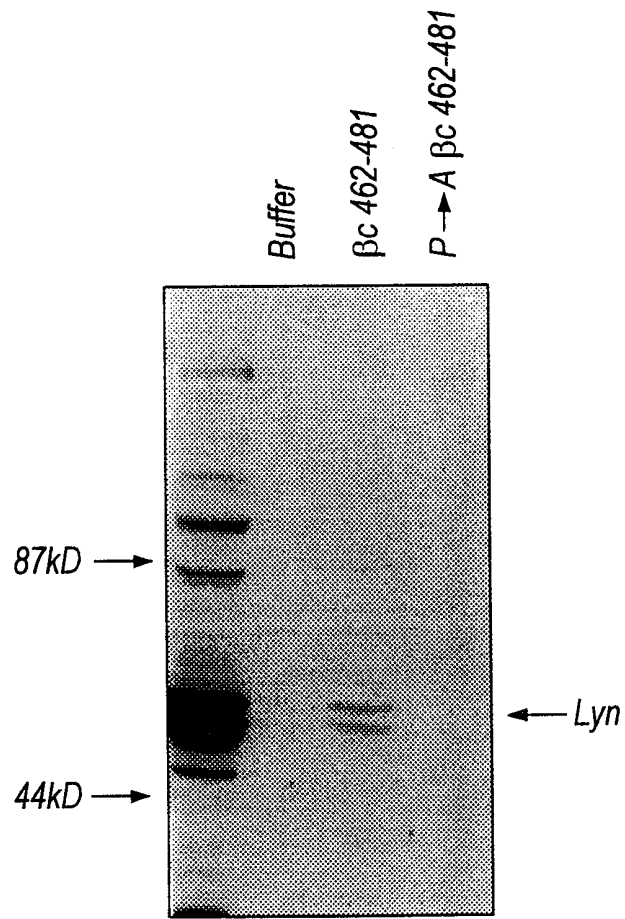


Fig. 1

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IP: Biotinylated Peptide
WB: Anti-Lyn

Fig.2

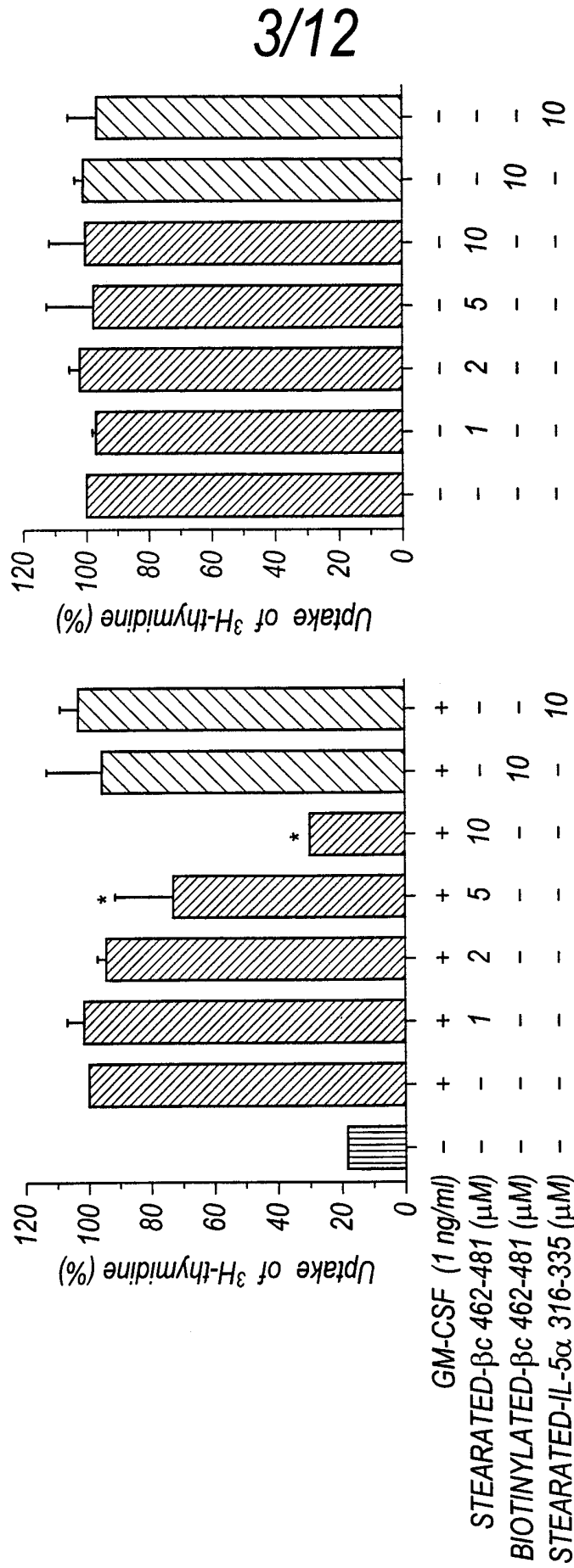
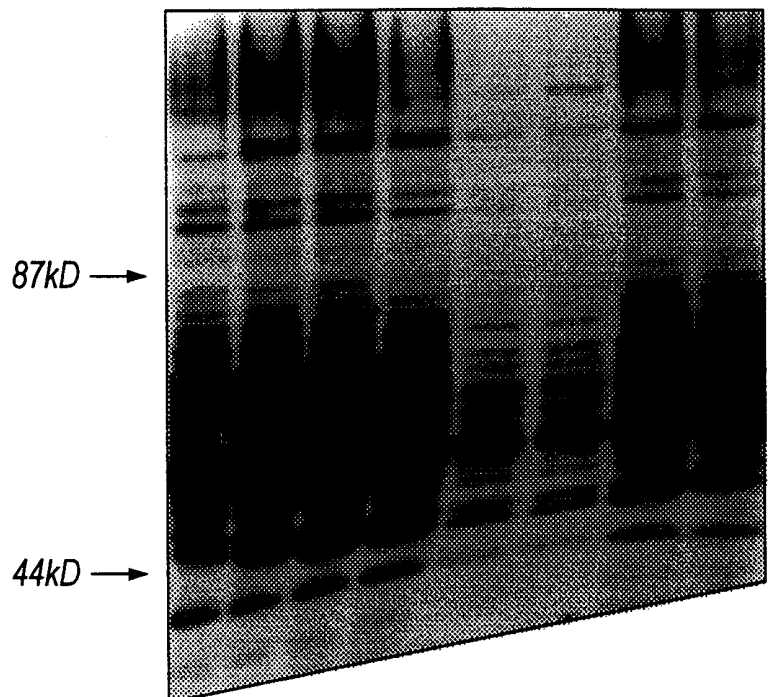


Fig.3(b)

Fig.3(a)

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WB: Anti-Phosphotyrosine



GM-CSF (10 ng/ml)	-	+	+	+	+	+	+	+
STEARATED- β c 462-481 (μ M)	-	-	2	5	10	20	-	-
BIOTINYLATED- β c 462-481 (μ M)	-	-	-	-	-	-	20	-
STEARATED-IL-5 α 316-335 (μ M)	-	-	-	-	-	-	-	20

Fig.4

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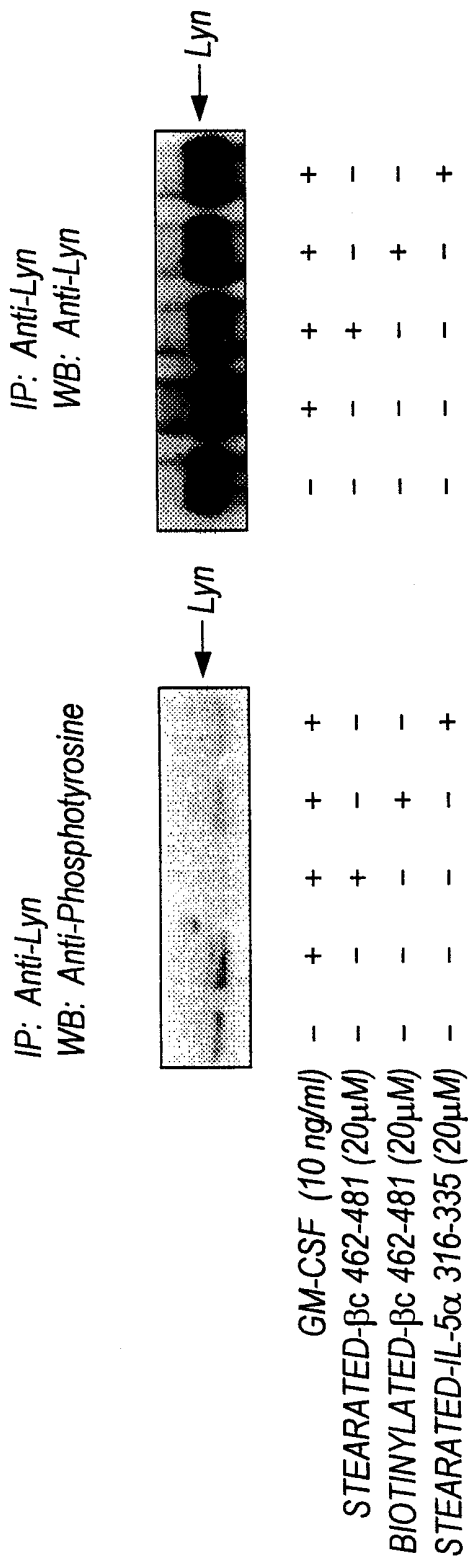


Fig.5

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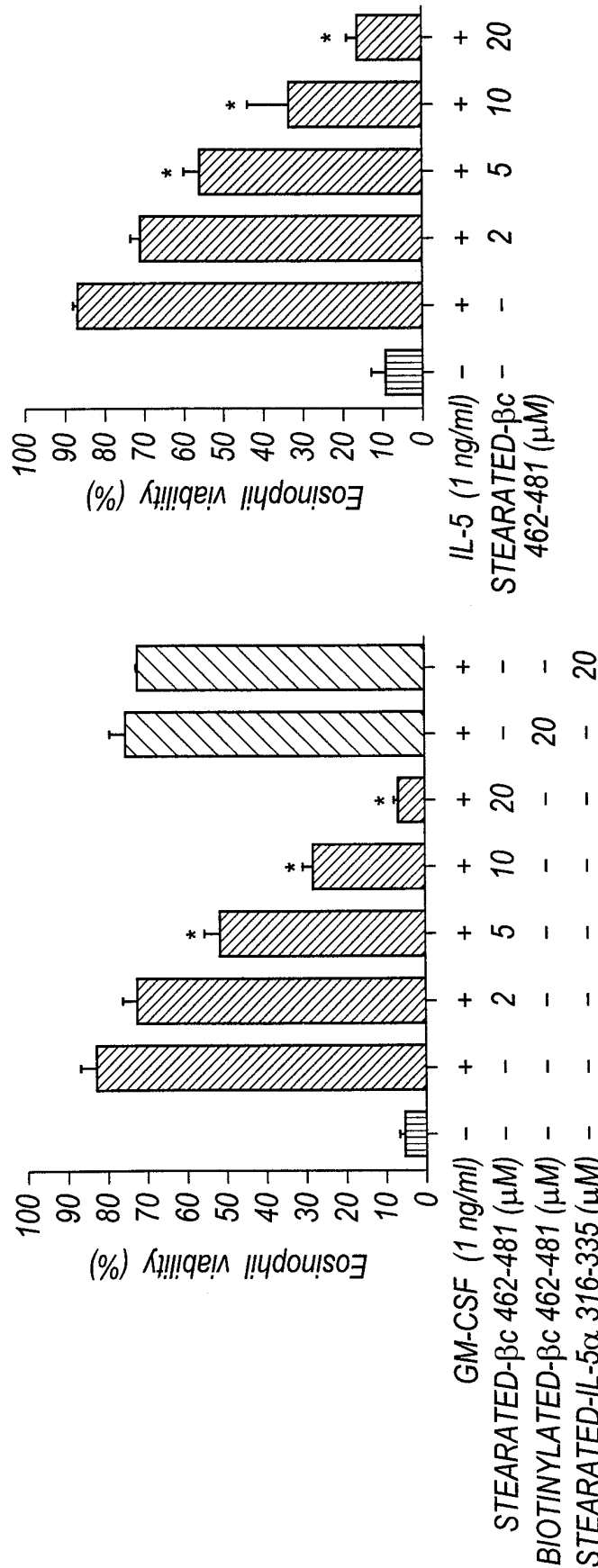


Fig. 6(b)

Fig. 6(a)

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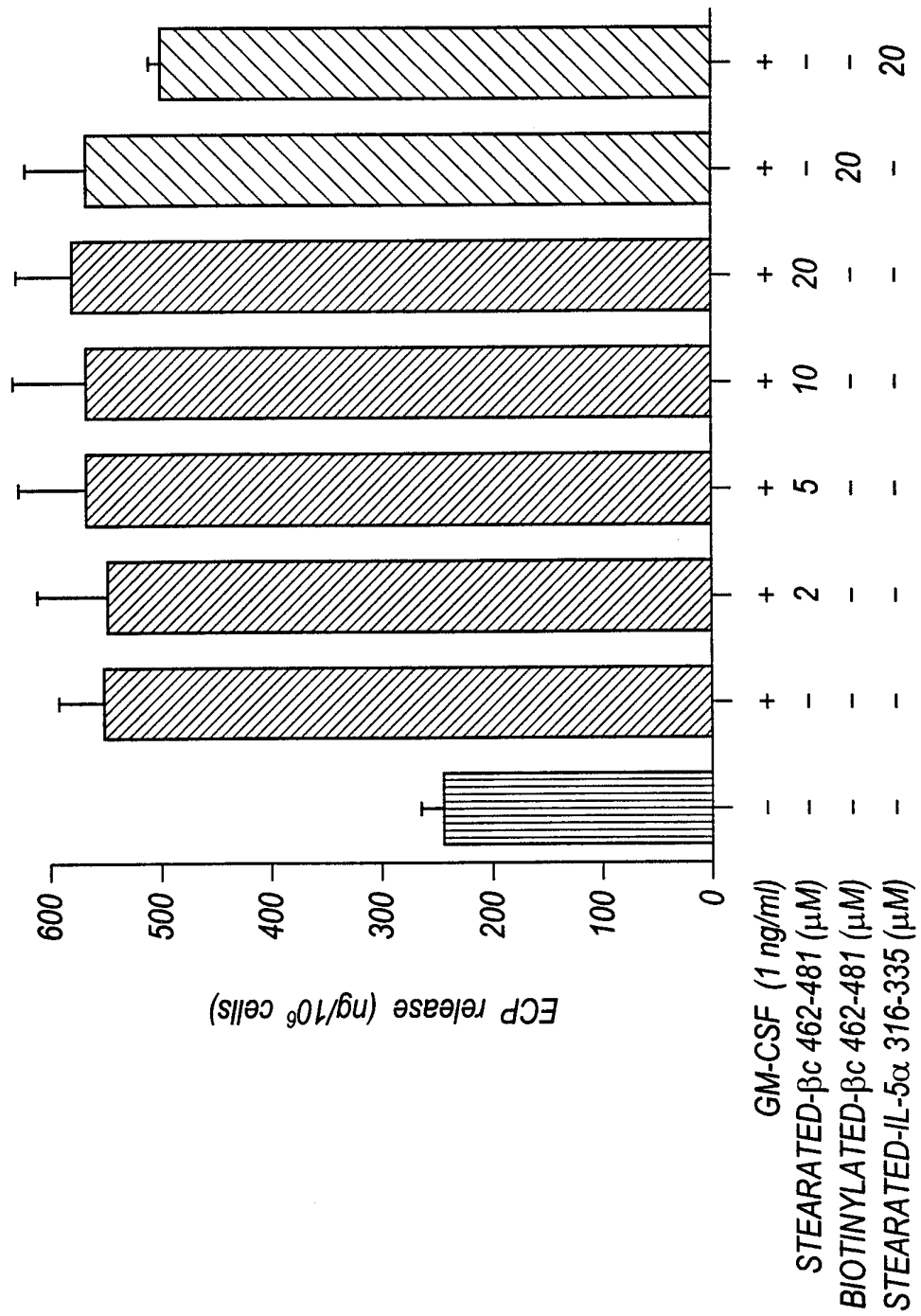


Fig.7

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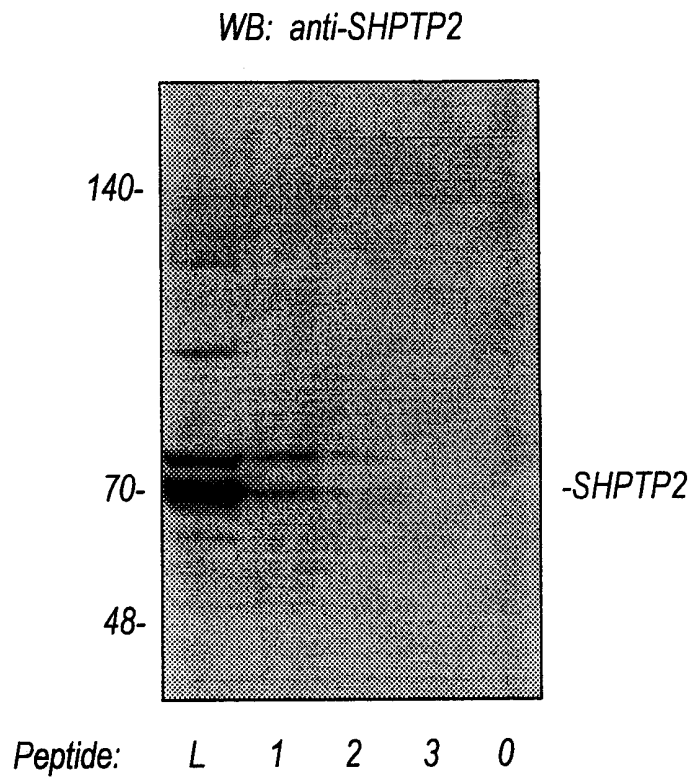


Fig.8

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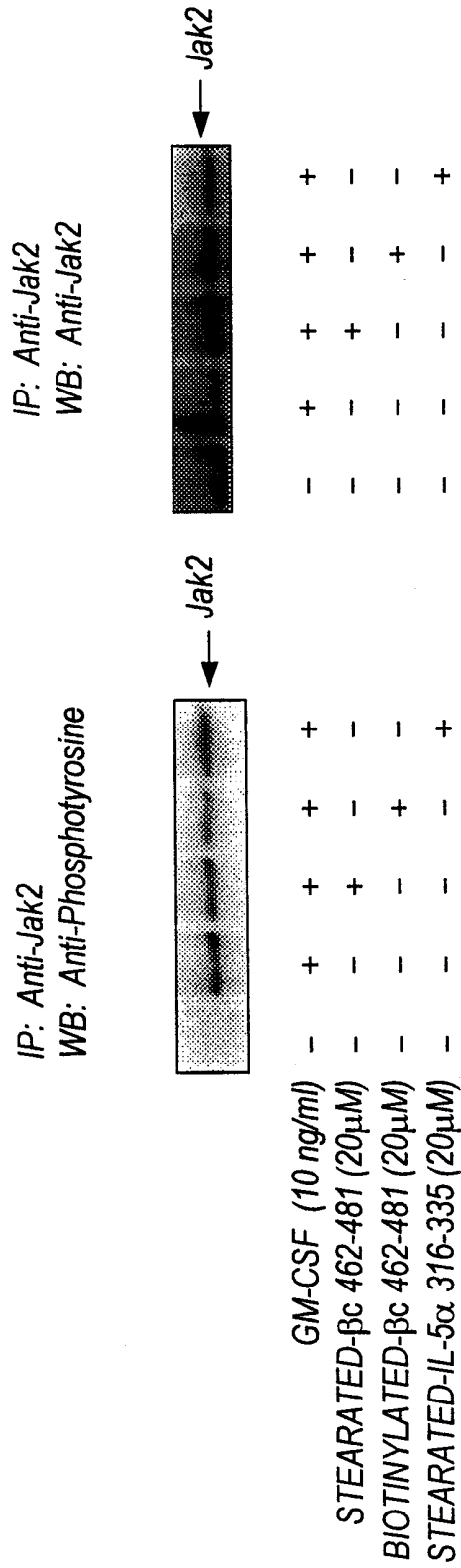


Fig.9

10/12

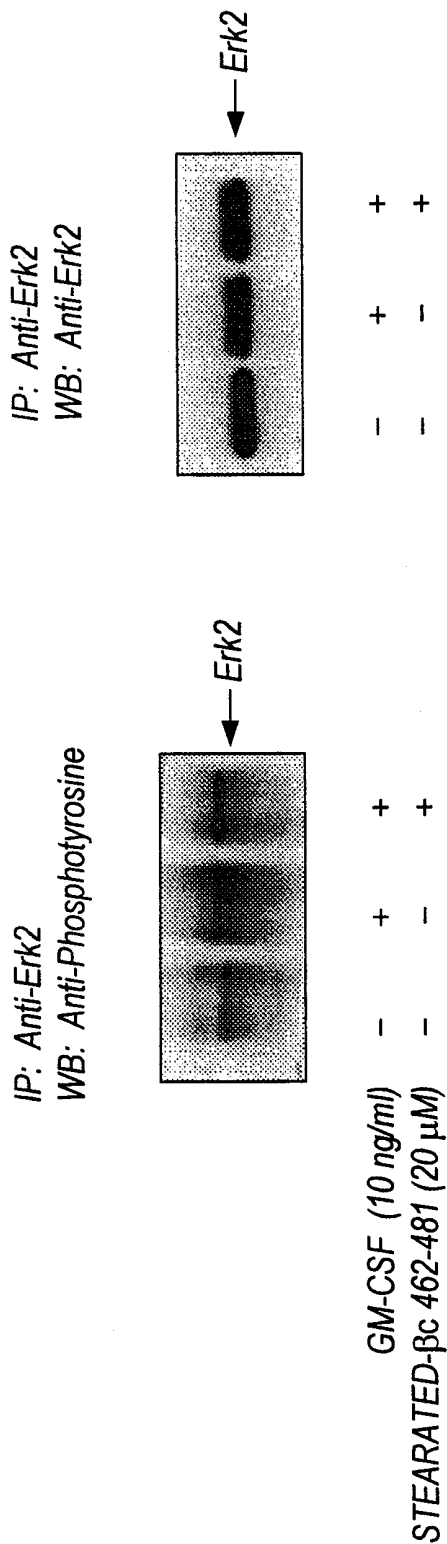


Fig. 10

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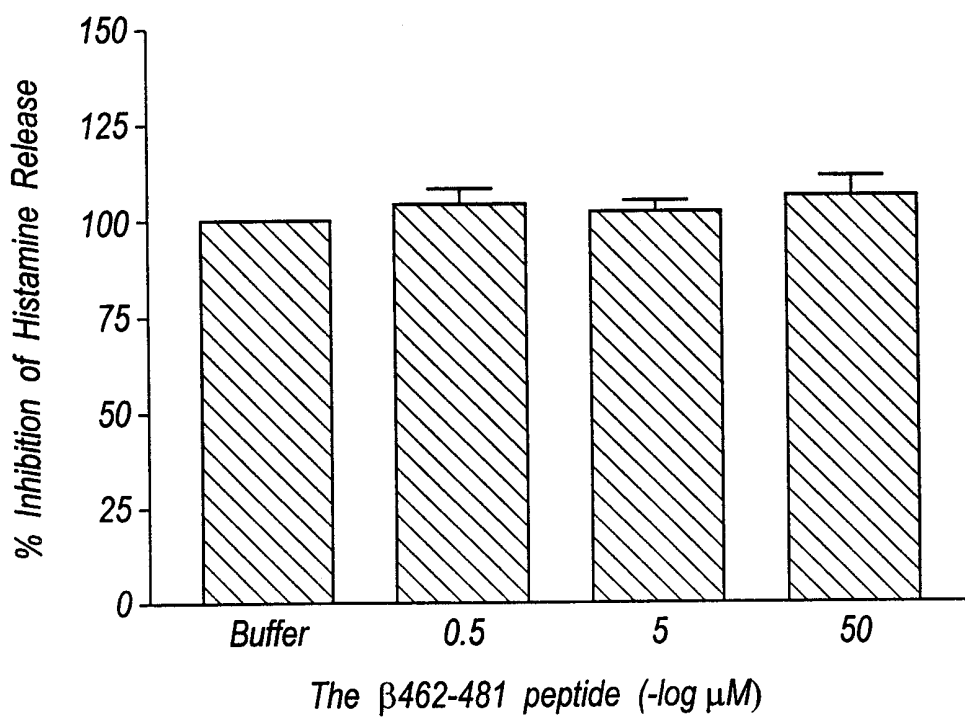


Fig.11

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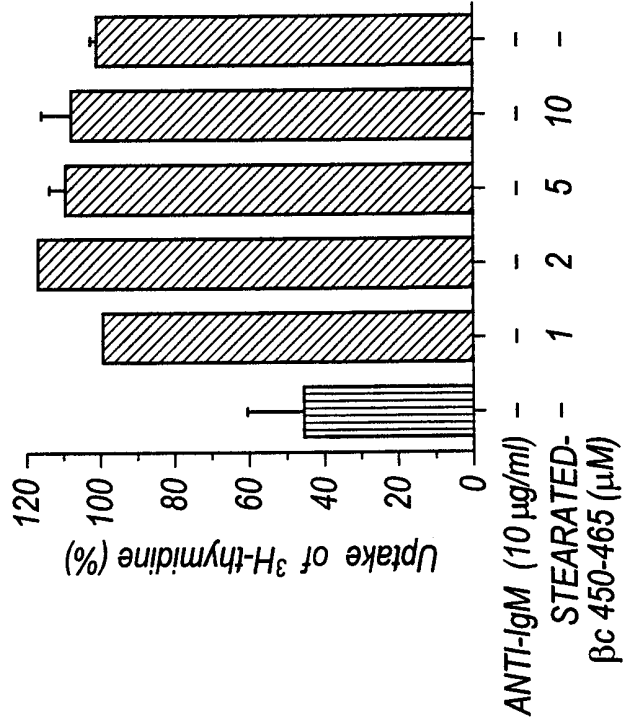


Fig. 12(a)

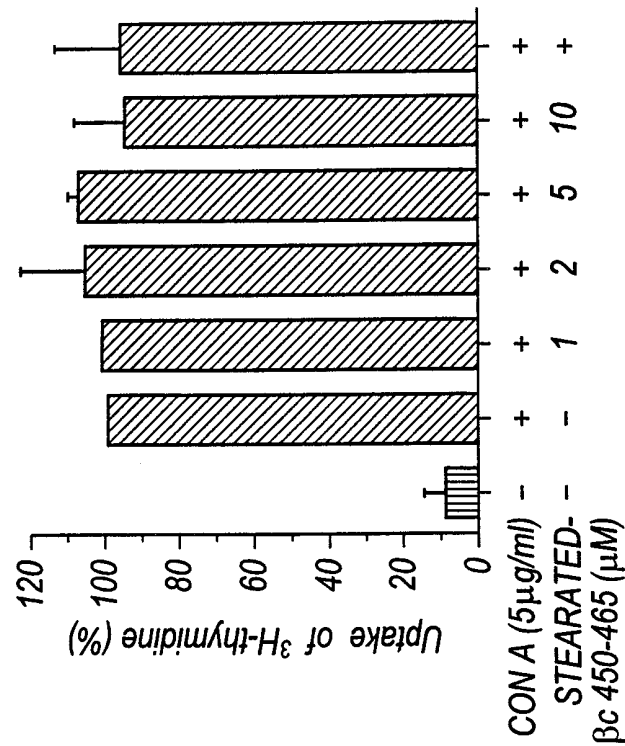


Fig. 12(b)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/01687

A. CLASSIFICATION OF SUBJECT MATTER		
IPC6: C07K 14/715, A61K 38/19, C12N 9/12 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)		
IPC6: C07K, A61K, C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JBC -- Rao and Mufson, Volume 270, No 12, March 1995, Padmini Rao et al, "A Membrane Proximal Domain of the Human Interleukin-3 Receptor beta Subunit That Signals DNA Synthesis in NIH 3T3 Cells Specifically Binds a Complex of Src and Janus Family Tyrosine Kinases and Phosphatidylnu.....", page 6886 - page 6893, see the whole document	1-2,5-6
A	--	3-4,7
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
<p>* Special categories of cited documents:</p> <p>"A" document: defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"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)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to underpin the principle or theory underlying the invention</p> <p>"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</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
Date of the actual completion of the international search		Date of mailing of the international search report
15 January 1999		22 -01- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer Patrick Andersson

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/01687

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Biological Chemistry, Volume 272, No 22, May 1997, Heather Bone et al, "SHP1 and SHP2 Protein-tyrosine Phosphatases Associate with Betac after Interleukin-3-induced Receptor Tyrosine Phosphorylation", page 14470 - page 14476, table 1, and the whole document	1-2,5-6
A	--	3-4,7
X	Journal of immunology, Volume 155, No 4, August 1995, Yan Li et al, "Association Between Lyn Protein Tyrosine Kinase (p53/56lyn) and the beta subunit of the Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) Receptors in a GM-CSF-Dependent Human Megakaryocytic Leukemia", page 2165 - page 2174, page 2172, column 1, lines 7-12, and the whole document	1,5,11-14
A	--	2-4,6-10
A	WO 9728190 A1 (MEDVET SCIENCE PTY. LTD.), 7 August 1997 (07.08.97), page 3, line 38 - page 4, line 1	1-14
	-- -----	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/01687

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)(a) for the following reasons:

1. Claims Nos.: 15-16
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39(iv). Methods for treatment of the human or animal body by therapy.
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.

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/12/98

International application No.

PCT/SE 98/01687

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9728190 A1	07/08/97	AU 1536697 A AU PN780096 D	22/08/97 00/00/00
