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HAEMOPOIETIC GROWTH FACTOR ANTAGONISTS AND USES THEREFOR

- 5 The present invention relates generally to variant recombinant forms of haemopoietic growth factors useful as antagonists to the corresponding native haemopoietic growth factor and their use in ameliorating aberrant effects caused by the native molecules and in the treatment of tumours and cancers and inflammation.
- 10 Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined following the description.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of
15 a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The rapidly increasing sophistication of recombinant DNA technology is greatly facilitating research and development in a range of industries. The medical and allied health fields in
20 particular have, and continue to, benefit from this developing technology. An area of substantial interest is the field of growth factors and cytokines.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is one member of a family of haemopoietic growth factors (HGFs) and exhibits a range of important activities such as
25 supporting survival of normal leukaemic and haemopoietic cells by suppressing programmed cell death, which is also known as apoptosis. GM-CSF binds to a heterodimeric receptor composed of a GM-CSF specific α chain and a β c chain which is shared with the receptors for interleukins (IL) 3 and 5. Both chains are required for GM-CSF mediated signalling.

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In International Patent Application No. PCT/AU94/00432 filed on 28 July, 1994, which is incorporated herein by reference, a series of HGF antagonists was described. It has now been surprisingly discovered that certain forms or types of these antagonists induce apoptosis in cells expressing HGF receptors. This property of the HGF antagonists provides *inter alia* a novel
5 approach to the treatment of a range of tumours and cancers and in particular myeloid leukaemias.

Accordingly, one aspect of the present invention contemplates a method for inducing apoptosis in cells carrying an HGF heterodimeric receptor comprising an HGF-specific α -chain and a β c
10 chain, said method comprising contacting said cells with an effective amount of an HGF antagonist for a time and under conditions sufficient to induce apoptosis wherein said HGF antagonist comprises a sequence of amino acids within a first α -helix of HGF wherein one or more exposed amino acids in said first α -helix of HGF having acidic properties is/are substituted with a basic amino acid residue or a non-acidic amino acid residue.

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The HGFs are preferably GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-13, IL-14, IL-15 and others of this family yet to be discovered, granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO) and thrombopoietin (TPO). Most preferably, the HGF is GM-CSF.

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According to this aspect of the present invention there is provided a method for inducing apoptosis in cells carrying an HGF heterodimeric receptor comprising an HGF-specific α -chain and a β c chain, said method comprising contacting said cells with an effective amount of an GM-CSF antagonist for a time and under conditions sufficient to induce apoptosis wherein said
25 GM-CSF antagonist comprises a sequence of amino acids within a first α -helix of HGF wherein one or more exposed amino acids in said first α -helix of HGF having acidic properties is/are substituted with a basic amino acid residue or a non-acidic amino acid residue.

The HGF antagonists are preferably produced in recombinant or synthetic form and, with the exception of the amino acid substitution(s) in the first α -helix, the amino acid sequence of the HGF may be the same as the naturally occurring molecule (i.e. native molecule) or may carry single or multiple amino acid substitutions, deletions and/or additions to the native sequence.

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In one particular embodiment, the HGF antagonists are:

- (i) in unglycosylated form;
- (ii) lack post-translational modification;
- (iii) produced in prokaryotic microorganisms; and/or
- 10 (iv) produced by chemical synthesis.

The antagonists may be derivatives of an HGF or may be any other molecule which selectively binds to the α -chain of the HGF receptor such as, but not limited to, antibodies or other small or large molecules resulting in apoptosis. The present invention is hereinafter
15 described with reference to GM-CSF antagonists and in particular human GM-CSF antagonists or mammalian GM-CSF antagonists capable of functioning in humans. This is done, however, with the understanding that the present invention extends to any HGF antagonist which is capable of inducing cell apoptosis by interaction with an HGF-specific α -chain of an HGF receptor. The present invention also extends to HGF antagonists which
20 interact with the β chain.

Although not intending to limit the present invention to any one theory or mode of action, it is proposed that the GM-CSF antagonist either blocks the action of wild-type GM-CSF by interacting selectively with the receptor α -chain or induces apoptosis by interacting with the
25 α - and β -chains of the receptor in a manner that leads to abnormal stimulation of the β -chain either qualitatively or quantitatively. Qualitative differences include recruitment of signalling molecules (for example, type of kinase or state). Quantitative differences include intensity of duration of signalling.

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The present invention is particularly directed to the use of a GM-CSF antagonist carrying a substitution of amino acid 21 (Glu) of human GM-CSF by Arg or Lys or any other basic or non-acidic amino acid residue. Such mutants are designated herein "E21R" and "E21K", respectively, which is based on a single letter designation of the amino acids involved in the substitution and the position of the substitution. The construction of vectors expressing E21R is described in the Examples. E21R is a fusion protein expressed in inclusion bodies as GM-CSF(E21R) preceded by a twelve amino acid leader sequence, namely, MFATSSSTGNDG

10 In accordance with the present invention, it has been surprisingly discovered that GM-CSF antagonist E21R binds to the GM-CSF-specific α -chain of the GM-CSF receptor and that such binding directly induces apoptosis of normal and malignant myeloid cells expressing the GM-CSF receptor. Apoptosis occurs even in the presence of the survival factors such as G-CSF and stem cell factor (SCF) but is prevented by engaging receptor chain β c with IL-3.

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Accordingly, in a particularly preferred embodiment, the present invention contemplates a method for inducing apoptosis of myeloid cells said method comprising contacting said cells with an apoptotic effective amount of a GM-CSF antagonist having a basic amino acid residue substituted at position 21 of the GM-CSF amino acid sequence for a time and under conditions sufficient for programmed cell death to initiate.

This method has particular relevance in the treatment of cancers such as myeloid leukaemias and inflammation, for example, rheumatoid arthritis and allergic conditions such as asthma. Preferred GM-CSF antagonists comprise amino acids Arg or Lys at position 21 of GM-CSF.

25 The most preferred antagonist comprises Arg at position 21 of GM-CSF and is referred to herein as E21R.

Accordingly, a further aspect of the present invention contemplates a method of inducing apoptosis in a cell carrying receptors for GM-CSF, said method comprising contacting said cell with a molecule capable of binding to the α -chain of the GM-CSF receptor or binding

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both to the α -chain and β -chain for a time and under conditions sufficient for programmed cell death to initiate.

Preferably, the molecule is a derivative of GM-CSF with amino residue 21 substituted by a basic amino acid.

Preferably, the basic amino acid residue is Arg or Lys. Most preferably, the basic amino acid residue is Arg.

10 Preferably, the molecules are:

- (i) in unglycosylated form;
- (ii) lack post-translational modification;
- (iii) produced in prokaryotic microorganisms; and/or
- (iv) produced by chemical synthesis.

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Another aspect of the present invention contemplates a method for treating myeloid leukaemia in a human subject, said method comprising administering to said human subject an effective amount of an HGF antagonist, said administration being for a time and under conditions sufficient to induce apoptosis of said myeloid leukaemic cells wherein said HGF antagonist
20 comprises a sequence of amino acids within a first α -helix of HGF wherein one or more exposed amino acids in said first α -helix having acidic properties is/are substituted with a basic amino acid residue or a non-acidic amino acid residue.

Preferably, the HGF receptor is a GM-CSF receptor.

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Preferably, the HGF antagonist is a GM-CSF antagonist.

Preferably, the GM-CSF antagonist comprises Arg or Lys at position 21 of the GM-CSF amino acid sequence.

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Preferably, the GM-CSF antagonist is E12R.

Induction of apoptosis will also be useful in enrichment of uncommitted progenitors for stem cell transplantation and gene transfer purposes. According to this aspect of the present invention there is provided a method for enriching uncommitted progenitor cells from bone marrow said method comprising contacting a population of cells from bone marrow with an effective amount of a molecule capable of stimulating the α -chain of an HGF receptor without concomitant stimulation of the β c chain of said receptor for a time and under conditions sufficient to induce apoptosis of cells carrying said α -chain.

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Preferably, the molecule is a derivative of an HGF having a sequence of amino acids within a first α -helix wherein one or more exposed amino acids in said first α -helix having acidic or acidic-like properties are substituted with a basic amino acid residue or non-acidic amino acid residue.

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Preferably, the molecule is a derivative of GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-13, IL-14, IL-15 and others of this family yet to be discovered, G-CSF, EPO or TPO.

20 Preferably, the molecule is GM-CSF having Arg or Lys at position 21 in place of Glu.

Most preferably, the molecule is E21R.

Preferably, apoptosis occurs with committed progenitors (CD34⁺ CD38⁺, CD34⁺ CD33⁺)
25 but not CD34⁺ CD38⁻ cells.

The present invention further contemplates genetic constructs useful in expressing E21R including constructs comprising the nucleotide sequence encoding the leader sequence MFATSSSTGNDG.

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The present invention also contemplates a method for purifying E21R with or without the leader sequence following expression of the above-mentioned genetic constructs.

Yet a further aspect of the present invention contemplates eukaryotic or prokaryotic organisms
5 carrying the above-mentioned genetic constructs.

Preferred aspects of these embodiments are described in the Examples.

The present invention is further described by reference to the following non-limiting Figures
10 and Examples.

In the Figures:

Figure 1 is a schematic representation of plasmid pBRE132. The DNA cassette has a unique
15 EcoRV cloning site and codes for MFATSSSTGND leader sequence.

Figure 2 is a schematic representation of plasmid pBRE133. *SmaI/HindIII* GM-E21R PCR
DNA was cloned into *EcoRV/HindIII* sites of pBRE132. pBRE133 encodes E21R
MFATSSSTGNDG-GM-CSF (E21R) protein.

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Figure 3 is a graphical representation showing competition of binding of E21R and 1nM ¹²⁵I-
GM-CSF to CHO cells transfected with GMR α (GM-CSF-specific α -chain of GM-CSF
receptor).

25 Figure 4 is a graphical representation showing antagonism of GM-CSF by E21R in a TF1
proliferation assay.

Figure 5 is a graphical representation showing antagonism of GM-CSF by E21R in a
neutrophil superoxide assay.

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Figure 6 is a graphical representation showing that E21R inhibits GM-CSF stimulated proliferation of AML cells.

Figure 7 is a graphical representation showing that E21R inhibits GM-CSF stimulated proliferation of CML cells.

Figure 8 is a graphical representation showing that E21R inhibits colony growth of JCML cells.

Figure 9 is a photographic representation showing that the E21R causes DNA fragmentation in myeloid cells.

Figure 10 are graphical representations showing that E21R induces apoptosis of $GMR\alpha^+$ myeloid leukaemic cells in a time- and dose-dependent manner.

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Figure 11 are graphic representations showing that E21R induces apoptosis of $CD34^+/GMR\alpha^+$ haemopoietic progenitors.

Figure 12 is a graphical representation showing that E21R-induced apoptosis requires transcription, protein synthesis and kinase activity. (A) Titration of genistein and staurosporine in the absence of (open symbols) of E21R (1 $\mu\text{g/ml}$). (B) Titration of actinomycin D and cycloheximide in the absence (open symbols) and presence (solid symbols) of E21R (1 $\mu\text{g/ml}$). Apoptosis was measured at the 48 hr time point. Data are means and SEM of triplicate samples from one AML case and are representative of four other cases.

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Figure 13 shows that the membrane proximal domains of both receptor chains are required for induction of apoptosis, while deletions of the more distal part of either chain did not affect E21R-mediated apoptosis.

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EXAMPLE 1**CONSTRUCTION OF pBRE132 AND pBRE133 FOR EXPRESSION OF
RECOMBINANT E21R PROTEIN****5 Construction of pBRE132 expression plasmid**

A synthetic DNA cassette was constructed by annealing two oligonucleotides, TATGTTTCGCTACTTCAAGCTCTACGGGGAACGATATCGCTGCAGCCA (SEQ ID NO:1) and AGCTTGGCTGCAGCGATATCGTTCCCCGTAGAGCTTGAAGTAGCGAACA (SEQ ID NO:2) at 65°C for 5 min in 200 mM NaCl. The synthetic DNA cassette was
10 cleaved with *NdeI/HindIII* and cloned into pEC611 expression vector (A. Brumby, 1987 "A vector for high expression of native proteins", Honours Thesis, University of Adelaide), generating pBRE132 (Figure 1).

Construction of pBRE133 (E21R expression vector)

15 GM-CSF (E21R) synthetic cDNA was amplified employing PCR primers with unique *SmaI* and *HindIII* sites. The PCR products were cleaved with *SmaI/HindIII* and cloned into the *EcoRV/HindIII* sites of pBRE132 generating pBRE133 (Figure 2).

Expression of pBRE133 in *Escherichia coli* MM294/pACYClac

20 Expression analysis demonstrated that pBRE133 in *Escherichia coli* MM294/pACYClac expressed inclusion bodies (IB) consisting of recombinant E21R protein, as judged by microscopy examination, standard SDS-PAGE and mass spectrometry analysis of the purified protein.

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EXAMPLE 2**PRODUCTION OF GRANULOCYTE MACROPHAGE COLONY
STIMULATING FACTOR ANTAGONIST E21R****1. FERMENTATION****5 A. Inoculation**

1. *Escherichia coli* MM294/pACYC1ac carrying pBRE133 was streaked out from a - 80°C glycerol stock onto a minimal medium plate containing ampicillin (100 µg/ml) and kanamycin (30 µg/ml) and grown overnight at 37°C.

10 2. Multiple colonies were assessed for GM-CSF (E21R) expression levels by microscopic examination of 20ml shake flask cultures containing inducer, or by colony size on agar plates with or without inducer, in this instance with IPTG.

3. The selected single colony from an agar plate was transferred into 20ml of minimal
15 medium containing ampicillin (100 µg/ml) + kanamycin (30 µg/ml). The culture was grown at 37°C overnight with agitation.

4. An amount of 10L of nitrogen-rich minimal medium, "C2", was sterilised in a 22 L fermenter and inoculated to a very low density with overnight culture. Growth was at 37°C
20 and a pH of 7.0 was maintained by the addition of 12.5 M NH₄OH.

5. Agitation was manually controlled and aeration automatically controlled with oxygen saturation levels remaining above 10% pO₂. Following depletion of glucose at 16-18 hours the cell mass was fed with a concentrated glucose solution containing additional salts.
25 Nutrient feed flow rate was determined by pH or oxygen saturated levels.

B. Induction

1. At an optical density of $A_{600} = 50-60$ the recombinant expression of E21R antagonist was induced, in this instance by the addition of IPTG. The culture was supplemented with
30 amino acids at the time of induction ($T=0$) and at $T=2$ hours. Induction continued for 4



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hours at 37°C, pH 7.0. Samples were removed at T = 0,1,2,3 & 4 hours and examined by microscopy for the presence of IB, their size being determined by disc centrifugation.

2. The culture was stored overnight at 4°C.

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2. PRIMARY ISOLATION OF GM-CSF (E21R) INCLUSION BODIES

1. *Homogenization* - Step 1

The culture was passed five times through a Gaulin 30CD homogenizer at 13500 psi, with homogenate being cooled between passes. Homogenate was then diluted with an equal 10 volume of RO H₂O.

The IB size was redetermined by disc centrifugation.

2. *Centrifugation* - Step 1

15 The homogenate was centrifuged in a Westfalia SB-7 separator with a constant speed of 9210 rpm at a flow rate determined by IB size.

The concentrate collected from this first step was diluted to $\leq 2.5\%$ v/v.

20 3. *Homogenization* - Step 2

The IB suspension was passed once through a Gaulin 30CD homogenizer at 13500 psi.

The IB size was again determined by disc centrifugation.

25 4. *Centrifugation* - Step 2

The homogenate was centrifuged in a Westfalia SA-1 separator with a constant speed of 9700 rpm at a flow rate determined by IB size.

EXAMPLE 3**GM-CSF ANTAGONIST (E21R) PURIFICATION**

The inclusion body (IB) solution is first washed with an equal volume of a mild buffered denaturant such as 2M GuHCl. The solution is stirred gently at 4°C for 30 minutes. The washed IB can then be recovered with a low speed centrifuge spin. The washed IB are then resuspended in a small volume of water (e.g. Milli Q), immediately prior to dissolution and refolding.

Dissolution, refolding and oxidation of the IB GM-CSF antagonist (E21R) is initiated by dissolving the washed IB in an unbuffered urea solution at an alkaline pH. To ensure complete dissolution of the IB, the concentration of urea should be between 2.5M and 4M and the pH between 11 and 12. The concentration of protein can be between 0.5 and 5 mg/ml. These three parameters can be varied depending on the purity of the IB preparation. The stirred solution is maintained at an alkaline pH with a sodium hydroxide solution (e.g. 2M NaOH), until it can be seen that refolding and oxidation has reached equilibrium. The extent of refolding to the correct fold for GM-CSF can be monitored by chromatographic means, e.g. reversed phase HPLC (RP-HPLC) or size-exclusion chromatography (SEC), comparing the chromatographic profile to that of a known folded standard of GM-CSF. Once refolding has reached equilibrium, the pH of the solution is lowered to a less alkaline pH, (e.g. pH 9.1) with a concentrated solution of a suitable buffer (e.g. 0.5M sodium borate pH 9.1).

The correctly refolded GM-CSF antagonist (E21R) can then be purified from incorrectly folded and aggregated forms using chromatography, e.g. anion-exchange chromatography (IEC) or RP-HPLC. For example, the refolding solution from above is loaded onto a anion-exchange column (e.g. Q-Sepharose Fast flow [Pharmacia]). Upon completion of loading, the column is washed with two column volumes of a low salt buffer. The correctly folded form of GM-CSF antagonist (E21R) is eluted from the column with a gradient of sodium chloride (e.g. 0-500 mM NaCl over ten column volumes). The column eluent can be analysed for GM-CSF antagonist (E21R) purity by either SEC (e.g. Superose 12 [Pharmacia]), or RP-HPLC (e.g. Brownlee butyl-silica column (100 mm x 2.1 mm i.d.)





employing 0.1% v/v TFA in water as Buffer A and 0.1% v/v TFA in acetonitrile as Buffer B).

The purified GM-CSF antagonist (E21R) can then be desalted (e.g. Sephadex G-25) into a suitable buffer (e.g. 5 mM Na_2HPO_4 pH 7.6) in preparation for formulation and lyophilisation. The desalted protein can be quantitated by its absorption at 280 nm in a spectrophotometer using a $a_{280}^{1\%}$ of 9.09. Following quantitation, 1 part glycine and 5 parts mannitol, by weight, are added to the desalted protein solution. The solution is stirred gently until all solids are dissolved. If necessary, the pH is adjusted to pH 7.6 with 1M NaOH or 25% w/v orthophosphoric acid. The solution is then sterilised by filtration (0.2 μm), aliquoted into sterile glassware and lyophilised.

EXAMPLE 4

E21R EXHIBITS ANTAGONISTIC PROPERTIES

Purified E21R bound the GM-CSF receptor α -chain with the same potency as wild type GM-CSF (Figure 3). In addition, E21R did not directly stimulate TF-1 cell proliferation (Figure 4) or neutrophil O_2^- release (Figure 5) but instead antagonised the stimulatory effect of GM-CSF in both functional assays (Figures 4 and 5). Using cells from patients with acute myeloid leukaemia (AML) the inventors found that E21R antagonised the stimulation of AML cell proliferation by GM-CSF in a dose-dependent manner (Figure 6). This was specific for GM-CSF in that E21R did not antagonise IL-3-mediated proliferation (Figure 6). E21R completely antagonised the proliferative effect of GM-CSF on cells from patients with chronic myeloid leukaemia (CML) (Figure 7).



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EXAMPLE 5**E21R INHIBITS COLONY FORMATION BY JUVENILE CHRONIC
MYELOID LEUKAEMIA (JCML) CELLS**

JCML is a rare disease which is generally diagnosed in children under 2 years. There is no
5 effective therapy for this condition and it is invariably fatal 2-3 years after diagnosis. Cells
from JCML patients grow spontaneously in culture probably due to autocrine production of
GM-CSF feeding back on these cells.

It was found that E21R inhibited the growth of JCML in a dose-dependent manner (Figure
10 8). This may be due to blocking autocrine GM-CSF, induction of apoptosis (see below), or
a different mechanism.

EXAMPLE 6

Since E21R binding to the α -chain antagonized GM-CSF stimulated cell function, the
15 inventors reasoned that E21R might interfere with cell survival. The life span of malignant
cells expressing the GM-CSF receptor from patients with either acute (AML) or chronic
(CML) myeloid leukaemia was studied. When these cells were incubated with E21R,
chromosomal DNA was degraded into low-molecular weight fragments, a salient feature of
apoptosis (Figure 9).

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E21R caused DNA from myeloid leukaemic cells to degrade. DNA degradation was absent
in cells incubated with either GM-CSF alone or medium only. The data are from one AML
case and representative of 20 other cases. Normal bone marrow (BM) cells expressing
CD34/GMR α , CD34/CD38/GMR α and CD34/CD33/GMR α also underwent apoptosis with
25 E21R. In the CD34⁺/CD38/GMR α population, DNA degradation was not present. The data
are from one BM case and representative of two other cases. Markers of known molecular
sizes in kilobases (kb) are shown. Peripheral blood cells from patients with AML (all FAB
subtypes) at diagnosis were Ficoll separated. The T lymphocytes were removed by incubation
with a mouse anti-human CD3 monoclonal antibody followed by a rabbit anti-mouse antibody
30 coupled to a magnetic bead (Miltenyi Biotech, Gladbach, Germany). The AML population

consisted of > 99% blasts. Bone marrow progenitors were collected from a healthy donor and the CD34⁺ subsets were sorted by flow cytometry (FACStar^{PLUS}, Becton-Dickinson, NJ). Cells that expressed the GMR α were isolated using an anti-GMR α monoclonal antibody and a magnetic bead procedure. The cells were incubated in RPMI 1640 medium with 10% v/v fetal calf serum (FCS), antibiotics, sodium bicarbonate, and L-glutamine for 48 h at 37°C. To the cultures either *E. coli*-derived E21R (1 μ g/ml) or GM-CSF (0.3 ng/ml, Genetics Institute, Cambridge, MA) were added. The medium used to culture bone marrow cells contained G-CSF (10 ng/ml, Amgen, Thousand Oaks, CA). DNA was extracted and analyzed after agarose gel electrophoresis.

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Incubation of the cells with either GM-CSF alone or medium only for up to 48 h caused no degradation of the DNA. Staining of DNA with propidium iodide from one AML case revealed that a 48-h incubation with E21R (1 μ g/ml) caused apoptosis of almost all the cells Figure 10 (a). The G0/G1, S and G2 regions represent cells in various phases of the cell cycle, while the subdiploid Ap peak denotes apoptotic cells. To quantify the number of apoptotic cells, the DNA binding of propidium iodide was measured. Initially about 75% of AML cells underwent apoptosis in the presence of E21R at 48 h. This proportion of apoptotic cells correlated well with the number of cells expressing the GM-CSF α receptor. E21R (1 μ g/ml) induced apoptosis in 21/21 AML cases (Figure 10b) and 14/14 CML cases (Figure 10c).

20

To examine whether the apoptotic effect of E21R was secondary to antagonism of GM-CSF present in the culture conditions or due to a direct effect to E21R, several controls were performed. First, it is known that while normal bone marrow haemopoietic cells do not express the GM-CSF mRNA, some AML cells can produce GM-CSF. However, GM-CSF was detected in supernatants of only 8 of 21 AML cases. The other 13 cases did not show detectable GM-CSF in the supernatants, and an RNase protection assay showed lack of mRNA for GM-CSF. Second, biologically active GM-CSF in the culture medium was not present: enumeration of apoptotic cells in serum-free medium yielded similar results as depicted in Figure 10b, and the neutralising anti-GM-CSF monoclonal antibody 4D4 did not induce apoptosis of the AML cells (Figure 10e). Finally, to determine whether any GM-CSF was



bound to the AML cells when transferred *ex vivo* to the *in vitro* cultures, the inventors measured the cell surface-associated binding of ^{125}I -labelled GM-CSF before and after acid elution. No differences were observed in specific GM-CSF binding, indicating that the cells were not carrying over receptor-bound GM-CSF. E21R did not induce apoptosis of myeloid cells
5 lacking GMR α indicating that E21R was not toxic.

A titration of E21R showed that full apoptotic effect was achieved with 1 $\mu\text{g/ml}$ (d). A GM-CSF concentration of 100 ng/ml effectively inhibited E21R (1 $\mu\text{g/ml}$) mediated apoptosis (d). Values are the mean and s.e.m. of three replicates from one AML case.

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When combined with G-CSF (10 ng/ml), but not with IL-3 (10 ng/ml, Genetics Institute), E21R induced apoptosis of nearly all the cells (e). Peripheral blood cells were collected from untreated CML patients (all Philadelphia chromosome positive) in blast crisis and processed as for the AML cells described above. Apoptosis was determined by flow cytometry as the
15 reduced amount of propidium iodide bound by DNA after incubating triplicate cultures of the cells overnight in a buffer containing 0.1% v/v Triton X-100 and propidium iodide (50 mg/ml).

EXAMPLE 7

Similar to normal haemopoiesis, myeloid leukaemic cells stem from a small pool of highly
20 proliferative progenitors that express the CD34 surface marker. To examine whether the effect of E21R was a general phenomenon related to the GMR α expression or restricted to malignant cells, the effect of E21R on the survival of normal CD34 $^{+}$ progenitors was studied. Bone marrow cells were fractionated by flow cytometry into total CD34 $^{+}$, CD34 $^{+}$ CD38 $^{+}$ (committed), and CD34 $^{+}$ CD33 $^{+}$ (myeloid committed) progenitors and the apoptotic fractions determined
25 after E21R treatment. DNA fragmentation was present in the CD34 $^{+}$ /GMR α^{+} , CD34 $^{+}$ CD38 $^{+}$ /GMR α^{+} , and CD34 $^{+}$ CD33 $^{+}$ /GMR α^{+} subsets, but not amongst the non-committed CD34 $^{+}$ CD38 $^{-}$ cells, the latter subset lacking GMR α (Figure 9). Analysis of DNA binding of propidium iodide revealed that E21R caused apoptosis of the cells in the CD34 $^{+}$ CD38 $^{+}$ /GMR α^{+} (Figure 11a,c), CD34 $^{+}$ /GMR α^{+} , and the CD34 $^{+}$ CD33 $^{+}$ /GMR α^{+} populations (Figure 11c), but
30 not on cells lacking the GM-CSF α chain.



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E21R in medium with G-CSF induced apoptosis of the CD34⁺CD38⁺/GMR α ⁺ committed progenitors (a), but not of the CD34⁺CD38⁺/GMR α cells (b). When combined with G-CSF and either GM-CSF or stem cell factor (SCF), E21R caused apoptosis of all CD34⁺/GMR α ⁺ subsets after 48h, while the addition of IL-3 prevented apoptosis (a,c). The non-committed
5 CD34⁺CD38⁺/GMR α cells were not affected by E21R treatment (b,c). Values are the mean and s.e.m. from three cases. Studies of colony formation by CD34⁺/GMR α ⁺ committed progenitors showed that E21R in combination with either G-CSF, or G-CSF plus GMCSF, or G-CSF plus SCF virtually abolished colony growth, while E21R plus G-CSF and IL-3, or E21R plus G-CSF, SCF and IL-3 did not inhibit colony growth of committed cells (d). Colony growth of
10 the non-committed CD34⁺CD38⁺/GMR α subset was not affected by E21R (d). Data from the three cases in (a), values are mean + s.e.m.

The bone marrow cells were collected and processed as described above. Apoptosis was measured as a reduced DNA binding of propidium iodide as outlined above. Colonies (>40
15 cells) of haemopoietic CD34⁺ progenitors in a methylcellulose assay were prepared and scored after 14 days. To triplicate cultures we added various combinations of E21R (1 μ g/ml), G-CSF (10 ng/ml), GM-CSF (10 ng/ml), and SCF (10 ng/ml, Amgen).

Upon purification of GMR α positive cells with an anti-GMR α monoclonal antibody, virtually
20 100% of the cells were apoptotic with E21R (Figure 10a). The results were observed in 21/21 AML cases (Figure 10b) and 14/14 CML cases (Figure 10c) when E21R was given at a dose of 1 μ g/ml, a concentration ensuring high GMR α occupancy. This concentration was based on a titration experiment where 1 μ g/ml of E21R yielded maximal apoptosis (Figure 10d). The effect was dependent on E21R binding to GMR α since a titration of GM-CSF against 1 μ g/ml
25 of E21R showed that at a concentration of 100 ng/ml GM-CSF totally abolished the apoptotic effect of E21R (Figure 10d).



EXAMPLE 8

To test the finding in Example 7, the effect of E21R combined with either G-CSF, whose receptor is a homodimer distinct from the GM-CSF receptor, or IL-3, whose receptor is a heterodimer in which βc is shared with the GM-CSF receptor was examined; leukaemic cells
5 expressing functional receptors for these cytokines were used. The leukaemic cells underwent apoptosis when they were incubated with E21R and G-CSF, but not with E21R and IL-3 (Figure 10e), indicating that involvement of the βc chain is essential for survival. Since only 6/21 AML and 3/14 CML cases expressed the IL-3 receptor, this example shows that E21R might be beneficial in the treatment of the majority of leukaemias.

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To rule out the possibility that the apoptotic effect of E21R was secondary to antagonism of GM-CSF present in the culture conditions, several controls were performed. First, it is known that some AML and CML cells can produce GM-CSF. With the use of a sensitive proliferation assay (lower detection limit 10 pg/ml), biologically active GM-CSF was not in the CD34⁺CD38⁻
15 /GMR α cells (Figure 11b,c). Apoptosis by E21R was seen even if two other survival factors, G-CSF and stem cell factor (SCF), were present (Figure 11a,c). However, when IL-3 was added to the medium, the haemopoietic progenitors expressing GMR α were rescued from apoptosis (Figure 11a,c).

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EXAMPLE 9

Since E21R directly induced apoptosis, the inventors examined the possible signaling requirements of this active process. A dose-response experiment showed that the specific tyrosine kinase inhibitor genistein did not affect E21R-induced apoptosis (Figure 12a). In separate control experiments genistein (0.1-10 μ g/ml) blocked GM-CSF (0.3-1 ng/ml)-mediated
25 stimulation of tyrosine phosphorylation in AML cells. The protein kinase C inhibitor staurosporine profoundly inhibited the E21R-induced apoptosis when given at a dose of 10 μ g/ml (Figure 12a), suggesting that serine/threonine kinases may be central to the apoptotic mechanism of E21R. Furthermore, the inventors found that inhibition of transcription by actinomycin D or of protein synthesis by cycloheximide greatly reduced E21R-induced
30 apoptosis of AML cells in a dose-dependent manner (Figure 12b).

EXAMPLE 10

The influence of E21R on growth of the progenitors was further determined in a colony assay. Various combinations of E21R and G-CSF with or without GM-CSF and SCF virtually abolished colony growth, while adding IL-3 overcame the inhibition of colony growth by E21R (Figure 11d), consistent with E21R inducing apoptosis of committed progenitors (Figure 11c vs. 11d). It is important to note that CD34⁺CD38⁻ cells are non-committed multipotential progenitors. The E21R-induced apoptosis of committed progenitors (CD34⁺CD38⁺, CD34⁺CD33⁺), but not of CD34⁺CD38⁻ cells will be useful for enrichment of uncommitted progenitors for stem cell transplantation and gene transfer purposes.

10

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, 15 individually or collectively, and any and all combinations of any two or more of said steps or features.



- 20 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BRESAGEN LIMITED and MEDVET SCIENCE PTY. LTD.

(ii) TITLE OF INVENTION: "HAEMOPOIETIC GROWTH FACTOR
ANTAGONISTS AND USES THEREFOR"

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: DAVIES COLLISON CAVE

(B) STREET: 1 LITTLE COLLINS STREET

(C) CITY: MELBOURNE

(D) STATE: VICTORIA

(E) COUNTRY: AUSTRALIA

(F) ZIP: 3000

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT INTERNATIONAL

(B) FILING DATE: 21-JUN-1996

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: PN3780/95

(B) FILING DATE: 23-JUN-1995

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: HUGHES DR, E JOHN L

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(ix) TELECOMMUNICATION INFORMATION:

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- 21 -

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 47 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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

TATGTTTCGCT ACTTCAAGCT CTACGGGGAA CGATATCGCT GCAGCCA

47

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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

AGCTTGGCTG CAGCGATATC GTTCCCCGTA GAGCTTGAAG TAGCGAACA

49

**CLAIMS:**

1. A method for inducing apoptosis in cells carrying a haemopoietic growth factor (HGF) heterodimeric receptor said receptor comprising an HGF-specific α -chain and a β c chain, said method comprising contacting said cells with an effective amount of an HGF antagonist for a time and under conditions sufficient to induce apoptosis wherein said HGF antagonist comprises a sequence of amino acids within a first α -helix wherein one or more exposed amino acids in said first α -helix of HGF having acidic properties is/are substituted with a basic amino acid residue or a non-acidic amino acid residue.
2. A method according to claim 1 wherein the HGF is an interleukin (IL).
3. A method according to claim 2 wherein the HGF is selected from IL-2, IL-3, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-13, IL-14 and IL-15.
4. A method according to claim 1 wherein the HGF is granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO) or thrombopoietin (TPO).
5. A method according to claim 4 wherein the HGF is GM-CSF.
6. A method according to claim 1 wherein the antagonist of HGF is derived from said HGF, said antagonist being capable of binding to the α -chain of the HGF receptor.
7. A method according to claim 6 wherein the antagonist is a derivative of GM-CSF.
8. A method according to claim 7 wherein the derivative of GM-CSF comprises an acidic amino acid residue of amino acid position 21 being substituted with a basic amino acid residue or a non-acidic amino acid residue.





9. A method according to claim 8 wherein the acidic amino acid residue is Glu or Asp, the basic amino acid residue is Lys or Arg and the non-acidic amino acid residue is Gln or Asn or derivatives thereof.
10. A method according to claim 9 wherein the HGF antagonist is E21R or E21K.
11. A method according to claim 10 wherein the HGF antagonist is E21R.
12. A method according to claim 1 wherein the cells in which apoptosis is induced are normal and malignant myeloid cells.
13. A method according to claim 12 wherein the myeloid cells are myeloid leukaemias.
14. A method for treating myeloid leukaemia in a human subject, said method comprising administering to said human subject an effective amount of an HGF antagonist, said administration being for a time and under conditions sufficient to induce apoptosis of said myeloid leukaemic cells wherein said HGF antagonist comprises a sequence of amino acids within a first α -helix wherein one or more exposed amino acids in said first α -helix having acidic properties is/are substituted with a basic amino acid residue or a non-acidic amino acid residue.
15. A method according to claim 14 wherein the HGF is an interleukin (IL).
16. A method according to claim 15 wherein the HGF is selected from IL-2, IL-3, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-13, IL-14 and IL-15.
17. A method according to claim 14 wherein the HGF is granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO) or thrombopoietin (TPO).





18. A method according to claim 17 wherein the HGF is GM-CSF.
19. A method according to claim 14 wherein the antagonist of HGF is derived from said HGF said antagonist being capable of binding to the α -chain of the HGF receptor.
20. A method according to claim 20 wherein the antagonist is a derivative of GM-CSF.
21. A method according to claim 20 wherein the derivatives of GM-CSF comprises an acidic amino acid residue at amino acid position 21 of HGF being substituted with a basic amino acid residue or a non-acidic amino acid residue.
22. A method according to claim 21 wherein the acidic amino acid residue is Glu or Asp, the basic amino acid residue is Lys or Arg and the non-acidic amino acid residue is Gln or Asn or derivatives thereof.
23. A method according to claim 22 wherein the HGF antagonist is E21R or E21K.
24. A method according to claim 23 wherein the HGF antagonist is E21R.
25. A method according to claim 14 wherein the cells in which apoptosis is induced are normal and malignant myeloid cells.
26. A method according to claim 25 wherein the myeloid cells are myeloid leukaemias.
27. A method for enriching uncommitted progenitor cells from bone marrow said method comprising contacting a population of cells from bone marrow with an effective amount of a molecule capable of binding to the α -chain of an HGF receptor for a time and under conditions sufficient to induce apoptosis of cells carrying said α -chain.





28. A method according to claim 27 wherein the molecule is a derivative of an HGF having a sequence of amino acids within a first α -helix of HGF wherein one or more exposed amino acids in first α -helix of HGF having acidic or acidic-like properties are substituted with a basic amino acid residue or a non-acidic amino acid residue.
29. A method according to claim 28 wherein the molecule is a derivative of GM-CSF, an interleukin G-CSF, EPO or TPO.
30. A method according to claim 29 wherein the molecule is a derivative of GM-CSF.
31. A method according to claim 30 wherein the molecule is E21R or E21K.
32. A method according to claim 30 wherein the molecule is E21R.
33. A method for producing recombinant molecules, said method comprising introducing a genetic construct capable of expressing a nucleotide sequence to produce said recombinant molecule into a cell, subjecting the cell to conditions to permit expression of said nucleotide sequence, collecting the expressed recombinant molecules in the form of inclusion bodies and subjecting said inclusion bodies to dissolution in a chaotropic agent under conditions of high pH sufficient to unfold said recombinant molecule, dilute said unfolded recombinant molecule and subjecting same to refolding conditions and then purifying said refolded recombinant molecule.
34. A method according to claim 33 wherein the chaotropic agent is urea.
35. A method according to claim 34 wherein the recombinant molecule is GM-CSF or a mutant thereof.
36. A method according to claim 33 wherein the nucleotide sequence further encodes for a leader sequence fused to said recombinant molecule.



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37. A method according to claim 36 wherein the leader sequence is the amino acid sequence MFATSSSTGND.

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* C L H S *

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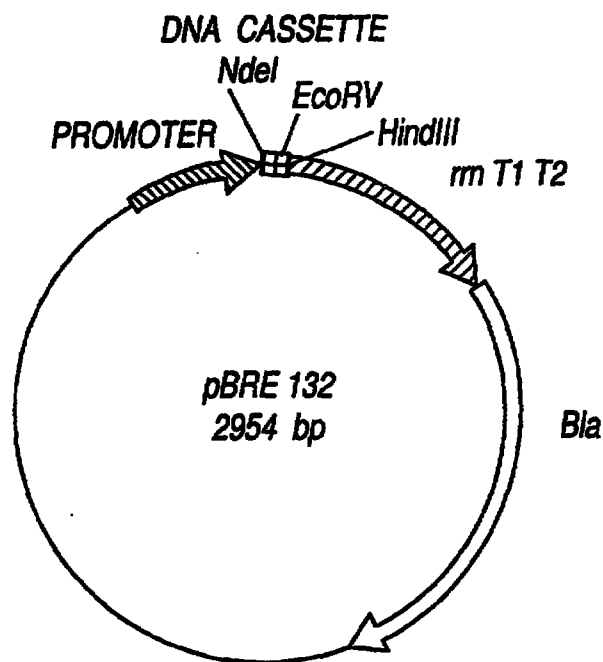


Fig.1

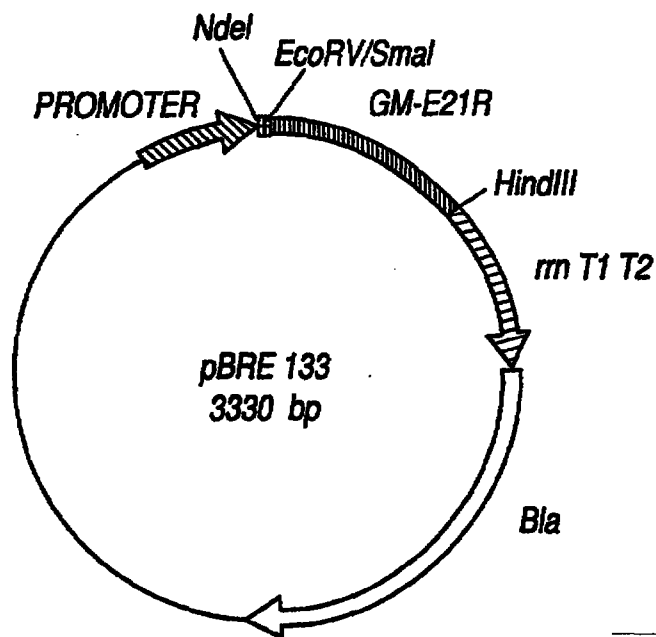


Fig.2

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COMPETITIVE BINDING TO CHO CELLS TRANSFECTED
WITH GMR α 1nM 125 I-GM-CSF

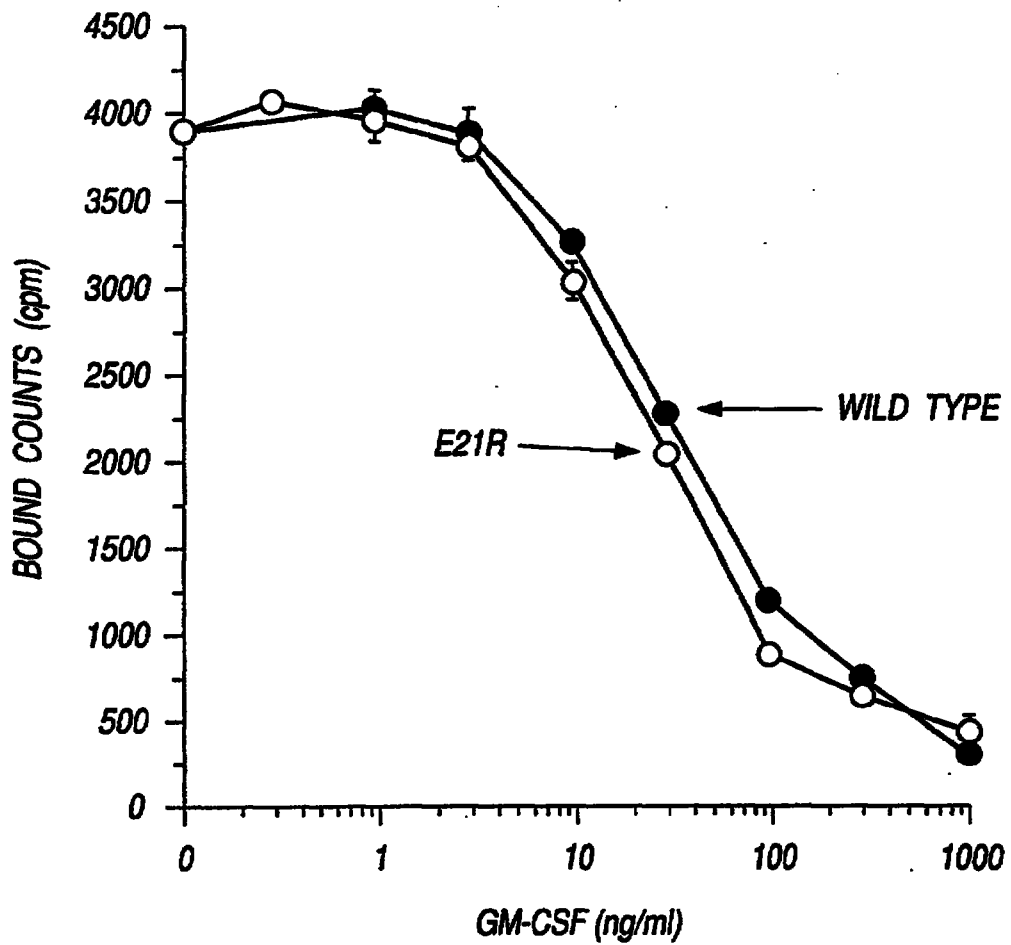


Fig. 3

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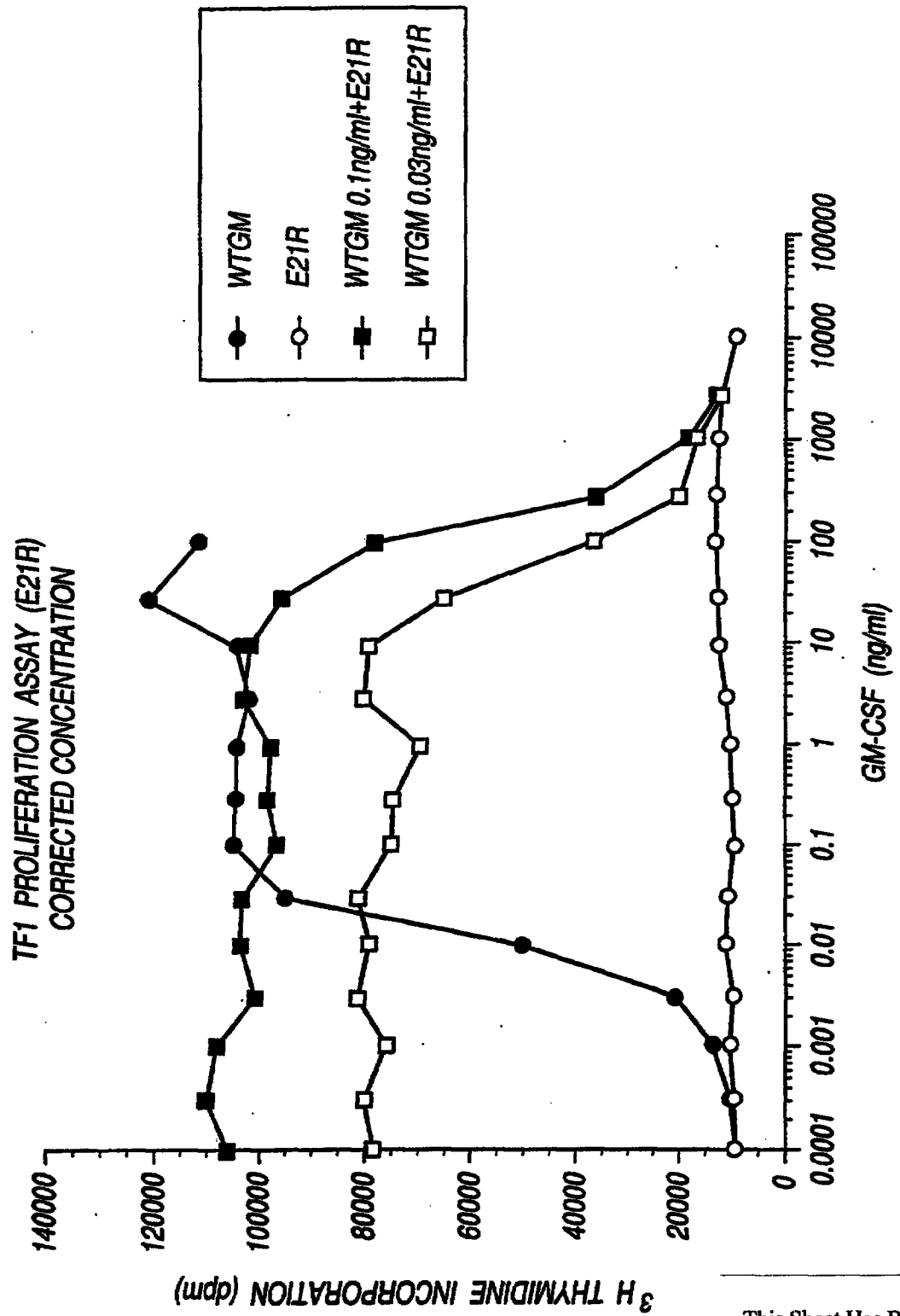


Fig. 4

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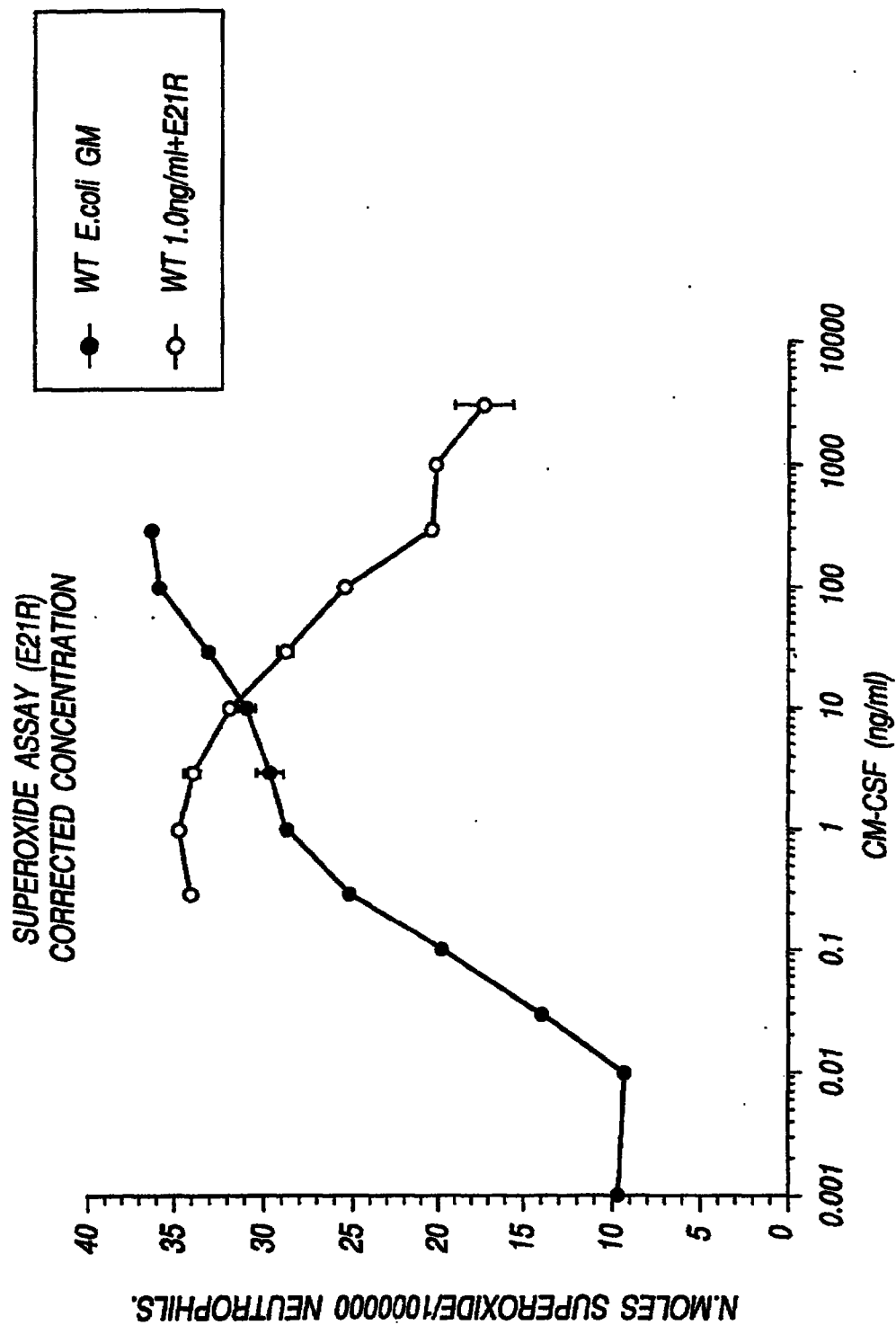


Fig. 5



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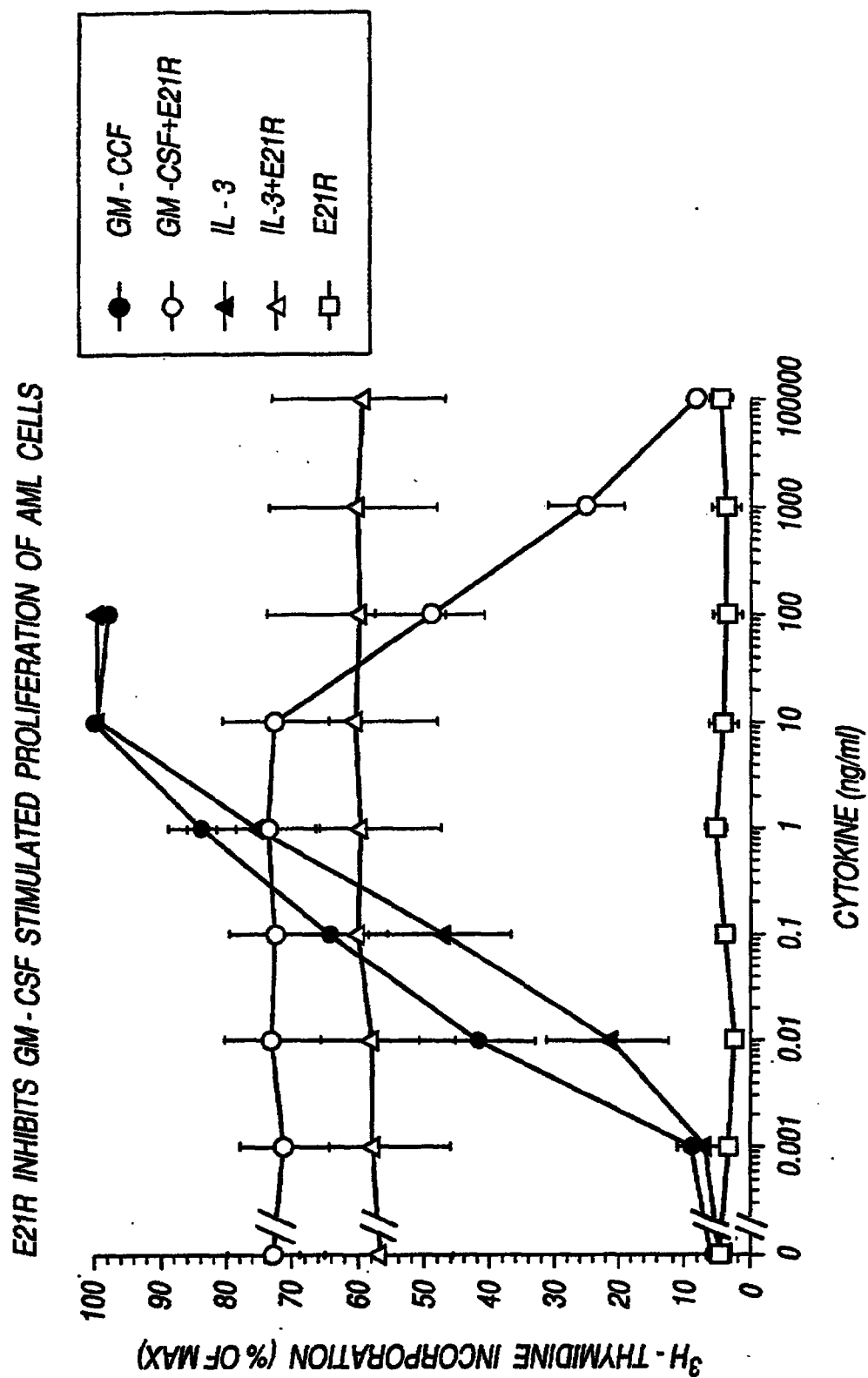


Fig. 6

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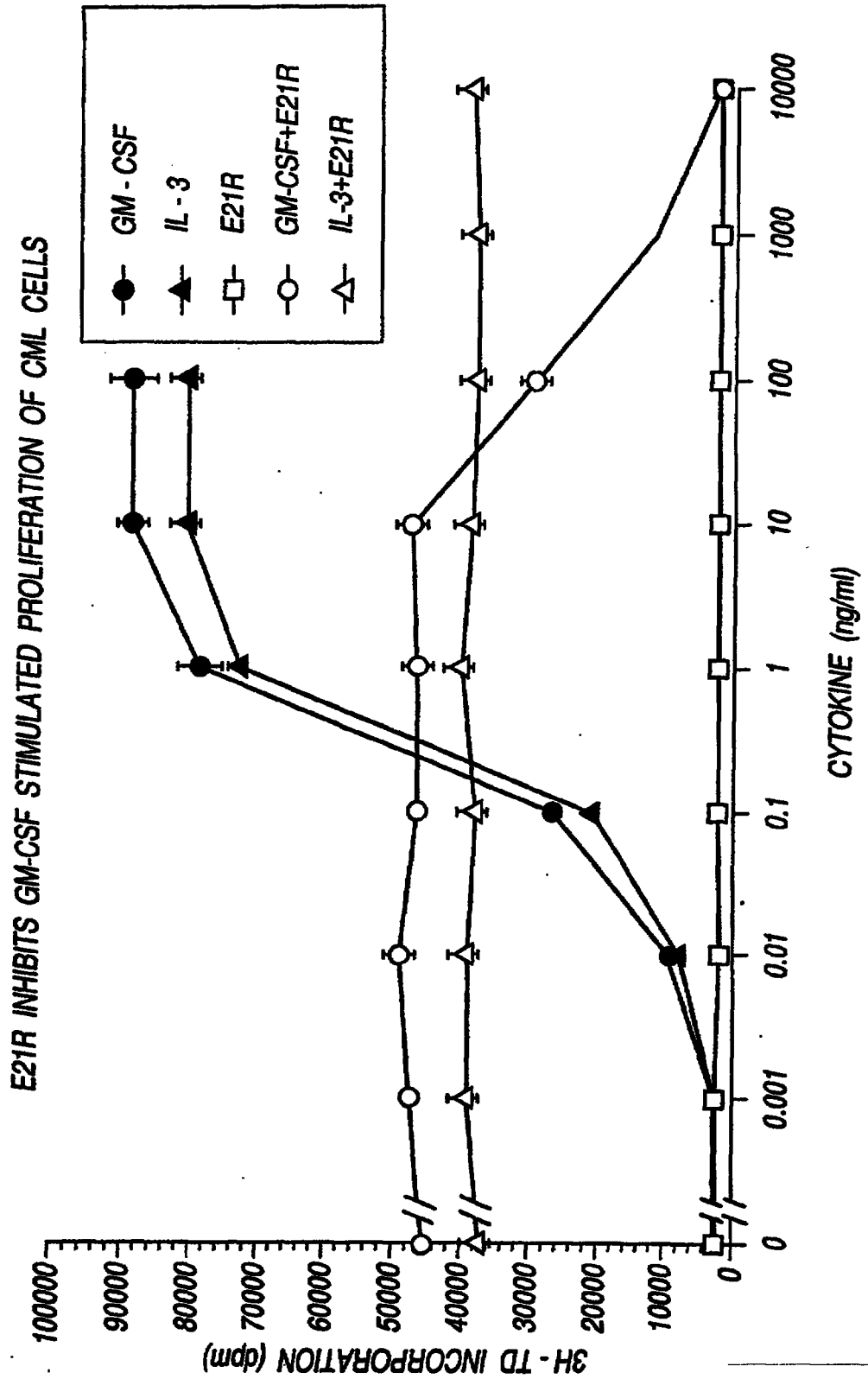


Fig. 7



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E21R INHIBITS COLONY GROWTH OF JCML CELLS

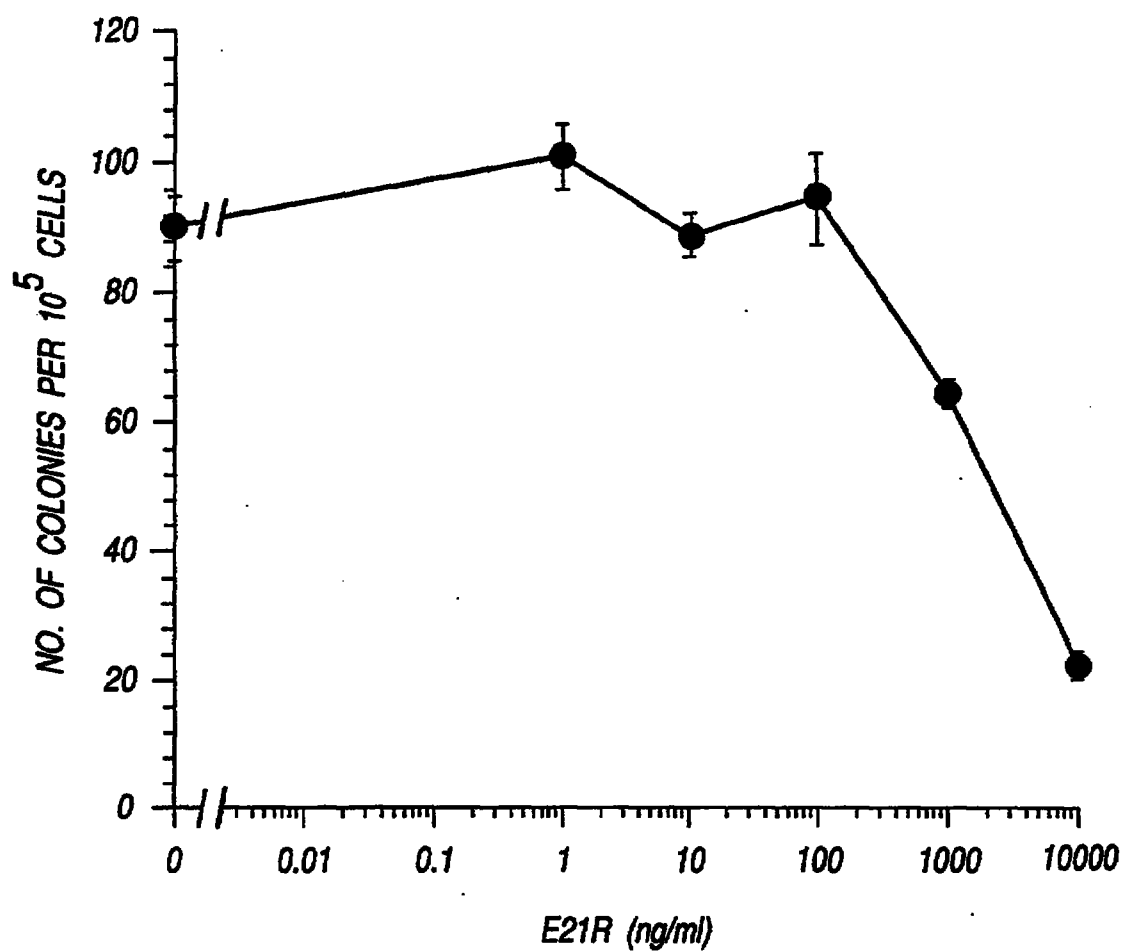


Fig. 8

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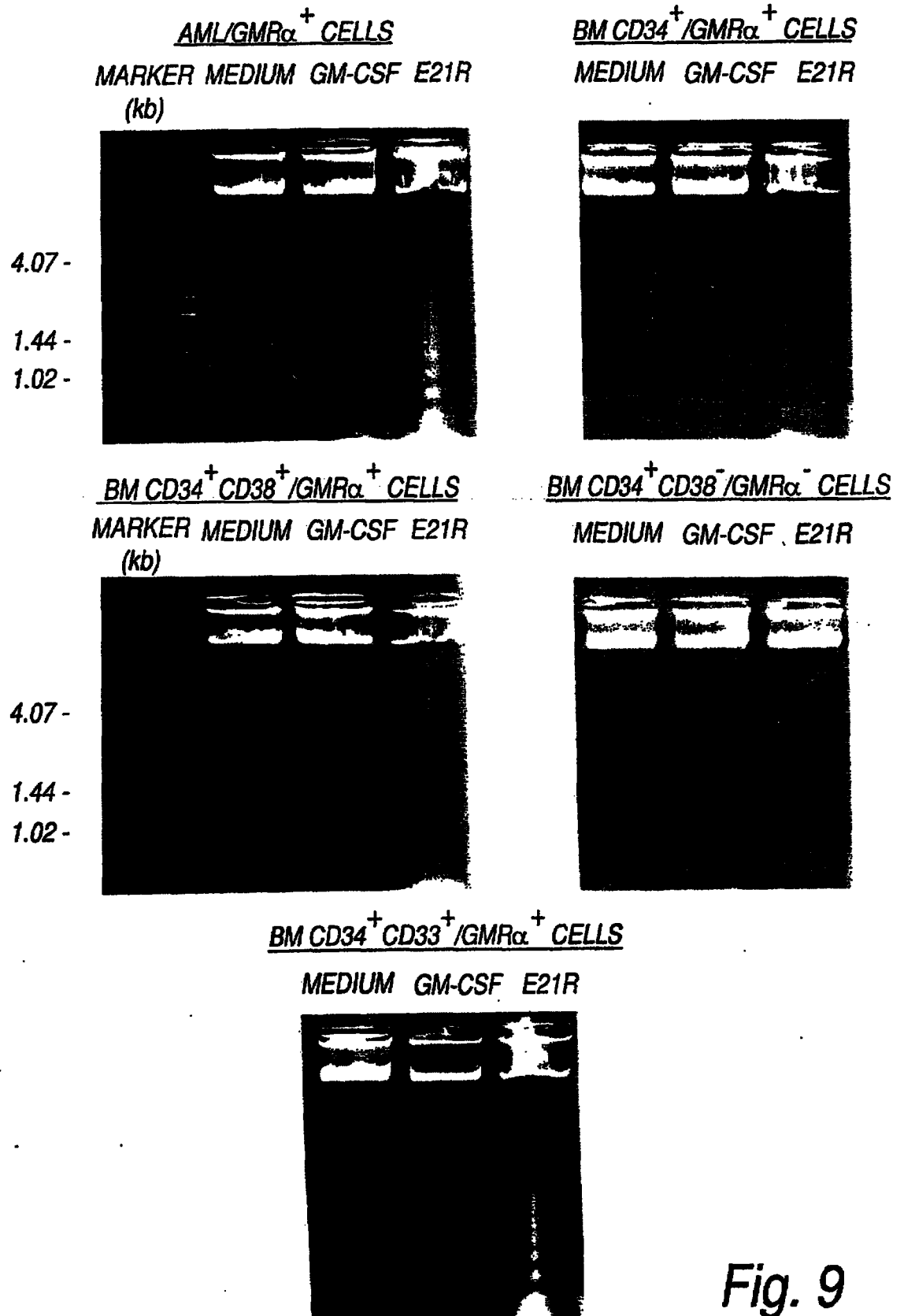


Fig. 9

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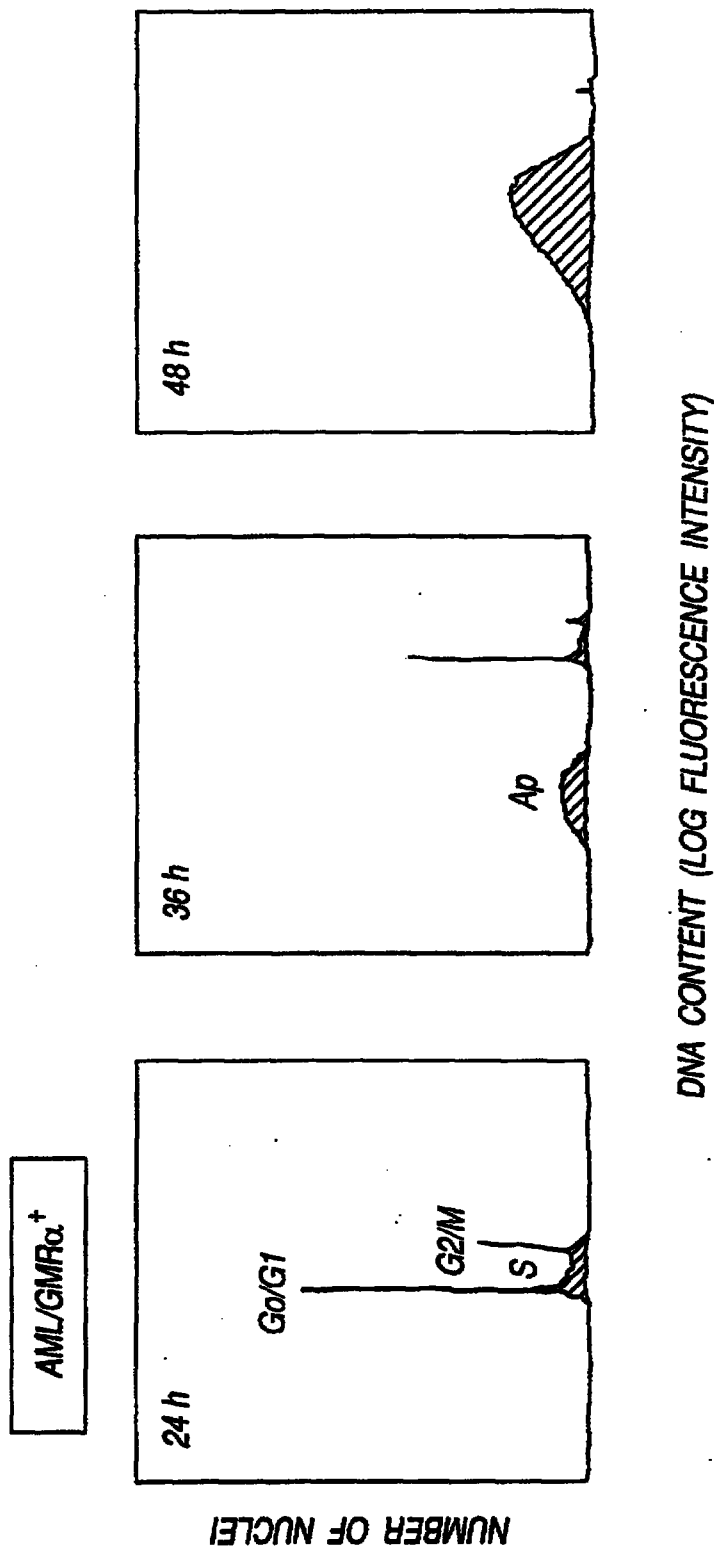


Fig. 10a

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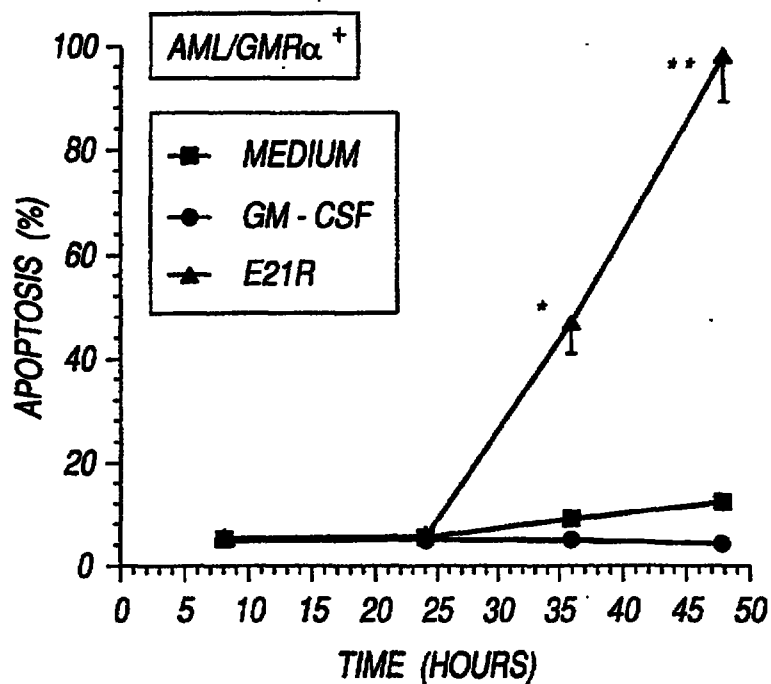


Fig. 10b

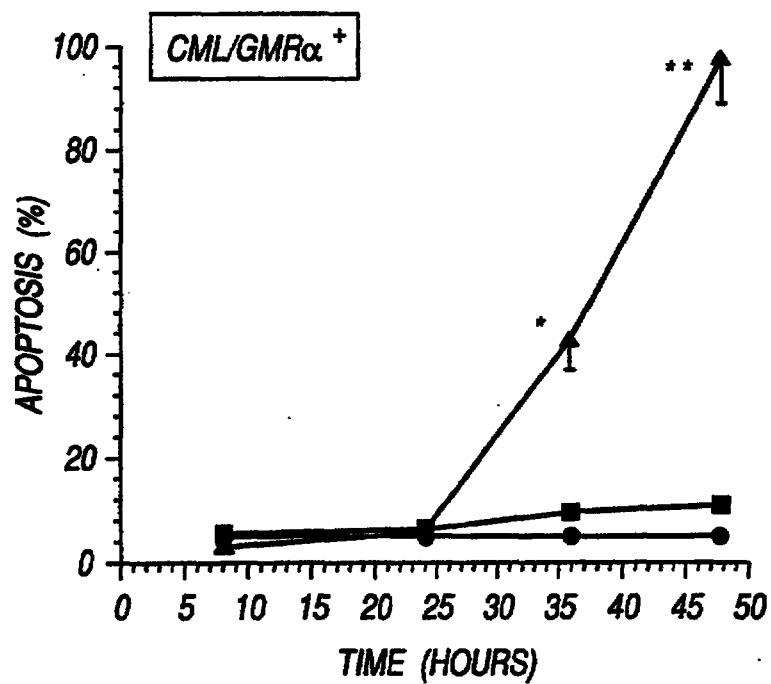


Fig. 10c

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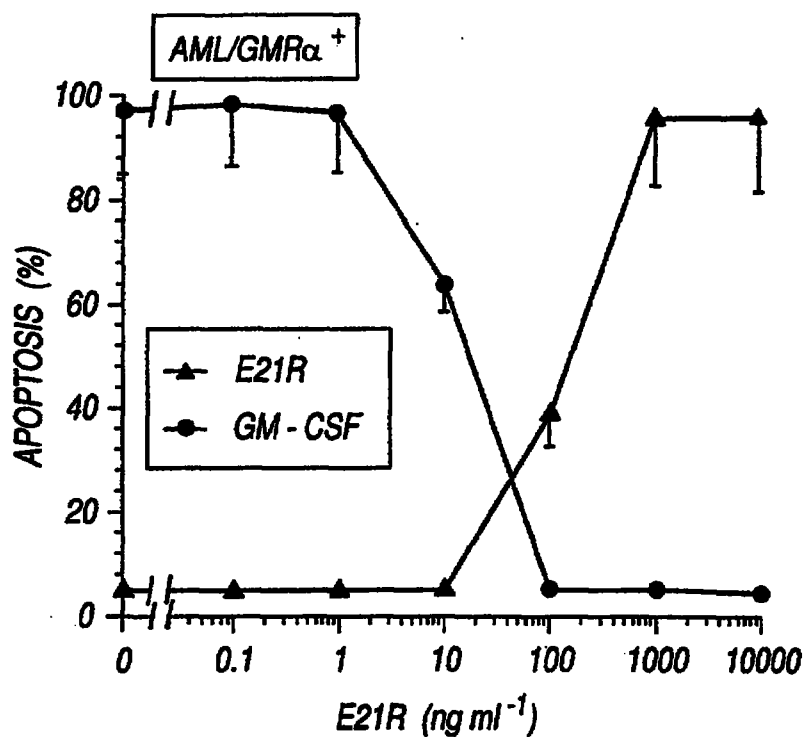


Fig. 10d

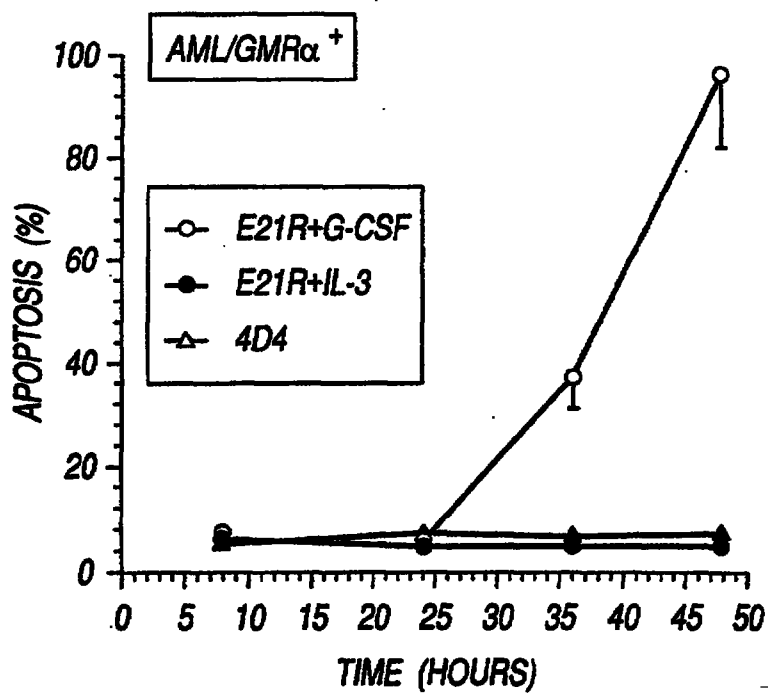


Fig. 10e

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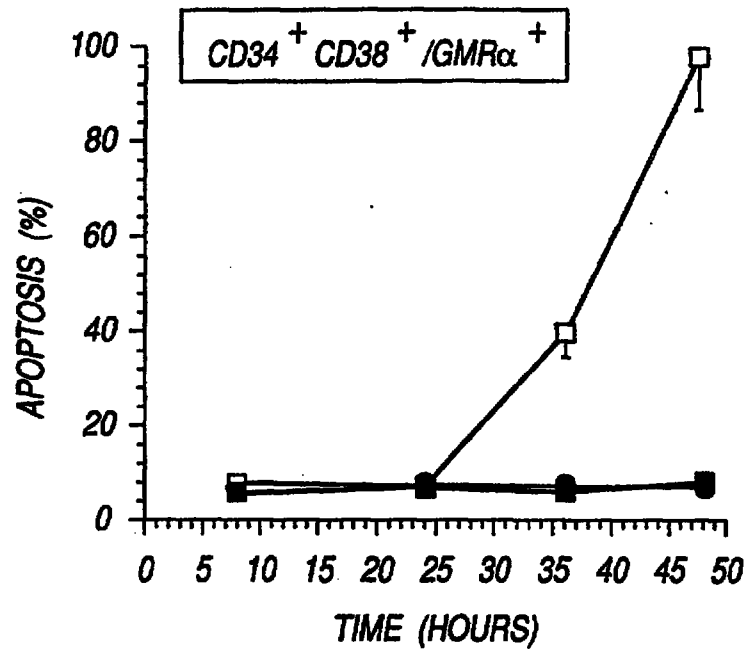


Fig.11a

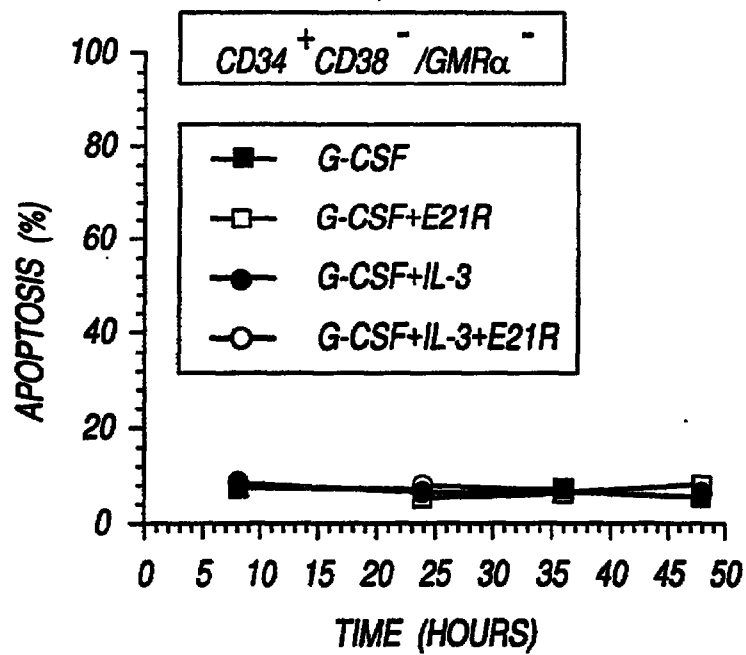


Fig.11b

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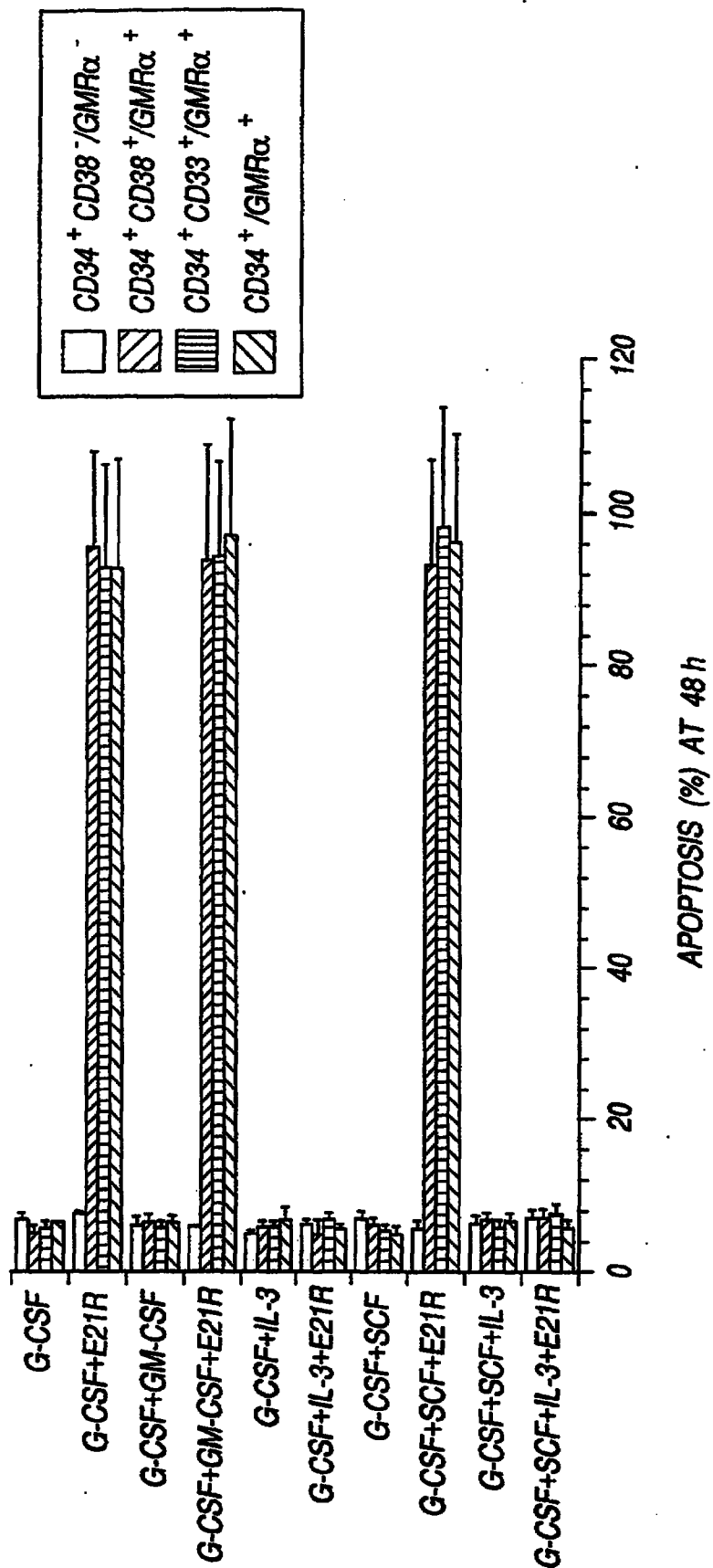


Fig. 11c

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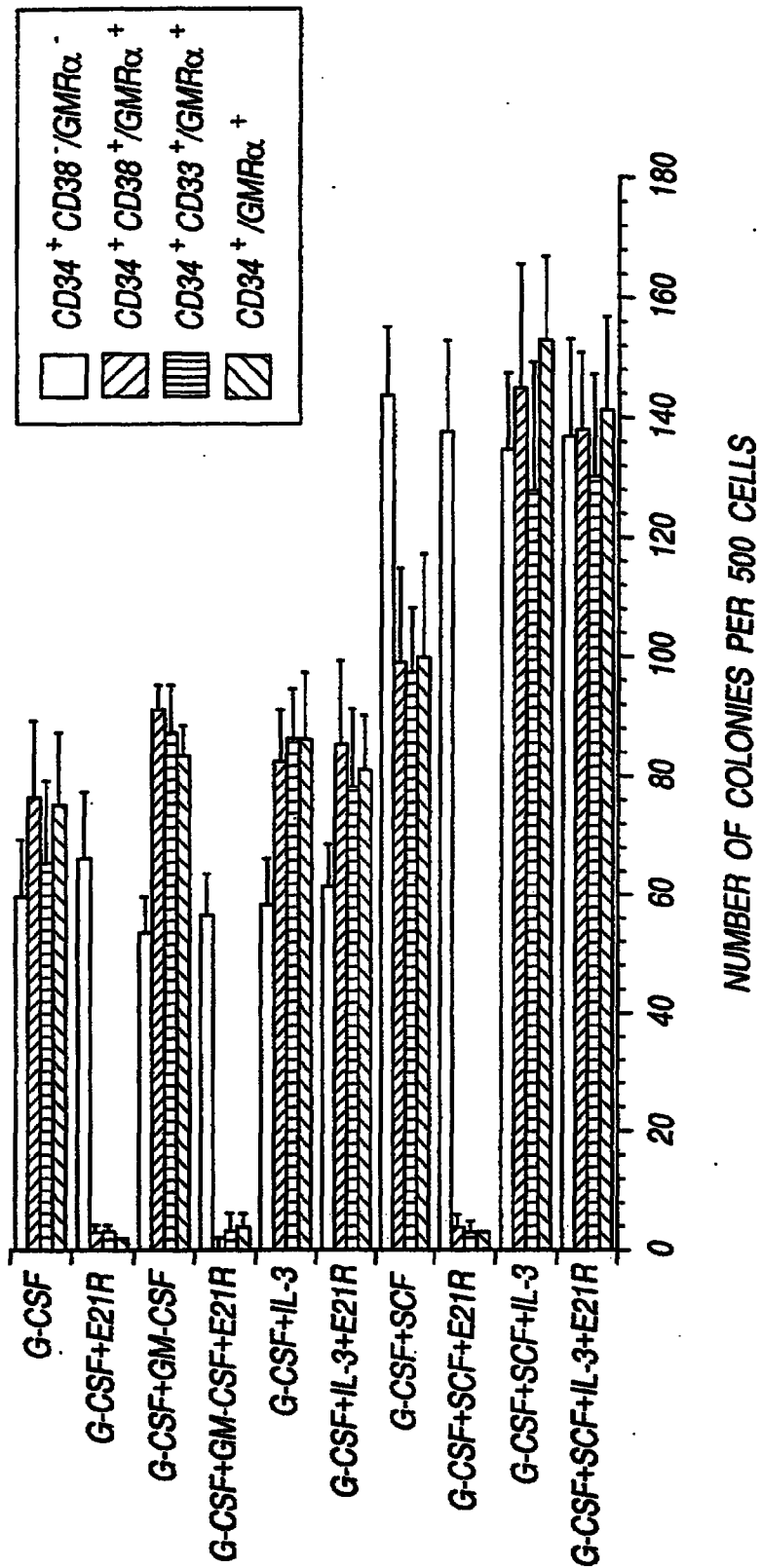


Fig. 11d

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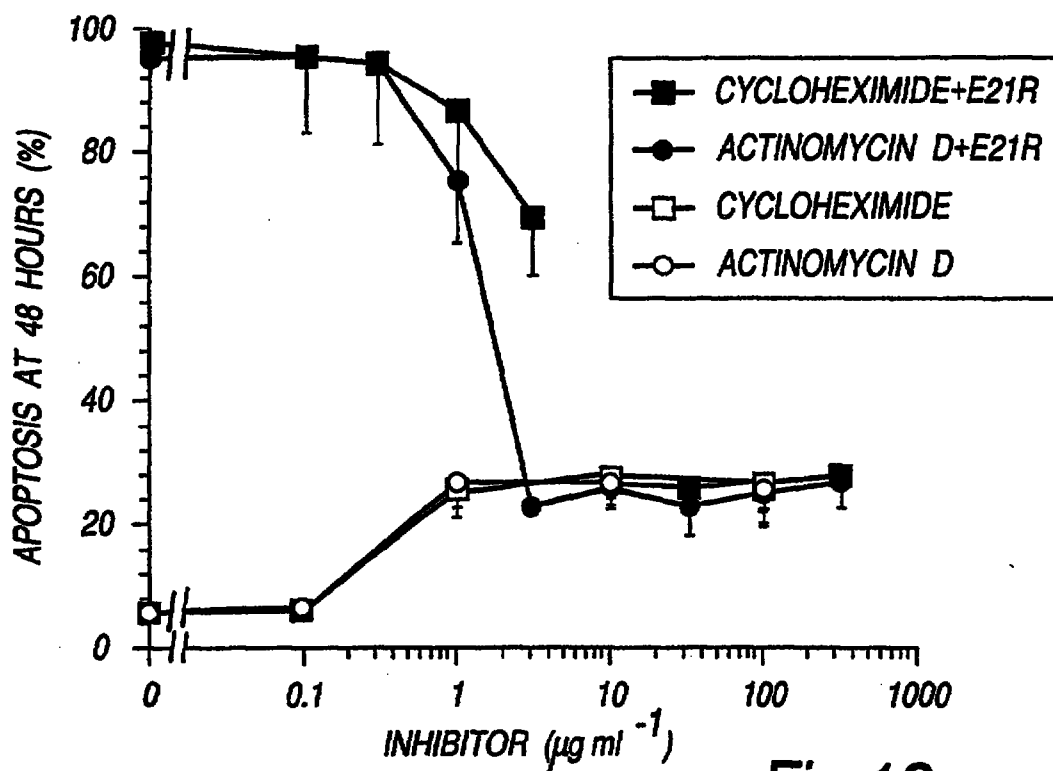


Fig. 12a

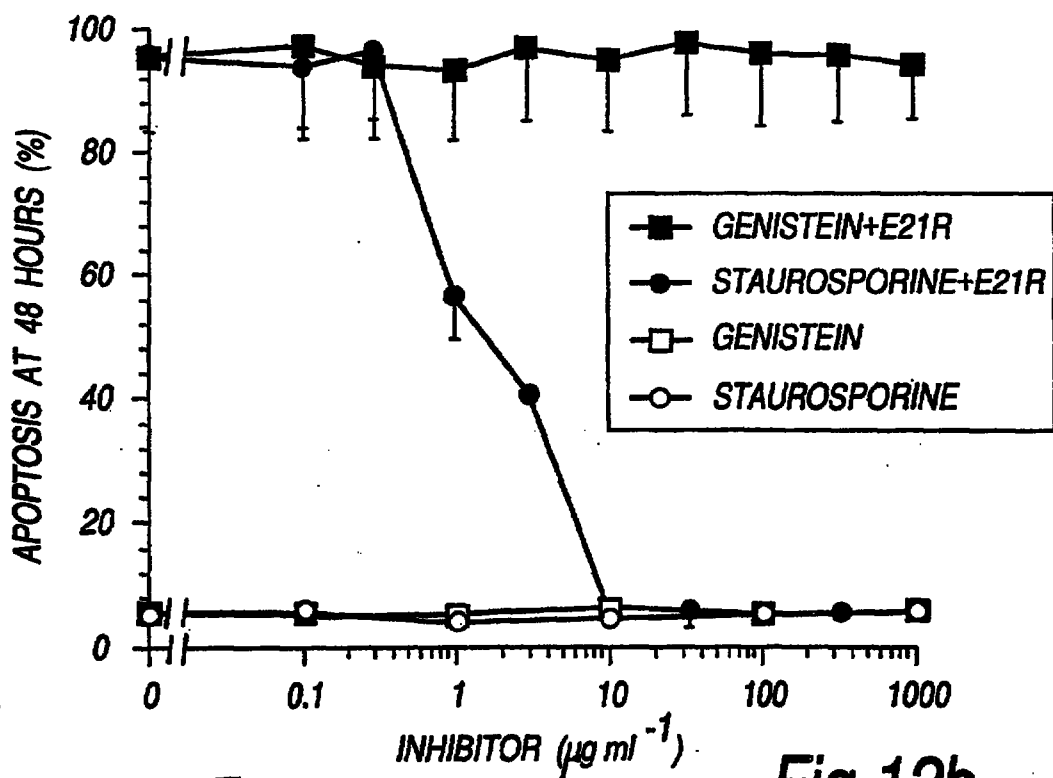


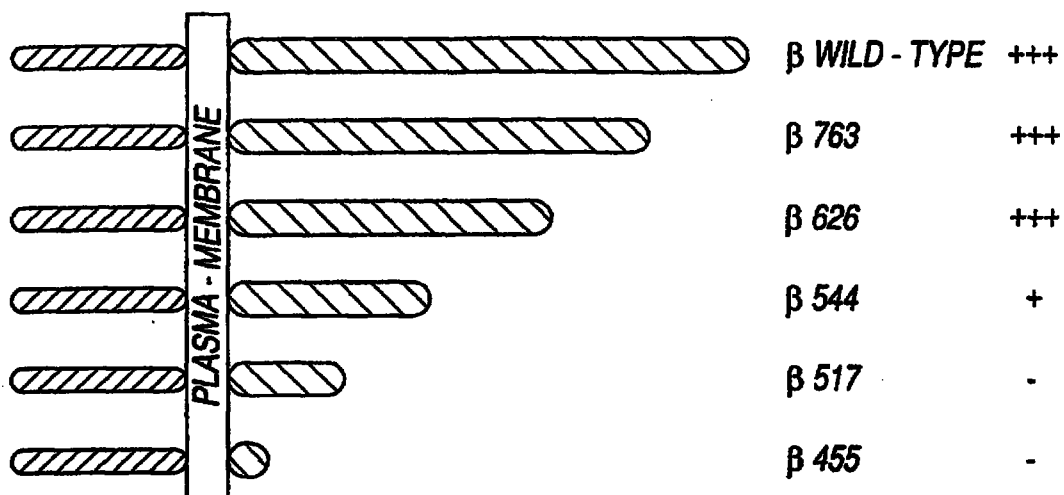
Fig. 12b

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EXTRACELLULAR CYTOPLASMIC β MUTANT APOPTOSIS



EXTRACELLULAR CYTOPLASMIC GMR α DELETIONS APOPTOSIS

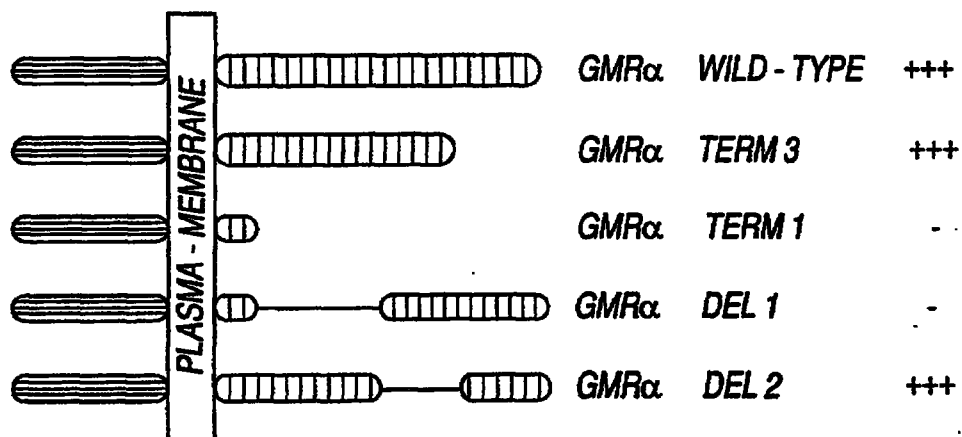


Fig. 13

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The HGF antagonists are preferably produced in recombinant or synthetic form and, with the exception of the amino acid substitution(s) in the first α -helix, the amino acid sequence of the HGF may be the same as the naturally occurring molecule (i.e. native molecule) or may carry single or multiple amino acid substitutions, deletions and/or additions to the native sequence.

5

In one particular embodiment, the HGF antagonists are:

- (i) in unglycosylated form;
- (ii) lack post-translational modification;
- (iii) produced in prokaryotic microorganisms; and/or
- 10 (iv) produced by chemical synthesis.

The antagonists may be derivatives of an HGF or may be any other molecule which selectively binds to the α -chain of the HGF receptor such as, but not limited to, antibodies or other small or large molecules resulting in apoptosis. The present invention is hereinafter
15 described with reference to GM-CSF antagonists and in particular human GM-CSF antagonists or mammalian GM-CSF antagonists capable of functioning in humans. This is done, however, with the understanding that the present invention extends to any HGF antagonist which is capable of inducing cell apoptosis by interaction with an HGF-specific α -chain of an HGF receptor. The present invention also extends to HGF antagonists which
20 interact with the β chain.

Although not intending to limit the present invention to any one theory or mode of action, it is proposed that the GM-CSF antagonist either blocks the action of wild-type GM-CSF by interacting selectively with the receptor α -chain or induces apoptosis by interacting with the
25 α - and β -chains of the receptor in a manner that leads to abnormal stimulation of the β -chain either qualitatively or quantitatively. Qualitative differences to wild-type GM-CSF include recruitment of signalling molecules (for example, type of kinase or state); quantitative differences include intensity of duration of signalling.

30

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Preferably, the GM-CSF antagonist is E12R.

Induction of apoptosis will also be useful in enrichment of uncommitted progenitors for stem cell transplantation and gene transfer purposes. According to this aspect of the present invention there is provided a method for enriching uncommitted progenitor cells from bone marrow said method comprising contacting a population of cells from bone marrow with an effective amount of a molecule capable of stimulating the α -chain of an HGF receptor or abnormally stimulating the β c chain of said receptor for a time and under conditions sufficient to induce apoptosis of cells carrying said α -chain.

10

Preferably, the molecule is a derivative of an HGF having a sequence of amino acids within a first α -helix wherein one or more exposed amino acids in said first α -helix having acidic or acidic-like properties are substituted with a basic amino acid residue or non-acidic amino acid residue.

15

Preferably, the molecule is a derivative of GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-13, IL-14, IL-15 and others of this family yet to be discovered, G-CSF, EPO or TPO.

20 Preferably, the molecule is GM-CSF having Arg or Lys at position 21 in place of Glu.

Most preferably, the molecule is E21R.

Preferably, apoptosis occurs with committed progenitors ($CD34^+ CD38^+$, $CD34^+ CD33^+$) but not $CD34^+ CD38^-$ cells.

25

The present invention further contemplates genetic constructs useful in expressing E21R including constructs comprising the nucleotide sequence encoding the leader sequence MFATSSSTGNDG.

30

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The present invention also contemplates a method for purifying E21R with or without the leader sequence following expression of the above-mentioned genetic constructs.

Yet a further aspect of the present invention contemplates eukaryotic or prokaryotic organisms
5 carrying the above-mentioned genetic constructs.

Preferred aspects of these embodiments are described in the Examples.

The present invention is further described by reference to the following non-limiting Figures
10 and Examples.

In the Figures:

Figure 1 is a schematic representation of plasmid pBRE132. The DNA cassette has a unique
15 EcoRV cloning site and codes for MFATSSSTGND leader sequence.

Figure 2 is a schematic representation of plasmid pBRE133. *SmaI/HindIII* GM-E21R PCR
DNA was cloned into *EcoRV/HindIII* sites of pBRE132. pBRE133 encodes
MFATSSSTGNDG-GM-CSF protein (designated herein "E21R").

20

Figure 3 is a graphical representation showing competition of binding of E21R and 1nM ¹²⁵I-
GM-CSF to CHO cells transfected with GMR α (GM-CSF-specific α -chain of GM-CSF
receptor).

25 Figure 4 is a graphical representation showing antagonism of GM-CSF by E21R in a human
erythroleukaemia cell line TF1 proliferation assay.

Figure 5 is a graphical representation showing antagonism of GM-CSF by E21R in a
neutrophil superoxide assay.

30

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Figure 6 is a graphical representation showing that E21R inhibits GM-CSF stimulated proliferation of AML cells.

Figure 7 is a graphical representation showing that E21R inhibits GM-CSF stimulated proliferation of CML cells.

Figure 8 is a graphical representation showing that E21R inhibits colony growth of JCML cells.

10 Figure 9 is a photographic representation showing that the E21R causes DNA fragmentation in myeloid cells.

Figure 10 are graphical representations showing that E21R induces apoptosis of $\text{GMR}\alpha^+$ myeloid leukaemic cells in a time- and dose-dependent manner.

15

Figure 11 are graphic representations showing that E21R induces apoptosis of $\text{CD34}^+/\text{GMR}\alpha^+$ haemopoietic progenitors.

Figure 12 is a graphical representation showing that E21R-induced apoptosis requires transcription, protein synthesis and kinase activity. (a) Titration of genistein and staurosporine in the absence (open symbols) and presence (solid symbols) of E21R ($1 \mu\text{g/ml}$). (b) Titration of actinomycin D and cycloheximide in the absence (open symbols) and presence (solid symbols) of E21R ($1 \mu\text{g/ml}$). Apoptosis was measured at the 48 hr time point. Data are means and SEM of triplicate samples from one AML case and are representative of four other cases.

25

Figure 13 shows that the membrane proximal domains of both receptor chains are required for induction of apoptosis, while deletions of the more distal part of either chain did not affect E21R-mediated apoptosis.

30

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EXAMPLE 1**CONSTRUCTION OF pBRE132 AND pBRE133 FOR EXPRESSION OF
RECOMBINANT E21R PROTEIN****5 Construction of pBRE132 expression plasmid**

A synthetic DNA cassette was constructed by annealing two oligonucleotides, TATGTTTCGCTACTTCAAGCTCTACGGGGAACGATATCGCTGCAGCCA (SEQ ID NO:1) and AGCTTGGCTGCAGCGATATCGTTCCCCGTAGAGCTTGAAGTAGCGAACA (SEQ ID NO:2) at 65°C for 5 min in 200 mM NaCl. The synthetic DNA cassette was
10 cleaved with *NdeI/HindIII* and cloned into pEC611 expression vector (A. Brumby, 1987 "A vector for high expression of native proteins", Honours Thesis, University of Adelaide), generating pBRE132 (Figure 1).

Construction of pBRE133 (E21R expression vector)

15 GM-CSF (E21R) cDNA was amplified employing PCR primers with unique *SmaI* and *HindIII* sites. The PCR products were cleaved with *SmaI/HindIII* and cloned into the *EcoRV/HindIII* sites of pBRE132 generating pBRE133 (Figure 2).

Expression of pBRE133 in *Escherichia coli* MM294/pACYClac

20 Expression analysis demonstrated that pBRE133 in *Escherichia coli* MM294/pACYClac expressed inclusion bodies (IB) consisting of recombinant E21R protein, as judged by microscopy examination, standard SDS-PAGE and mass spectrometry analysis of the purified protein.

25

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EXAMPLE 2
PRODUCTION OF GRANULOCYTE MACROPHAGE COLONY
STIMULATING FACTOR ANTAGONIST E21R

1. FERMENTATION

5 A. Inoculation

1. *Escherichia coli* MM294/pACYClac carrying pBRE133 was streaked out from a 80°C glycerol stock onto a minimal medium plate containing ampicillin (100µg/ml) and kanamycin (30µg/ml) and grown overnight at 37°C.
- 10 2. Multiple colonies were assessed for GM-CSF (E21R) expression levels by microscopic examination of 20ml shake flask cultures containing inducer, or by colony size on agar plates with or without inducer, in this instance with IPTG.
3. The selected single colony from an agar plate was transferred into 20ml of nitrogen-
15 rich minimal medium (2) containing ampicillin (100 µg/ml) + kanamycin (30 µg/ml). The culture was grown at 37°C overnight with agitation.
4. An amount of 10L of C2 was sterilised in a 22 L fermenter and inoculated to a very low density with overnight culture. Growth was at 37°C and a pH of 7.0 was maintained by
20 the addition of 12.5 M NH₄OH.
5. Agitation was manually controlled and aeration automatically controlled with oxygen saturation levels remaining above 10% pO₂. Following depletion of glucose at 16-18 hours the cell mass was fed with a concentrated glucose solution containing additional salts.
25 Nutrient feed flow rate was determined by pH or oxygen saturated levels.

B. Induction

1. At an optical density of $A_{600} = 50-60$ the recombinant expression of E21R antagonist was induced, in this instance by the addition of IPTG. The culture was supplemented with
30 amino acids at the time of induction (T=0) and at T=2 hours. Induction continued for 4

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EXAMPLE 3**GM-CSF ANTAGONIST (E21R) PURIFICATION**

The inclusion body (IB) solution is first washed with an equal volume of a mild buffered denaturant such as 2M guanidine hydrochloride (GuHCl). The solution is stirred gently at 4°C for 30 minutes. The washed IB can then be recovered with a low speed centrifuge spin. The washed IB are then resuspended in a small volume of water (e.g. Milli Q), immediately prior to dissolution and refolding.

Dissolution, refolding and oxidation of the GM-CSF antagonist (E21R) IB is initiated by dissolving the washed IB in an unbuffered urea solution at an alkaline pH. To ensure complete dissolution of the IB, the concentration of urea should be between 2.5M and 4M and the pH between 11 and 12. The concentration of protein can be between 0.5 and 5 mg/ml. These three parameters can be varied depending on the purity of the IB preparation. The stirred solution is maintained at an alkaline pH with a sodium hydroxide solution (e.g. 2M NaOH), until it can be seen that refolding and oxidation has reached equilibrium. The extent of refolding to the correct fold for GM-CSF can be monitored by chromatographic means, e.g. reversed phase HPLC (RP-HPLC) or size-exclusion chromatography (SEC), comparing the chromatographic profile to that of a known folded standard of GM-CSF. Once refolding has reached equilibrium, the pH of the solution is lowered to a less alkaline pH, (e.g. pH 9.1) with a concentrated solution of a suitable buffer (e.g. 0.5M sodium borate pH 9.1).

The correctly refolded GM-CSF antagonist (E21R) can then be purified from incorrectly folded and aggregated forms using chromatography, e.g. anion-exchange chromatography (IEC) or RP-HPLC. For example, the refolding solution from above is loaded onto a anion-exchange column (e.g. Q-Sepharose Fast flow [Pharmacia]). Upon completion of loading, the column is washed with two column volumes of a low salt buffer. The correctly folded form of GM-CSF antagonist (E21R) is eluted from the column with a gradient of sodium chloride (e.g. 0-500 mM NaCl over ten column volumes). The column eluent can be analysed for GM-CSF antagonist (E21R) purity by either SEC (e.g. Superose 12 [Pharmacia]), or RP-HPLC (e.g. Brownlee butyl-silica column (100 mm x 2.1 mm i.d.)

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employing 0.1 % v/v TFA in water as Buffer A and 0.1 % v/v TFA in acetonitrile as Buffer B).

The purified GM-CSF antagonist (E21R) can then be desalted (e.g. Sephadex G-25) into a suitable buffer (e.g. 5 mM Na_2HPO_4 pH 7.6) in preparation for formulation and lyophilisation. The desalted protein can be quantitated by its absorption at 280 nm in a spectrophotometer using a $\epsilon_{280}^{\text{A}^{1\%}}$ of 9.09. Following quantitation, 1 part glycine and 5 parts mannitol, by weight, are added to the desalted protein solution. The solution is stirred gently until all solids are dissolved. If necessary, the pH is adjusted to pH 7.6 with 1M NaOH or 25 % w/v orthophosphoric acid. The solution is then sterilised by filtration (0.2 μm), aliquoted into sterile glassware and lyophilised.

EXAMPLE 4

E21R EXHIBITS ANTAGONISTIC PROPERTIES

Purified E21R bound the GM-CSF receptor α -chain with the equivalent potency as wild type GM-CSF (Figure 3). In addition, E21R did not directly stimulate TF-1 cell proliferation (Figure 4) or neutrophil O_2^- release but instead antagonised the stimulatory effect of GM-CSF in both functional assays (Figures 4 and 5). Using cells from patients with acute myeloid leukaemia (AML) the inventors found that E21R antagonised the stimulation of AML cell proliferation by GM-CSF in a dose-dependent manner (Figure 6). This was specific for GM-CSF in that E21R did not antagonise IL-3-mediated proliferation (Figure 6). E21R completely antagonised the proliferative effect of GM-CSF on cells from patients with chronic myeloid leukaemia (CML) (Figure 7).

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consisted of > 99% blasts. Bone marrow progenitors were collected from a healthy donor and the CD34⁺ subsets were sorted by flow cytometry (FACStar^{PLUS}, Becton-Dickinson, NJ). Cells that expressed the GMR α were isolated using an anti-GMR α monoclonal antibody and a magnetic bead procedure. The cells were incubated in RPMI 1640 medium with 10% v/v fetal calf serum (FCS), antibiotics, sodium bicarbonate, and L-glutamine for 48 h at 37°C. To the cultures either *E. coli*-derived E21R (1 μ g/ml) or GM-CSF (0.3 ng/ml, Genetics Institute, Cambridge, MA) were added. The medium used to culture bone marrow cells contained G-CSF (10 ng/ml, Amgen, Thousand Oaks, CA). DNA was extracted and analyzed after agarose gel electrophoresis.

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Incubation of the cells with either GM-CSF alone or medium only for up to 48 h caused no degradation of the DNA. Staining of DNA with propidium iodide from one AML case revealed that a 48-h incubation with E21R (1 μ g/ml) caused apoptosis of almost all the cells Figure 10 (a). The G0/G1, S and G2/M regions represent cells in various phases of the cell cycle, while the subdiploid Ap peak denotes apoptotic cells. To quantify the number of apoptotic cells, the DNA binding of propidium iodide was measured. Initially about 75% of AML cells underwent apoptosis in the presence of E21R at 48 h. This proportion of apoptotic cells correlated well with the number of cells expressing the GM-CSF α receptor. E21R (1 μ g/ml) induced apoptosis in 21/21 AML cases (Figure 10b) and 14/14 CML cases (Figure 10c).

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To examine whether the apoptotic effect of E21R was secondary to antagonism of GM-CSF present in the culture conditions or due to a direct effect to E21R, several controls were performed. First, it is known that while normal bone marrow haemopoietic cells do not express the GM-CSF mRNA, some AML cells can produce GM-CSF. However, GM-CSF was detected in supernatants of only 8 out of 21 AML cases. The other 13 cases did not show detectable GM-CSF in the supernatants, and an RNase protection assay showed lack of mRNA for GM-CSF. Second, biologically active GM-CSF in the culture medium was not present: enumeration of apoptotic cells in serum-free medium yielded similar results as depicted in Figure 10b, and the neutralising anti-GM-CSF monoclonal antibody 4D4 did not induce apoptosis of the AML cells (Figure 10e). Finally, to determine whether any GM-CSF was

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bound to the AML cells when transferred *ex vivo* to the *in vitro* cultures, the inventors measured the cell surface-associated binding of ^{125}I -labelled GM-CSF before and after acid elution. No differences were observed in specific GM-CSF binding, indicating that the cells were not carrying over receptor-bound GM-CSF. E21R did not induce apoptosis of myeloid cells
5 lacking GMR α indicating that E21R was not toxic.

A titration of E21R showed that full apoptotic effect was achieved with 1 $\mu\text{g/ml}$ Figure 10 (d). A GM-CSF concentration of 100 ng/ml effectively inhibited E21R (1 $\mu\text{g/ml}$) mediated apoptosis Figure 10 (d). Values are the mean and s.e.m. of three replicates from one AML case.

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When combined with G-CSF (10 ng/ml), but not with IL-3 (10 ng/ml, Genetics Institute), E21R induced apoptosis of nearly all the cells Figure 10 (e). Peripheral blood cells were collected from untreated CML patients (all Philadelphia chromosome positive) in blast crisis and processed as for the AML cells described above. Apoptosis was determined by flow cytometry
15 as the reduced amount of propidium iodide bound by DNA after incubating triplicate cultures of the cells overnight in a buffer containing 0.1% v/v Triton X-100 and propidium iodide (50 mg/ml).

EXAMPLE 7

Similar to normal haemopoiesis, myeloid leukaemic cells stem from a small pool of highly
20 proliferative progenitors that express the CD34 surface marker. To examine whether the effect of E21R was a general phenomenon related to the GMR α expression or restricted to malignant cells, the effect of E21R on the survival of normal CD34 $^{+}$ progenitors was studied. Bone marrow cells were fractionated by flow cytometry into total CD34 $^{+}$, CD34 $^{+}$ CD38 $^{+}$ (committed), and CD34 $^{+}$ CD33 $^{+}$ (myeloid committed) progenitors and the apoptotic fractions determined
25 after E21R treatment. DNA fragmentation was present in the CD34 $^{+}$ /GMR α^{+} , CD34 $^{+}$ CD38 $^{+}$ /GMR α^{+} , and CD34 $^{+}$ CD33 $^{+}$ /GMR α^{+} subsets, but not amongst the non-committed CD34 $^{+}$ CD38 $^{-}$ cells, the latter subset lacking GMR α (Figure 9). Analysis of DNA binding of propidium iodide revealed that E21R caused apoptosis of the cells in the CD34 $^{+}$ CD38 $^{+}$ /GMR α^{+} (Figure 11a,c), CD34 $^{+}$ /GMR α^{+} , and the CD34 $^{+}$ CD33 $^{+}$ /GMR α^{+} populations (Figure 11c), but
30 not on cells lacking the GM-CSFR α chain (Figure 11b, c).

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E21R in medium with G-CSF induced apoptosis of the CD34⁺CD38⁺GMR α ⁺ committed progenitors Figure 11 (a), but not of the CD34⁺CD38⁺GMR α ⁻ cells Figure 11 (b). When combined with G-CSF and either GM-CSF or stem cell factor (SCF), E21R caused apoptosis of all CD34⁺GMR α ⁺ subsets after 48h, while the addition of IL-3 prevented apoptosis Figure 11 (a,c). The non-committed CD34⁺CD38⁺GMR α ⁻ cells were not affected by E21R treatment Figure 11 (b,c). Values are the mean and s.e.m. from three cases. Studies of colony formation by CD34⁺GMR α ⁺ committed progenitors showed that E21R in combination with either G-CSF, or G-CSF plus GMCSF, or G-CSF plus SCF virtually abolished colony growth, while E21R plus G-CSF and IL-3, or E21R plus G-CSF, SCF and IL-3 did not inhibit colony growth of committed cells Figure 11 (d). Colony growth of the non-committed CD34⁺CD38⁺GMR α ⁻ subset was not affected by E21R Figure 11 (d). Data from the three cases in Figure 11 (a), values are mean + s.e.m.

The bone marrow cells were collected and processed as described above. Apoptosis was measured as a reduced DNA binding of propidium iodide as outlined above. Colonies (>40 cells) of haemopoietic CD34⁺ progenitors in a methylcellulose assay were prepared and scored after 14 days. To triplicate cultures the inventors added various combinations of E21R (1 μ g/ml), G-CSF (10 ng/ml), GM-CSF (10 ng/ml), and SCF (10 ng/ml, Amgen).

Upon purification of GMR α positive cells with an anti-GMR α monoclonal antibody, virtually 100% of the cells were apoptotic with E21R (Figure 10a). The results were observed in 21/21 AML cases (Figure 10b) and 14/14 CML cases (Figure 10c) when E21R was given at a dose of 1 μ g/ml, a concentration ensuring high GMR α occupancy. This concentration was based on a titration experiment where 1 μ g/ml of E21R yielded maximal apoptosis (Figure 10d). The effect was dependent on E21R binding to GMR α since a titration of GM-CSF against 1 μ g/ml of E21R showed that at a concentration of 100 ng/ml GM-CSF totally abolished the apoptotic effect of E21R (Figure 10d).

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EXAMPLE 10

The influence of E21R on growth of the progenitors was further determined in a colony assay. Various combinations of E21R and G-CSF with or without GM-CSF and SCF virtually abolished colony growth, while adding IL-3 overcame the inhibition of colony growth by E21R (Figure 11d), consistent with E21R inducing apoptosis of committed progenitors (Figure 11c vs. 11d). It is important to note that CD34⁺CD38⁻ cells are non-committed multipotential progenitors. The E21R-induced apoptosis of committed progenitors (CD34⁺CD38⁺, CD34⁺CD33⁺), but not of CD34⁺CD38⁻ cells will be useful for enrichment of uncommitted progenitors for stem cell transplantation and gene transfer purposes. Figure 13 shows that the membrane proximal domains of both receptor chains is important for inducing apoptosis.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.



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18. A method according to claim 17 wherein the HGF is GM-CSF.
19. A method according to claim 14 wherein the antagonist of HGF is derived from said HGF said antagonist being capable of binding to the α -chain of the HGF receptor.
20. A method according to claim 19 wherein the antagonist is a derivative of GM-CSF.
21. A method according to claim 20 wherein the derivatives of GM-CSF comprises an acidic amino acid residue at amino acid position 21 of HGF being substituted with a basic amino acid residue or a non-acidic amino acid residue.
22. A method according to claim 21 wherein the acidic amino acid residue is Glu or Asp, the basic amino acid residue is Lys or Arg and the non-acidic amino acid residue is Gln or Asn or derivatives thereof.
23. A method according to claim 22 wherein the HGF antagonist is E21R or E21K.
24. A method according to claim 23 wherein the HGF antagonist is E21R.
25. A method according to claim 14 wherein the cells in which apoptosis is induced are normal and malignant myeloid cells.
26. A method according to claim 25 wherein the myeloid cells are myeloid leukaemias.
27. A method for enriching uncommitted progenitor cells from bone marrow said method comprising contacting a population of cells from bone marrow with an effective amount of a molecule capable of binding to the α -chain of an HGF receptor for a time and under conditions sufficient to induce apoptosis of cells carrying said α -chain.

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37. A method according to claim 36 wherein the leader sequence is the amino acid sequence MFATSSSTGNDG.

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* C L M S *

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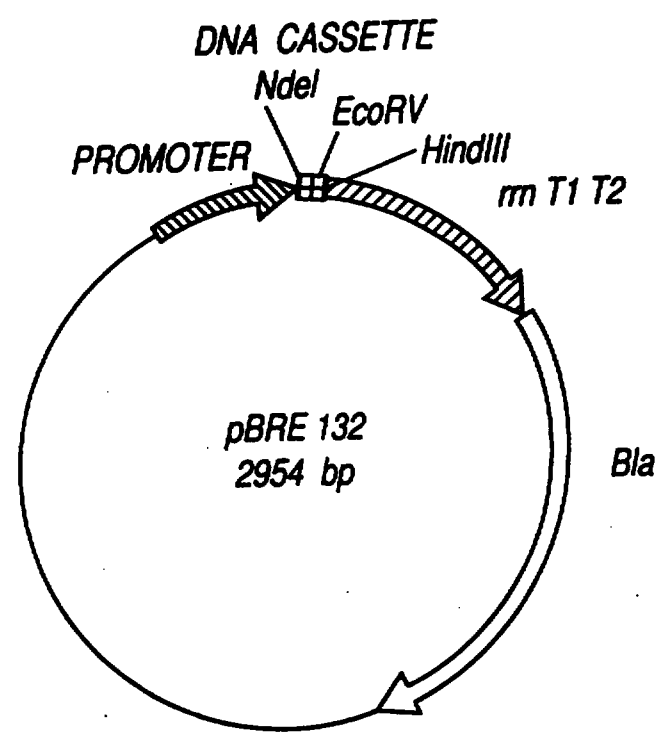


Fig. 1

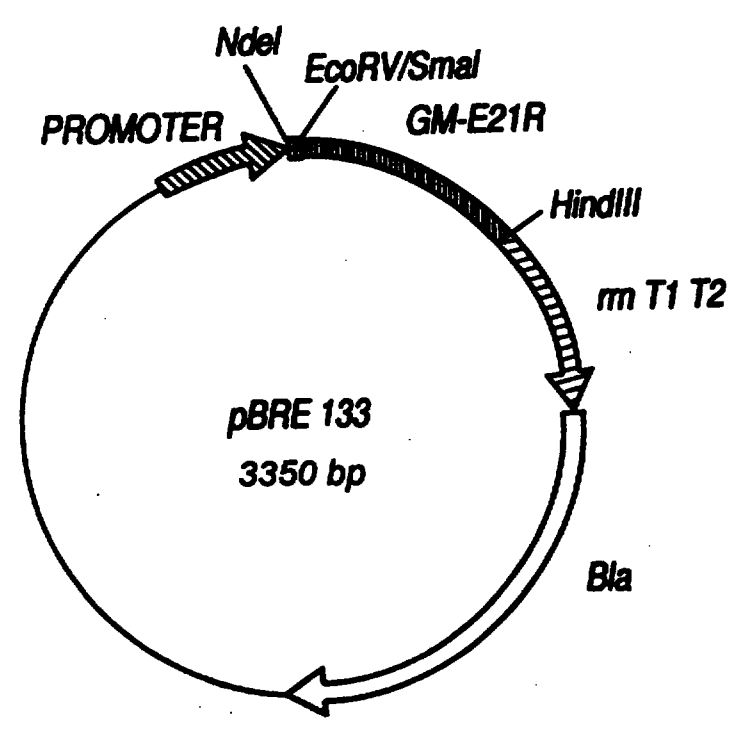


Fig. 2

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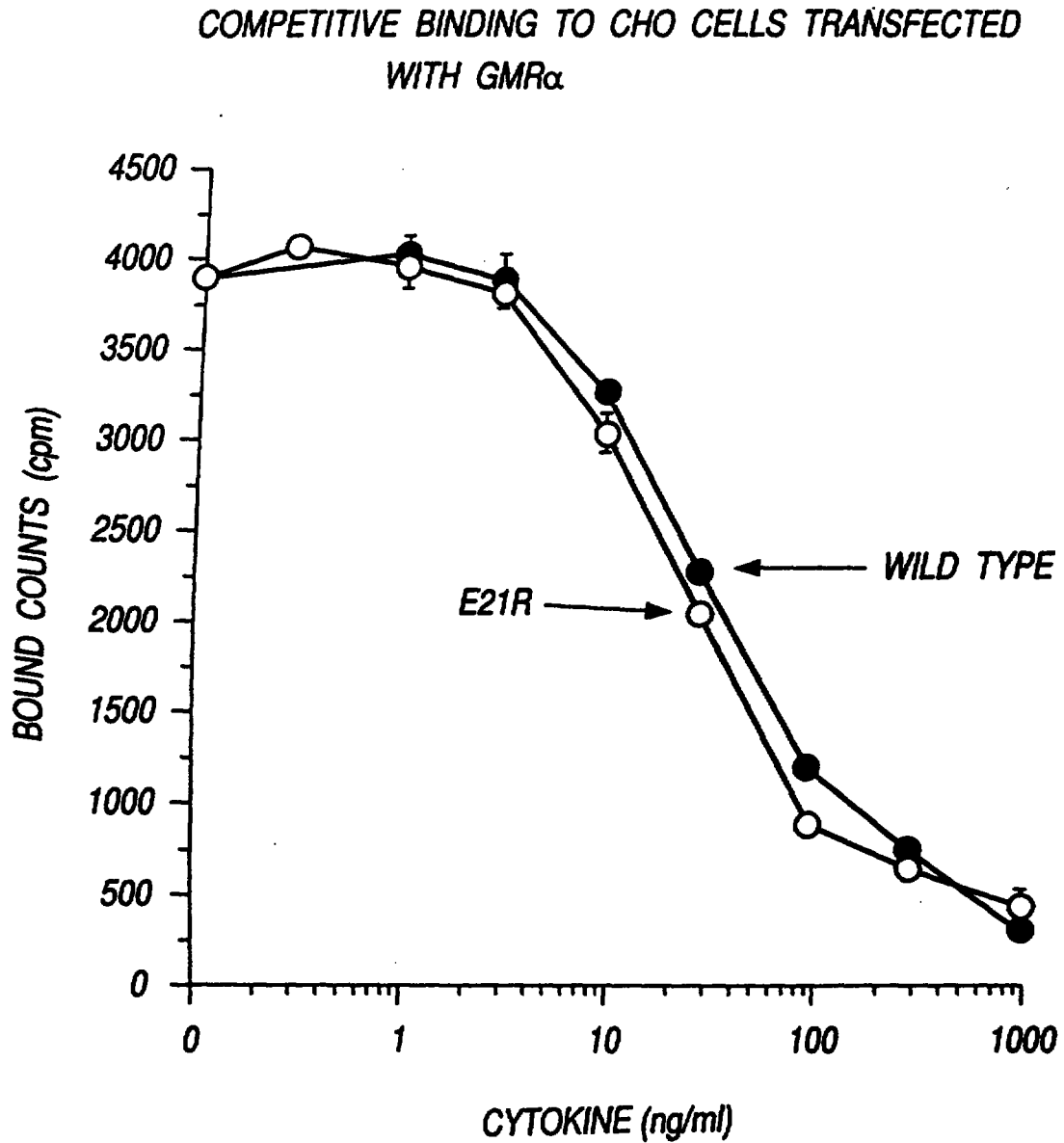


Fig. 3

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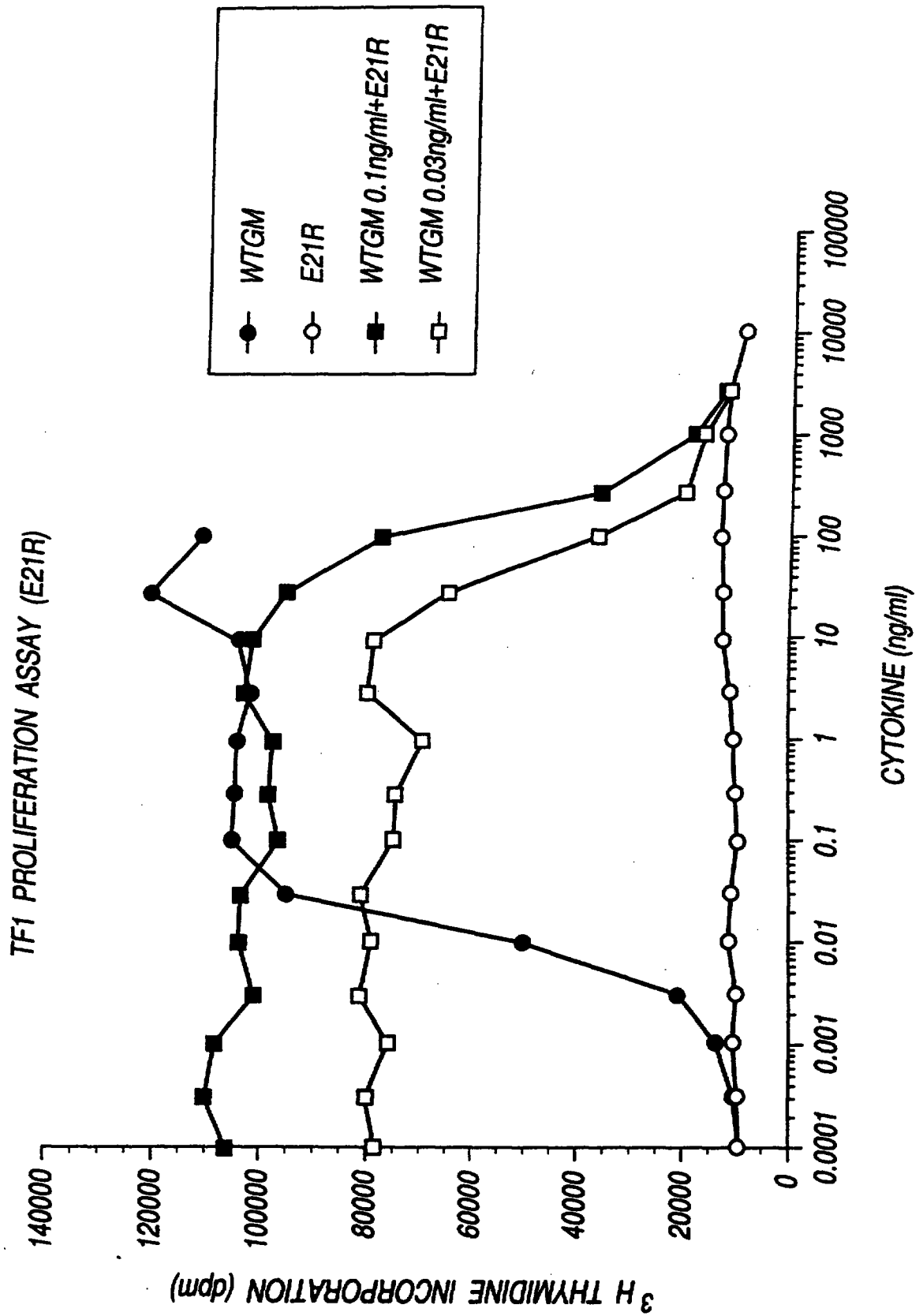


Fig. 4

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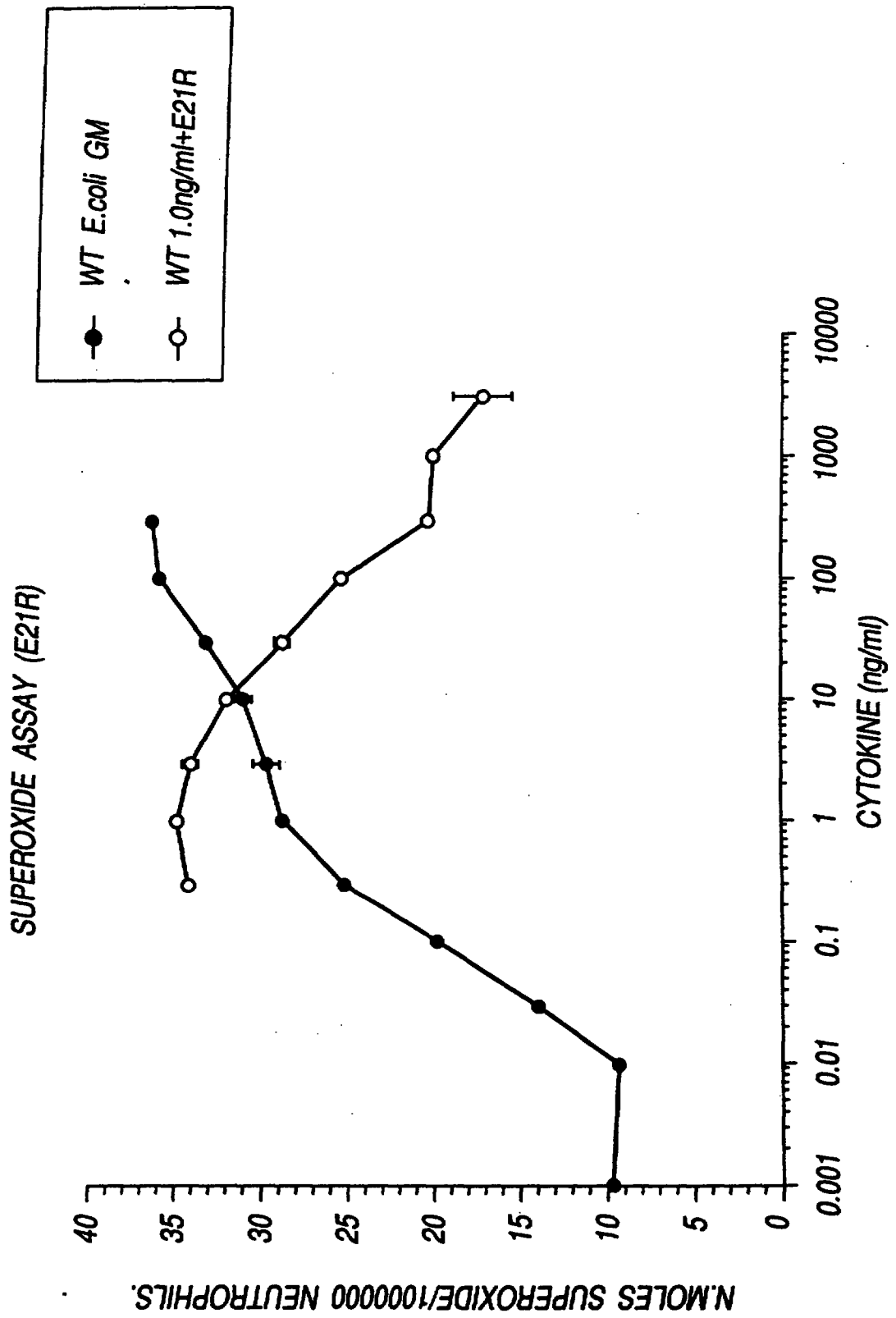


Fig. 5

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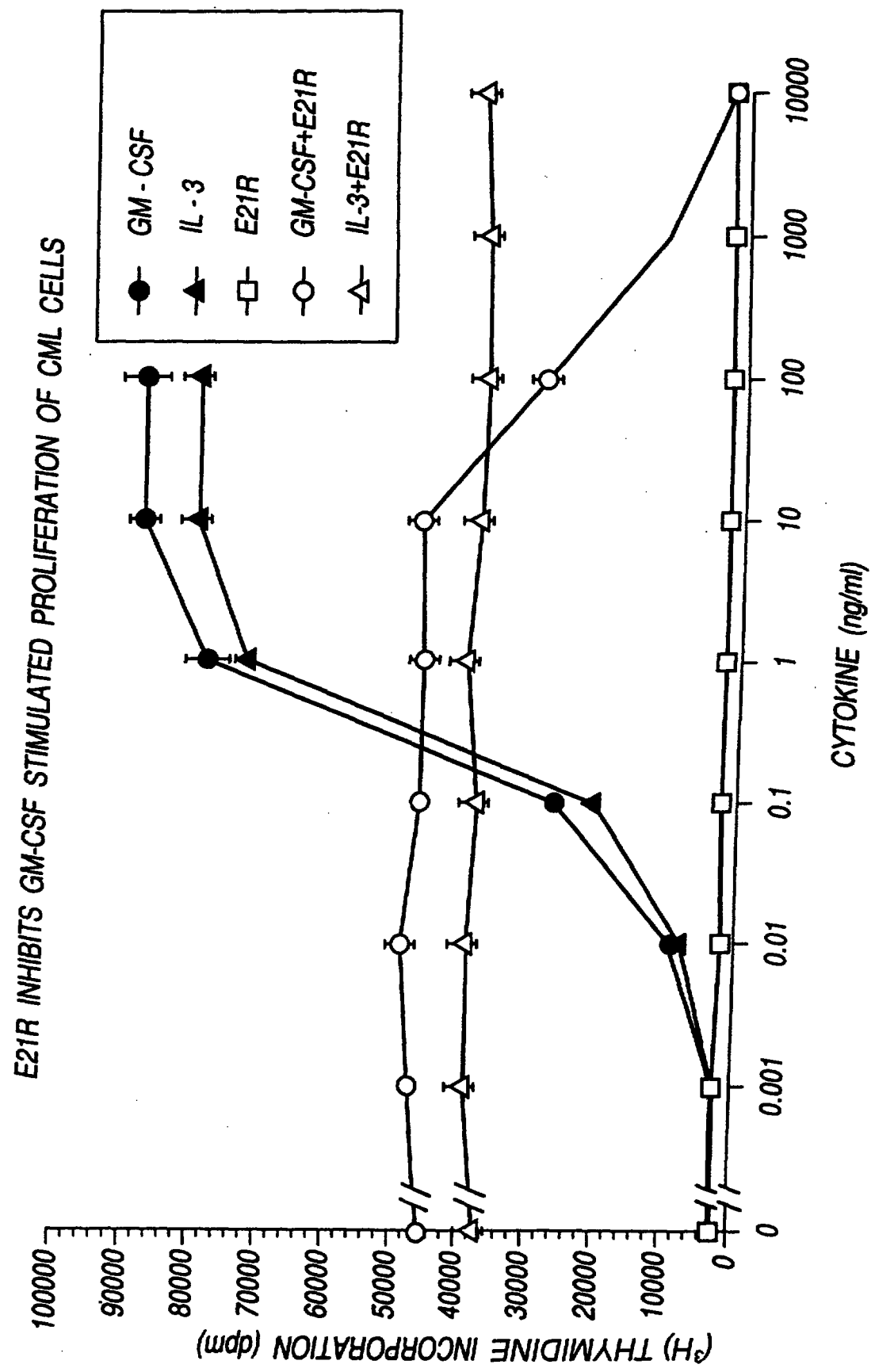


Fig. 7

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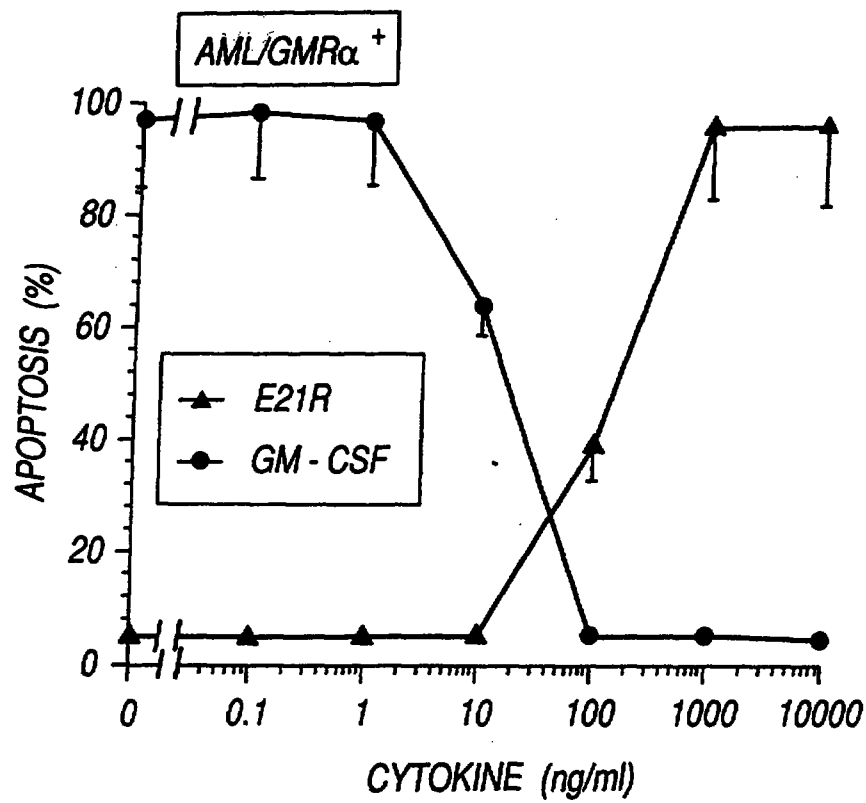


Fig.10d

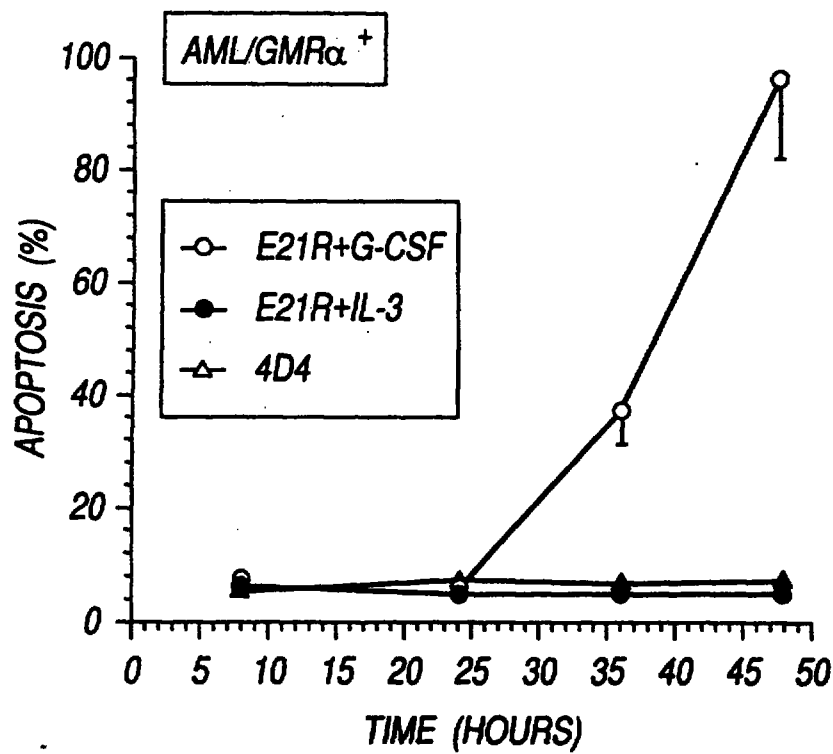


Fig.10e

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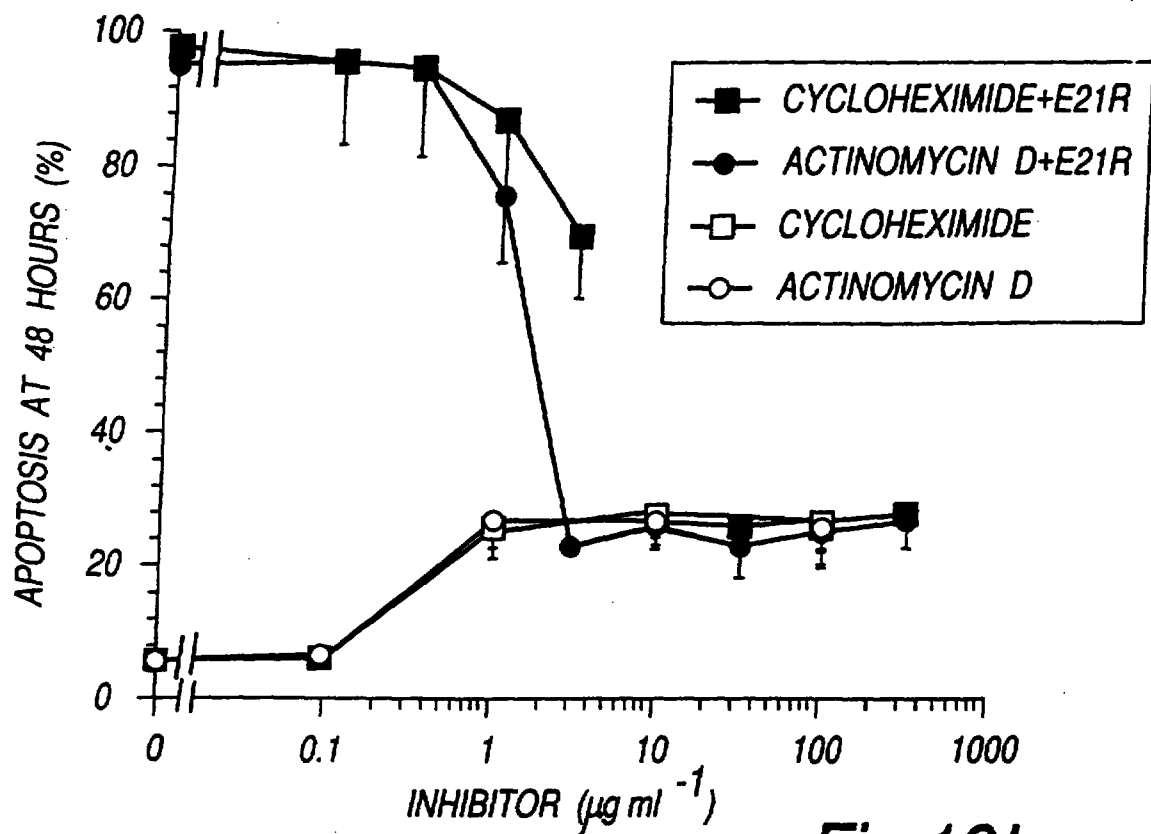


Fig.12b

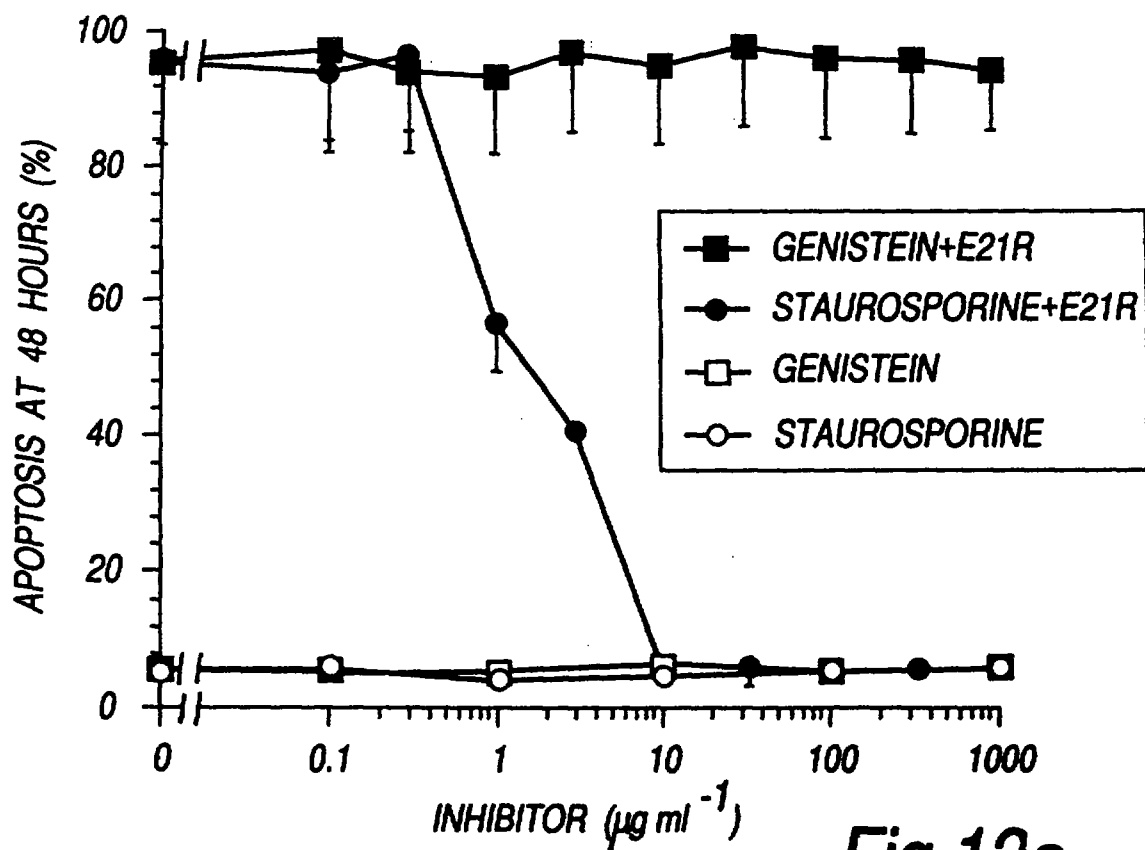


Fig.12a

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/AU96/00382 (22) International Filing Date: 21 June 1996 (21.06.96) (30) Priority Data: PN 3780 23 June 1995 (23.06.95) AU (71) Applicants (for all designated States except US): BRESAGEN LIMITED [AU/AU]; 38-39 Winwood Street, Thebarton, S.A. 5031 (AU). MEDVET SCIENCE PTY. LTD. [AU/AU]; Frome Road, Adelaide, S.A. 5000 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only): VADAS, Mathew, Alexander [AU/AU]; 8 Branch Road, Stirling, S.A. 5152 (AU). LOPEZ, Angel, Francisco [AU/AU]; 142 Stanley Street, North Adelaide, S.A. 5006 (AU). SHANNON, Mary, Frances [IE/AU]; 8 The Crescent, Crafrers, S.A. 5152 (AU). CHEAH, Keat-Chye [AU/AU]; 10 Musgrave Avenue, Aberfoyle Park, S.A. 5159 (AU). SENN, Carol, Ruth [AU/AU]; Stentiford Road, Forest Range, S.A. 5139 (AU). BASTIRAS, Stan [AU/AU]; 21 Bonython Close, Adelaide, S.A. 5000 (AU). ROBINS, Allan [AU/AU]; Lot 8 Barker Road, Waterloo Corner, S.A. 5110 (AU).	(74) Agents: HUGHES, E., John, L. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU). (81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report.	
(54) Title: HAEMOPOIETIC GROWTH FACTOR ANTAGONISTS AND USES THEREFOR		
(57) Abstract The present invention relates generally to variant recombinant forms of haemopoietic growth factors useful as antagonists to the corresponding native haemopoietic growth factor and their use in ameliorating aberrant effects caused by the native molecules and in the treatment of tumours and cancers and inflammation.		

REPLACED SHEETS SG

- ☐ Description : paged to paged
☒ Claim(s) : paged 22 to paged 36
☐ Drawing(s) : paged to paged
☐ Abstract : paged to paged