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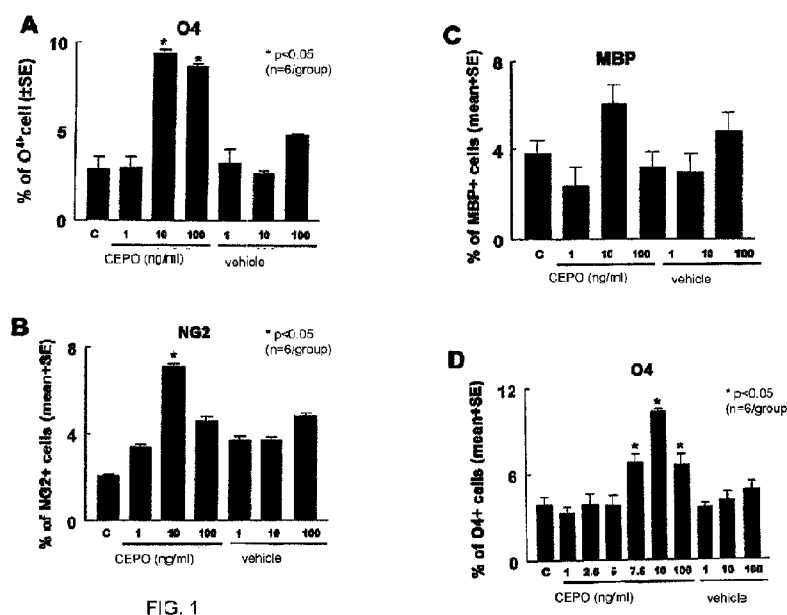
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(54) Title: BIOASSAY FOR THE MEASUREMENT OF CEPO CONCENTRATION AND ACTIVITY



(57) Abstract: The present invention relates to abioassay for detecting the effective concentra- tion of carbamylated erythropoietin (CEPO) and estimating the specific activity using neural progenitor cells, as well as the use of these neural progenitor cells for determining the concentration or activityof CEPO in a sample.

Bioassay for the measurement of CEPO concentration and activity

FIELD OF THE INVENTION

The present invention relates to a bioassay for detecting the effective concentration of carbamylated erythropoietin and estimating the specific activity using neural progenitor cells.

BACKGROUND OF THE INVENTION

The hormone erythropoietin (EPO) has been known for decades for its ability to stimulate proliferation and survival of erythroid cells in the bone marrow and spleen. Various forms of *in vitro* and *in vivo* bioassays exist to measure the levels of EPO in the biological fluids as well as in pharmaceutical preparations. The biological activity of EPO is measured in international units, conventionally as milliunits per millilitre (mU/mL), by using an *in vivo* bioassay which measures the ability of EPO containing samples to stimulate erythroid cells in comparison to a stimulation using 5 micromoles of cobalt.

Recently, it has become known that EPO also has other biological functions. It has been discovered that EPO has tissue-protective properties in for example the heart and brain tissues. New derivatives of EPO have been developed which retain the protective properties of EPO but which do not have EPO's erythropoietic effect. As one example, it has been described that by modification of the lysine residues of EPO through carbamylation, an EPO derivative can be obtained which does not have an effect on the hemopoietic system (Leist et al. *Derivatives of Erythropoietin That Are Tissue Protective But Not Erythropoietic*. Science. 2004. Vol. 305. no. 5681, pp. 239 -242). This carbamoylated EPO (CEPO) still have the ability to mediate cytoprotection e.g. in neural tissue, even though CEPO does not bind to the classical EPO receptor (EPOR) (Leist et al. *Derivatives of Erythropoietin That Are Tissue Protective But Not Erythropoietic*. Science. 2004. Vol. 305. no. 5681, pp. 239 -242). More specifically, it has recently been shown that CEPO enhances the proliferation of neural progenitor cells derived from the subventricular zone in the brain of adult mice, and their differentiation into neurons (Wang et al. *The Sonic Hedgehog Pathway Mediates Carbamylated*

Erythropoietin-enhanced Proliferation and Differentiation of Adult. JBC. 2007. 282, 32462-32470).

The inventors of the present invention have in particular discovered that the
5 CEPO mediates a dosage dependent effect on proliferation and maturation re-
sponse of oligodendrocytes derived from the SVZ (subventricular zone) in rats.
The invention describes effective concentrations, conditions and possible read-
outs applicable for determining the bioactivity of CEPO in an oligodendrocyte-
based *in vitro* system. The implication is therefore to use this finding as a bioas-
10 say for determining the effective concentration and activity of CEPO in a given
sample.

CEPO is currently in clinical development for stroke and Friedreich's ataxia, how-
ever, no bioassay currently exist for CEPO. As CEPO does not have any effect
15 on the erythroid cells, existing bioassays for EPO cannot be used. It is important
to have a bioassay in place for a new biological product to be launched on the
market because the biological activity of the final product can vary due to small
variations in the production process.

20 The present invention discloses a novel bioassay for CEPO.

SUMMARY OF THE INVENTION

The present invention relates to a method for determining the concentration or
activity of carbamylated erythropoietin (CEPO) in a sample comprising the steps
25 of,

- i) Contacting a sample comprising CEPO with neural progenitor cells
of a mammalian species, such as a rodent,
- ii) Adding a differentiation medium essentially free of external added
growth factors,
- 30 iii) Determining the expression of specific markers for the oligoden-
drogenesis, and
- iv) Correlating said expression with that of a CEPO standard or refer-
ence.

The present invention also relates to the use of said neural progenitor cells for determining the concentration or activity of CEPO in a sample.

BRIEF DESCRIPTION OF DRAWINGS

- 5 Fig. 1 shows the dosage dependent response on the neural progenitor cells when CEPO is added at different concentrations. Numbers in the vehicle groups represents different vehicle volumes used as the same volume for CEPO at corresponding concentrations * $p < 0.05$ vs control.
- 10 Fig. 2 Western blot shows levels of MBP, CNPase, and beta actin in N20.1 cells treated with CEPO 1, (CE1), 10, (CE10), and 100 (CE100) ng/ml. Beta actin was used as an internal control. C = control.

DETAILED DESCRIPTION OF THE INVENTION

- 15 The central nervous system (CNS) is composed of many types of neurons and glial cells. These cells are generated from multipotent common precursors located in e.g. the subventricular zone. As part of the differentiation process, progenitor cells in the SVZ become gradually restricted in their differentiation potential through progressive series of decisions culminating with their commitment to
- 20 a single cell lineage (Levinson and Goldman. *Multipotential and lineage restricted precursors coexist in the mammalian perinatal subventricular zone*. J. Neurosci. Res. 48 1997 (2). 83-94). Differentiation of multipotent progenitor cells is a highly complex and regulated process linked to developmental changes, response to stress situations, e.g. injury, and general change in presence of trophic factors
- 25 and cytokines. The multipotent progenitor cell can as such develop into either neuronal or glia phenotype depending on the external environment. In the progressive development of the progenitor cell, the glia progenitor cells may mature into astrocyte or oligodendrocytes phenotype. Part of the differentiation into e.g. a oligodendrocyte can be determined or monitored by the expression of specific
- 30 markers of proteins which characterize the particular cell type.

Oligodendrocytes are a major class of the CNS glial cells involved in myelination of the axons. Mature oligodendrocytes in the adult CNS are derived from non-myelinating oligodendrocyte progenitor cells (OPCs) that are present in the cor-

pus callosum and the striatum. Neural progenitor cells in the subventricular zone (SVZ) of the lateral ventricles also give rise to OPCs that disperse throughout the corpus callosum and striatum. The markers of OPCs include ganglioside GD3, the NG2 chondroitin sulfate proteoglycan, O4, and the platelet-derived growth factor-alpha receptor subunit PDGFR α . Mature oligodendrocytes express MBP and CNPase. The inventors of the present invention have discovered that CEPO stimulates the proliferation and differentiation of neural progenitor cells into oligodendrocytic lineage cells and promotes maturation of OPCs to myelinating oligodendrocytes in a dose-dependent manner and thus can be used in a bioassay to determine the potency in a sample, such as in a pharmaceutical composition.

In vitro neural progenitor cells can be harvested from, for example, the subventricular zone in the brains of various species of mammals, for example rodents, such as mice, rats, squirrels, porcupines, beavers, chipmunks, guinea pigs, and voles. Particular preferred are mice, rats or rabbits. It is envisaged that cultured stem cells, as such, can also be used.

Once harvested these neural progenitor cells may be plated in the presence of a growth medium comprising epidermal growth factor and basic fibroblast growth factor. According to one embodiment about 2×10^4 SVZ cells/ml are plated in a growth medium containing Dulbecco's Modified Eagle's Medium F-12 (DMEM-F-12), about 20 ng/ml of epidermal growth factor and about 20 ng/ml basic fibroblast growth factor.

To investigate the presence of CEPO, e.g. in the form of concentration or activity, in a given sample, CEPO may be added to the growth medium. To establish a reference or standard, the concentration of CEPO used may range from about 0 to about 100 ng/ml. According to one embodiment CEPO may be added at the following concentrations; about 0 ng/ml, about 1 ng/ml, about 5 ng/ml, about 7.5 ng/ml, about 1 ng/ml 10 and about 100 ng/mL. The cells will be left standing for about 5 to about 10 days, such as about 7 days.

The cells may then be transferred to a differentiation medium comprising about 2 to about 5 % fetal bovine serum (FBS), without growth factors. According to one embodiment the amount of FBS used is about 2%.

- 5 The cells will then be left standing for about 10 to about 20 days, such as about 14 days, where after detection of specific markers will be performed. It is envisaged that any specific marker can be used which show a dosage dependent effect of CEPO on the differentiation of the neural progenitor cells to mature oligodendrocytes, called oligodendrogenesis. According to one embodiment the
- 10 markers may be selected from one or more, or all, of O4, NG2, MBP and CNpase and may be quantified using immunostaining as shown in Example 1.

According to one aspect, the invention thus relate to a method, in particular an *in vitro* method, for determining the concentration or activity of carbamylated erythropoietin (CEPO) in a sample comprising the steps of,

15

- i) Contacting a sample comprising CEPO with neural progenitor cells from a mammalian species, such as a rodent,
- ii) Adding a differentiation medium essentially free of external added growth factors,
- 20 iii) Determining the expression of specific markers for the oligodendrogenesis, and
- iv) Correlating said expression with that of a CEPO standard or reference.

- 25 According to another aspect, the invention also relates to these neural progenitor cells for determining the concentration or activity of CEPO in a sample.

Further embodiments of the invention may be found in the claims and examples.

- 30 In the present invention, the term CEPO is intended to include any variant or derivative of carbamylated EPO (e.g described in US 2004157293 or Science, Vol. 35, pp 239-242 or WO 2006/050819 hereby incorporated by reference in their entirety), that is a variant or derivative of EPO in which at least one of the primary-amino groups (the lysines and the N-terminal group) of the protein is car-

bamylated. In particular, the invention relates to CEPO with an amino acid sequence as depicted below in table 1 (SEQ ID NO 2) or comprising an additional arginine in the C-terminal end (SEQ ID NO 1), or a sequence which is 95%, 98% or 99% identical to SEQ ID NO 1 or 2.

5

The percent identity between two amino acid sequences is a function of the number of identical positions shared by the sequences (i.e., % homology = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences may be accomplished using a mathematical algorithm, such as the BLAST program (e.g., BLAST 2.2.8 available through the NCBI using standard settings BLOSUM62, Open Gap= 11 and Extended Gap= 1).

15

Table 1

1	A	P	P	R	L	I	C	D	S	R	V	L	E	R	Y	L	L	E	A	K
21	E	A	E	N	I	T	T	G	C	A	E	H	C	S	L	N	E	N	I	T
41	V	P	D	T	K	V	N	F	Y	A	W	K	R	M	E	V	G	Q	Q	A
61	V	E	V	W	Q	G	L	A	L	L	S	E	A	V	L	R	G	Q	A	L
81	L	V	N	S	S	Q	P	W	E	P	L	Q	L	H	V	D	K	A	V	S
101	G	L	R	S	L	T	T	L	L	R	A	L	G	A	Q	K	E	A	I	S
121	P	P	D	A	A	S	A	A	P	L	R	T	I	T	A	D	T	F	R	K
141	L	F	R	V	Y	S	N	F	L	R	G	K	L	K	L	Y	T	G	E	A
161	C	R	T	G	D															

Table 1: Potential carbamylation sites are shown in bold and conventional amino acids in arial font.

20

There are nine potential carbamylation sites as shown in table 1: Alanine at position 1 and Lysine at positions 20, 45, 52, 97, 116, 140, 152 and 154. Accordingly, the invention relates to CEPO in which at least one or more, e.g. at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7 or all nine, of the of the amino acids selected from the group comprising alanine at position 1 and ly-

25

sine at positions 20, 45, 52, 97, 116, 140, 152 and 154 (as shown in table 1) are carbamylated.

- 5 CEPO may be produced by carbamylating EPO e.g. as disclosed in WO2006/002646. Briefly, purified human EPO (or alternatively recombinant human EPO or biologically or chemically modified human EPO) can be mixed with an approximately equal volume of 1M potassium cyanate/0.25M potassium tetraborate, pH about 9.0, and incubating at about 29°C for about 24-48 hours.
- 10 The reaction can be stopped by cooling to room temperature, adding 3M ammonium sulphate/150mM Tris-HCl, pH 7.5 and hydrophobic interaction chromatography (HIC).

EXAMPLES

Example 1

- 5 Materials: Primary SVZ neural progenitor cells were isolated from adult Wistar rats.

Methods:

- To investigate whether CEPO enhances proliferation and differentiation in neural
10 progenitor cells, SVZ neural progenitor cells were used.

Isolation of SVZ cells:

- Briefly, the anesthetized adult Wistar rats (3-4 months old) were killed by decapi-
tation and the brains were rapidly removed from severed heads. Under a surgical
15 microscope, SVZ tissue was dissected along the lateral wall of the lateral ventri-
cle, under the corpus callosum down to the ventral tip of the lateral ventricle from
approximately 1.5-2.0 mm thick parasagittal sections. The excised tissue was
collected in Hanks' buffered saline solution (HBSS; GIBCO) and dissociated into
a single-cell suspension by incubation in 0.25% trypsin and titration with a de-
20 scending series of fire-polished glass pipettes. The cells were washed several
times and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) supple-
mented with 10% fetal bovine serum (FBS), 5% horse serum, 1% antibiotic anti-
mycotic, and bFGF 20 ng/ml (R&D System, Minneapolis).

- 25 Experimental protocol:

- SVZ cells were plated at a density of approximately 2×10^4 cells/ml in the pres-
ence growth medium that contains Dulbecco's Modified Eagle's Medium-F-12
(DMEM-F-12) medium, 20 ng/ml of epidermal growth factor and 20 ng/ml basic
fibroblast growth factor. CEPO at concentrations of 0, 1, 7.5, 10, and 100 ng/ml
30 were added and left standing for 7 days. Bromodeoxyuridine (BrdU, 20 μ g/ml)
was added to the culture medium at 6 days to label proliferating cells. The cells
were subsequently transferred to a differentiation medium that contained 2% fetal
bovine serum (FBS), but the growth factors were eliminated. The cells were cul-
tured for another 14 days.

Immunostaining and quantification of immunoreactive cells:

Immunofluorescent staining of cultured cells was performed. The following primary antibodies were used in the present study: rabbit anti-NG2 (1:200, Chemicon, CA), mouse anti-O4 (1:100, Chemicon, CA), mouse anti-CNPase (1:400, Chemicon, CA), and rabbit anti-MBP (1:400, Dako). Cultured cells were fixed in 4% paraformaldehyde for 15-20 min at room temperature. Nonspecific binding sites were blocked with 5% normal goat serum for 60 min at room temperature. The cells were then incubated with the primary antibodies listed above and with CY3-conjugated or FITC-conjugated secondary antibodies. Nuclei were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). The number of positive cells and total DAPI cell number were counted in randomly selected 5 microscopic fields under 20x objective and the percentage of immunoreactive cells within the total number of DAPI positive cells were quantified.

Results: The effect of CEPO on proliferation and differentiation of SVZ neural progenitor cells

CEPO at 10 and 100 ng/ml significantly increased the number of O4 positive oligodendrocyte progenitor cells with a peak at 10 ng/ml (Fig. 1A). Using additional doses it was found that incubation of neural progenitor cells with CEPO at 7.5, 10, and 100 ng/ml significantly increased the number of O4 positive oligodendrocyte progenitor cells with a peak at 10 ng/ml (Fig. 1D). CEPO at 10 ng/ml also significantly increased NG2 positive oligodendrocyte progenitor cells (Fig. 1B). Moreover, CEPO at 7.5, 10, and 100 ng/ml significantly increased the number CNpase positive myelinating oligodendrocytes, with a peak at 10 ng/ml (Fig. 1E). However, although we detected an increase in MBP positive myelinating oligodendrocytes at 10 ng/ml, (Fig. 1C) it did not reach statistical significance (Fig. 1C).

EXAMPLE 2

To investigate whether CEPO enhances maturation of OPCs to mature oligodendrocytes, the N20.1 cell line, mouse oligodendrocyte immortalised with SV40 T antigen, was used, which was generously provided by Dr. Anthony Campagnoni, University of California at Los Angeles. The N20.1 cells were cultured in DMEM/
5 Ham's F12 medium contains 10% FBS (Fetal Bovine Serum) and G418 (100ul/ml) at 37°C. The cells were passed once a week after trypsinization (0.05% trypsin-ethylenediaminetetraacetic acid; Life Technology).

10 Experimental protocol:

The N20.1 cells were treated with CEPO (0, 1, 10 and 100 ng/ml) in DMEM/Ham's F12 containing 1% FBS but without G418 at 39°C for 9 days.

Western blot analysis:

15 Briefly, mouse oligodendrocytes were lysed and equal amounts of proteins were loaded on 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes and the blots were subsequently probed with the following primary antibodies: mouse polyclonal anti-CNPase (1:1000, Millipore) and rat polyclonal anti-MBP (1:1000, Millipore). For detection, horseradish peroxidase (HRP-) conjugated secondary antibodies were used (1:2000) fol-
20 lowed by enhanced chemiluminescence (ECL) development (Pierce Biotechnology, Inc.). Normalization of results was ensured by running parallel Western blots with the β -actin antibody used as an internal control. The optical density was quantified using an image processing and analysis program (Scion image, Eder-
25 ick, MA).

Results: The effect of CEPO on maturation of oligodendrocytes of N20.1 cells
CEPO at 1, 10 and 100 ng/ml substantially increased CNPase protein levels
30 while CEPO at 1 and 10 ng/ml augmented MBP protein levels (Fig. 2). Mature oligodendrocytes express MBP and CNPase genes. Thus, these data indicate that CEPO promotes maturation of oligodendrocytes.

CLAIMS

1. A method for determining the concentration or activity of carbamylated erythropoietin (CEPO) in a sample comprising the steps of,
 - 5 i) Contacting a sample comprising CEPO with neural progenitor cells of a mammalian species, such as a rodent,
 - ii) Adding a differentiation medium essentially free of external added growth factors,
 - iii) Determining the expression of specific markers for the oligodendrogenesis, and
 - 10 iv) Correlating said expression with that of a CEPO standard or reference.
2. The method according to claim 1, wherein said neural progenitor cells in step i) are isolated from rats or mice and incubated in a growth medium
15 for about 5 to about 10 days together with the sample comprising CEPO before proceeding to step ii).
3. The method according to any one of claims 1 or 2, wherein the neural progenitor cells used in step i) have a density of about 2×10^4 cells/ml.
4. The method according to claim 2, wherein said growth medium comprises
20 about 20 ng/ml basic fibroblast growth factor and about 20 ng/ml epidermal growth factor.
5. The method according to any one of the previous claims, wherein said cells in step ii) are incubated for time period of about 10 to about 20 days.
6. The method according to any one of the previous claims, wherein said differentiation medium in step ii) comprises about 2% to about 5% % serum,
25 such as fetal bovine serum.
7. The method according to any one of the previous claims, wherein said markers in step iii) are selected from NG2, MBP, O4 and CNapase.
8. The method according to claim 1, wherein step iv) comprises comparing
30 the expression with a CEPO standard or reference curve.
9. The method according to any one of the previous claims, wherein said CEPO standard or reference in step iv) is made using a dosage range from about 0 to about 100 ng/ml of CEPO.

10. Use of neural progenitor cells from a mammalian species, such as a rodent, for determining the concentration or activity of CEPO in a sample.
11. Use of a neural progenitor cell according to claim 10 in a method according to any one of claims 1-9.

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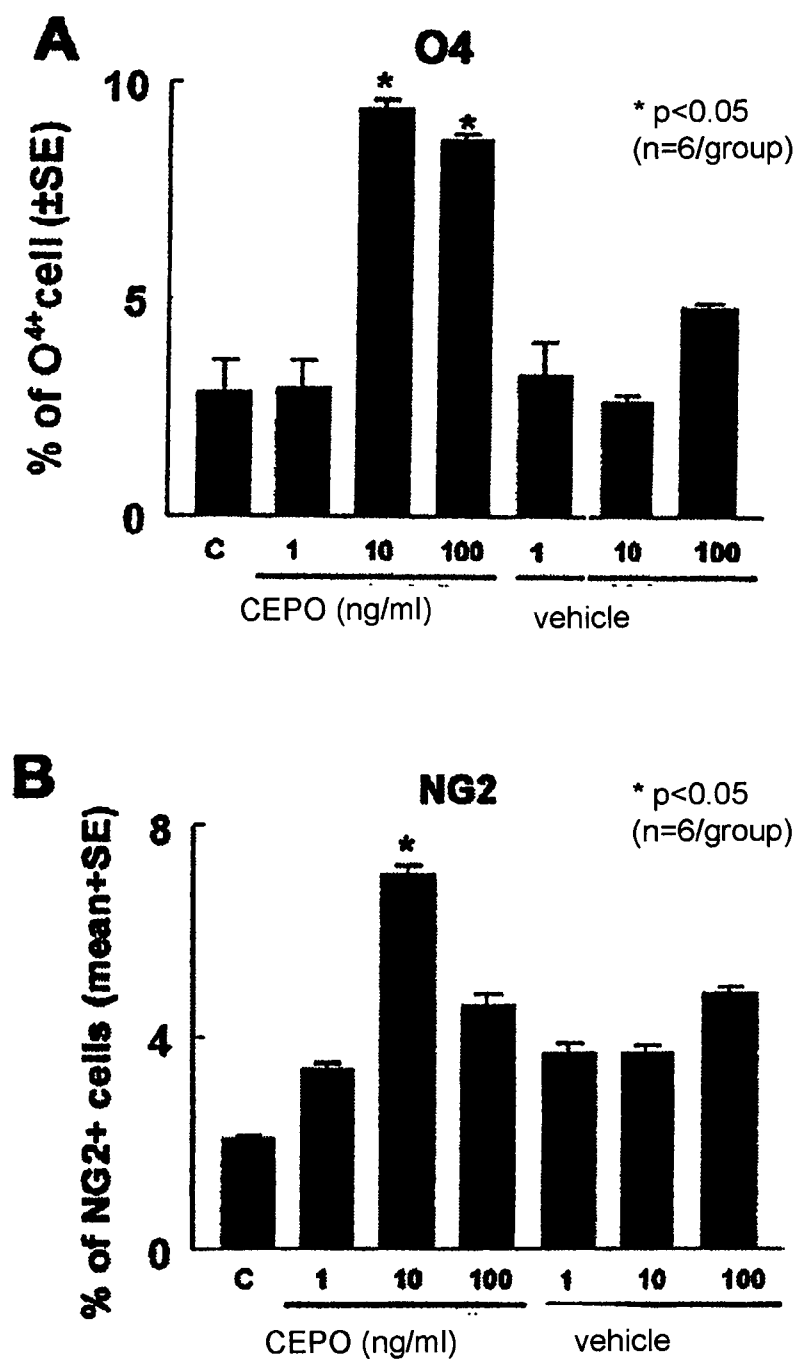


FIG. 1

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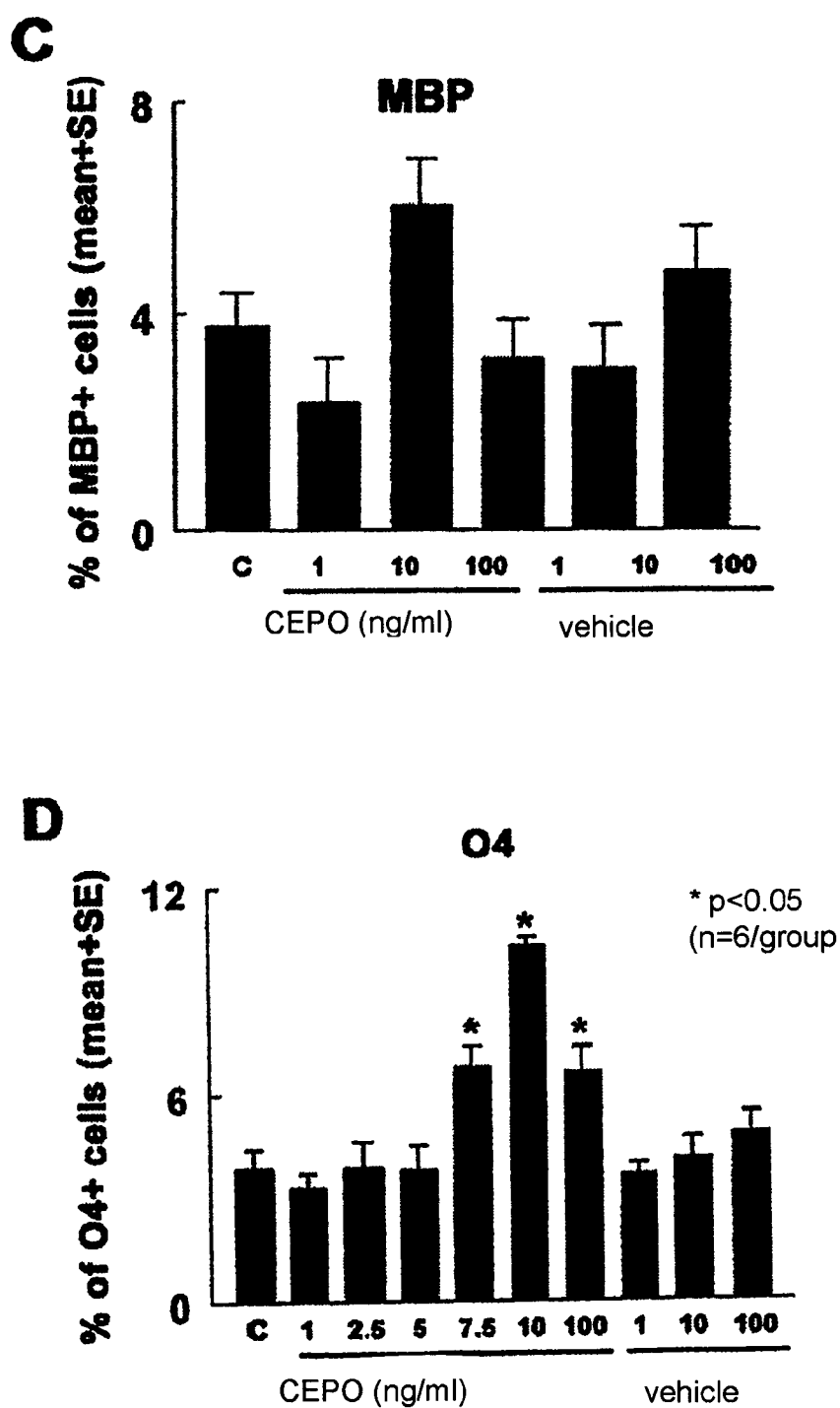


FIG. 1

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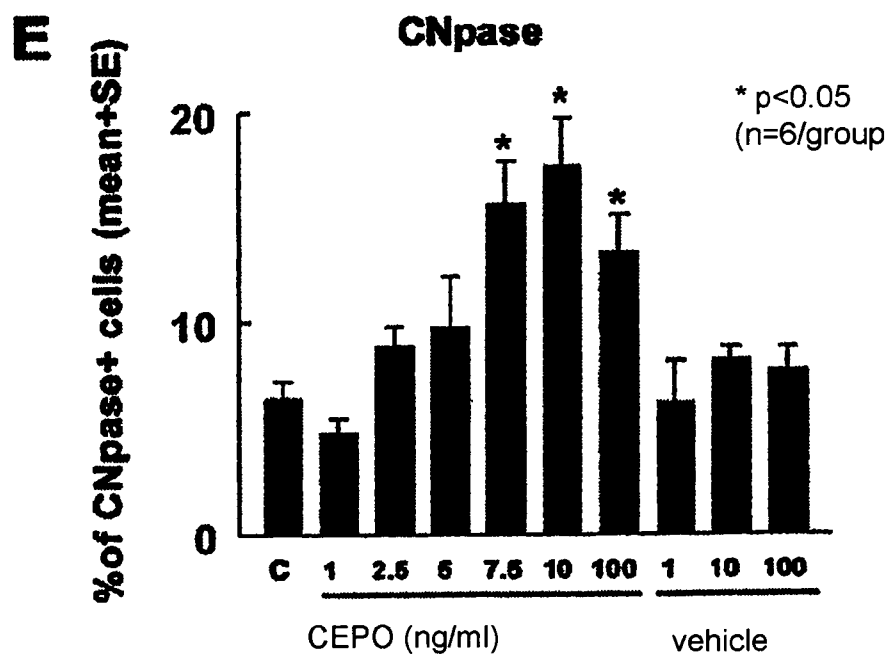


FIG. 1

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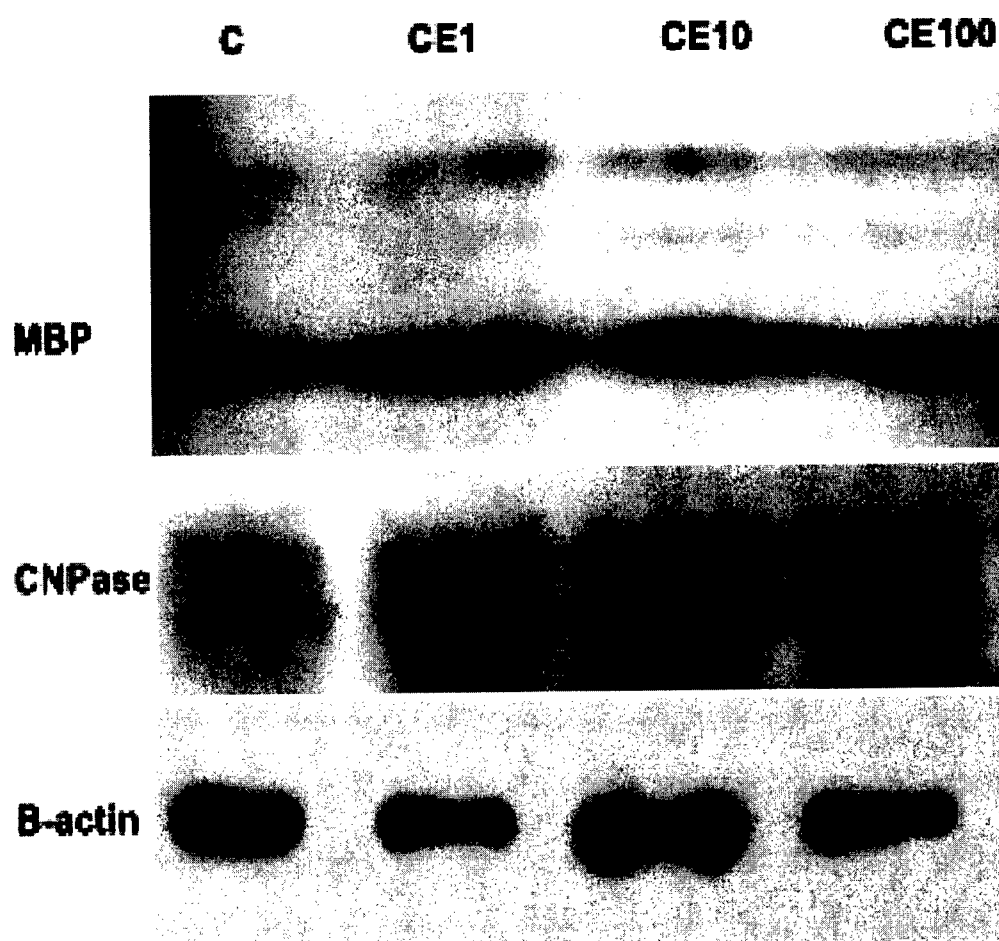


Fig. 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2011/050285

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/74 G01N33/50
ADD.

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)
G01N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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A	the whole document, in particular abstract; page 32464, paragraph spanning columns 1 and 2; figure 1 ----- -/--	1-9,11



Further documents are listed in the continuation of Box C.



See patent family annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search

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Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2011/050285

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2006/002646 A2 (LUNDBECK & CO AS H; CHRISTENSEN S; FOLDAGER L) 12 January 2006 (2006-01-12) the whole document, in particular page 38, lines 9-13 -----	1-11
A	EP 0 183 404 A2 (YISSUM RES DEV CO) 4 June 1986 (1986-06-04) the whole document, in particular page 4, line 20 to page 5, line 18; claim 1 -----	1-11
A	US 5 908 754 A (LISI P J ET AL) 1 June 1999 (1999-06-01) the whole document, in particular column 4, lines 19-46; claims 1, 2 -----	1-11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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