

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
7 December 2006 (07.12.2006)

PCT

(10) International Publication Number
WO 2006/129110 A2

(51) International Patent Classification:
G01N 33/68 (2006.01)

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(21) International Application Number:
PCT/GB2006/002030

(81) Designated States (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV,
LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI,
NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG,
SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US,
UZ, VC, VN, YU, ZA, ZM, ZW.

(22) International Filing Date: 2 June 2006 (02.06.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0511279.2 2 June 2005 (02.06.2005) GB

(84) Designated States (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT,
RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA,
GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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Published:

— *without international search report and to be republished
upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: ASSAY

(57) Abstract: The invention relates to a method for screening for pre-eclampsia in a mammal, such as a human, by determining the amount of ACE2 (Angiotensin-converting Enzyme 2), variant, or fragment of ACE2, or mRNA encoding the ACE2. Additionally a new fragment of ACE2 has been identified and isolated. Reagents and kits for carrying out the method are also provided.



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Assay

The invention relates to a method for screening for pre-eclampsia in a mammal, such as a human, by determining the amount of ACE2 (Angiotensin-converting Enzyme 2), variant, or fragment of ACE2, or mRNA encoding the ACE2. Additionally a new fragment of ACE2 has been identified and isolated. Reagents and kits for carrying out the method are also provided.

Pre-eclampsia is a disorder unique to pregnancy (affecting about 10% of pregnancies), characterised by high blood pressure i.e. blood pressure of $> 140/90$ mmHg (on at least two occasions 6 hours apart) and the presence of protein in the urine. In some cases (1-2% of pregnancies), convulsions or coma or both may develop resulting in eclampsia. It endangers both the mother and foetus and along with other hypertensive pregnancy disorders, is one of the main causes of maternal and perinatal morbidity and mortality. In the developed world, pre-eclampsia is estimated to play a role in almost 1 out of every 5 maternal deaths and accounts for some 15% of premature births. The costs associated with managing pre-eclampsia have been estimated to be in the region of 10 billion US dollars per year with a similar figure being suggested in coping for disease after birth resulting from pre-eclampsia during pregnancy. These later effects include the psychological and physical effects on the affected mother (cerebral haemorrhage and adult respiratory distress syndrome) and many infant conditions associated with premature birth and intrauterine growth restriction due to pre-eclampsia, ranging from respiratory distress in premature babies to cerebral palsy, blindness, epilepsy, deafness, and learning disabilities. In severe cases, intrauterine death may occur. The detrimental effects of pre-eclampsia upon the health of women and children all over the world has prompted the World Health Organisation to launch a global program to combat this disorder.

Pre-eclampsia is a rapidly progressive disorder affecting multiple organ systems. In severe cases, a multidisciplinary approach in an intensive care setting is absolutely crucial in the successful care of these patients. The current management of pre-eclamptic

patients concentrates upon intensive maternal and foetal surveillance employing a wide range of blood tests, urinalysis and ultrasonography (Doppler). The use of anti-hypertensives (methyldopa, nifedipine, hydralazine and labetalol) is well recognised, particularly in the prevention of cerebro-vascular accidents. The ultimate treatment of pre-eclampsia is the delivery of the placenta (and the baby) that invariably abates the progression of this disease.

The pathophysiology of pre-eclampsia has been well studied. The underlying abnormality is generalised vasoconstriction of the arterioles and enhanced sensitivity of these blood vessels to vasopressor peptides and amines. It has been proposed by some investigators that an imbalance of prostaglandins i.e. Prostacyclin (vasodilator and platelet aggregator inhibitor) and Thromboxane A₂ (vasoconstrictor and platelet aggregator) is central in the development of pre-eclampsia. Other investigators have found that there is decreased production of nitric oxide, an endogenous vasodilator, in pre-eclampsia. However, despite extensive research, the exact aetiology of pre-eclampsia remains an enigma. In relation to this, the placenta has been identified as the organ with a pivotal role in the pathogenesis of pre-eclampsia. Essentially, in pre-eclampsia, placentation and trophoblast invasion is abnormal. This compromises the utero-placental circulation and results in placental ischaemia.

Pre-eclampsia assays are known.

WO 91/16633 shows a pre-eclampsia marker based on A134-binding cell marker. This is assayed using an anti-(cellular fibronectin) antibody.

US 5,198,366 discloses pp-13 as a marker for pre-eclampsia, intra-uterine growth retardation and pre-term delivery.

Cytokines have been implicated in pre-eclampsia. Hence, M-CSF levels have been suggested as an assay target and therapeutic agent (US 5,543,138).

Other markers that have been tried to be assayed include Insulin-like Growth Factor Binding Protein 1 (US 5,712,103), Marinobutagenin (WO 2004/071273), defensins (WO 99/42826) and free albumin: non-esterified fatty acid ratios (WO 01/77675). Mitogens have also been assayed (US 5,238,819), as have phosphatidyl choline (US 6,461,830).

Syncytin levels have been used as targets for pre-eclampsia drugs (WO 02/04678 and US 2002/0102530).

US 5,849,474 discloses an assay method which looks for haemoglobin variants, haemoglobin variant precursors or red blood cell glycolytic enzymes or precursors of such enzymes from the blood of a pregnant female mammal. The assayed compounds are produced within the female mammal's red blood cells. The document suggests that reduced levels of 2,3 diphosphoglyceric acid (2,3-DPG) could indicate an interruption in glycolysis, resulting in decreased ATP production and increased haemolysis. 2,3-DPG increase in normal pregnancy causes a shift in the oxyhaemoglobin dissociation curve for the mother's blood which increases the supply of oxygen made available to not only maternal tissues, but also for transport to the foetus.

There is a complicated enzymatic cascade effecting the production of 2,3-DPG involving a large number of different enzymes. US 5,849,474 suggested that one or more of these enzymes from the mother might be involved in affecting 2,3-DPG levels. A problem with this approach is that there are a number of different factors which affect the 2,3-DPG production in mothers, including stress factors as diverse as the altitude at which the mother lives and whether the mother smokes.

Although there are several different pre-eclampsia assays known, there is still a need to produce improved assays for determining the presence of the condition as early as possible during pregnancy, to allow treatment of the condition before further harm is done to either the mother or the foetus.

The renin-angiotensin system (RAS) has been known for many years. It has a central role in the control of blood pressure. Within the RAS angiotensinogen is converted to a 10 amino acid long peptide (angiotensin I - Ang I) by renin. This in turn is converted into angiotensin II (Ang II), an 8 amino acid long peptide by Angiotensin Converting Enzyme (ACE). This is a zinc metallopeptidase. ACE has been identified as a fundamental regulator of RAS in humans and is an important target for blood pressure homeostasis. ACE inhibitors such as captopril and lisinopril have been proven to be effective anti-hypertensive therapeutic agents. Ang II is directly involved in causing vasoconstriction, cell proliferation and hypertrophy.

In 2000 a human homologue of ACE was discovered (see the review by Warner F.J., *et al.*, *Cell. Mol. Life Sci.* (2004), Vol. 61, pages 2704-2713). This human homologue is known as ACE H or ACE2. ACE2 produces a 7 amino acid peptide, Ang(1-7) via two pathways: it converts Ang I to a 9 amino acid peptide Ang(1-9) which is converted to Ang(1-7) by ACE; and it converts Ang II directly to Ang(1-7). Ang(1-7) is thought to be involved in vasodilation, anti-proliferation and apoptosis. Hence, it decreases blood pressure in humans.

ACE2 has been found in rat, mouse and human genomes, displaying about 86% and 82% identity to the human sequence. The human ACE2 gene consists of 18 exons, the first 12 of which being of a similar size to the first 11 exons of the ACE. The gene comprises a zinc-binding motif within exon 9. Only a single ACE2 species appears to have been reported. ACE gives two isoforms: sACE with two catalytic domains and tACE with only one such domain. The gene also contains a portion encoding a transmembrane region.

Work on animal models indicates that ACE2 is associated with hypertension. Tissue-specific expression of ACE2 mRNA has been observed in kidney and heart. Immunohistochemical studies have localised ACE2 to the endothelion of ventricular intramyocardial vessels and over the smooth muscle and adventitia of larger vessels. ACE2 activity has been observed to increase in idiopathic dilated cardiomyopathy

patients. In kidney ACE2 has been observed in the endothelium of intrarenal arteries and epithelia of proximal tubules. It has also been observed in testes and the gastrointestinal tract. The presence of ACE2 in placentae has not been reported.

The ACE2 gene encodes an open reading frame of an 805 amino acid polypeptide. This includes a potential signal peptide at the N-terminus and a 22 amino acid hydrophobic region near the C-terminus, thought to be a transmembrane region. The gene contains a number of glycosylation sites.

Towler P., *et al.* (*J. Biol. Chem.* (2004), Vol. 279(1), pages 17996-18007) discusses the study of X-ray crystals in combination with an ACE2 inhibitor MLN-4760 ((S,S)-2-[1-carboxy-2-[3-(3,5-dichlorobenzyl)-3H-imidazol-4-yl] ethylamino]-4-methylpentanoic acid). Huentelman M.J., *et al.* (*Hypertension* (2004), Vol. 44, pages 903-906) discusses the identification of an ACE2 inhibitor N-(2-aminoethyl)-1-aziridine-ethanamine. This inhibitor is also implicated in the inhibition of SARS coronavirus, for which ACE2 appears to be a target.

Brosnihan K.B. (*Braz. J. Med. Biol. Res.* (2004), Vol. 37(8), pages 1255-1262) shows that Ang(1-7) is increased in pregnancy. In pre-eclamptic subjects plasma Ang(1-7) was suppressed compared to normal levels. Kidney and urinary levels of Ang(1-7) were increased in pregnant rats. This coincided with enhanced detection and expression of ACE2 in kidneys in pregnant rats. This led the authors of the paper to speculate that ACE2 may play a role in the renal production of Ang(1-7) in pregnancy.

No detection of ACE2 in placentae has previously been reported. The Applicants have unexpectedly found that ACE2 mRNA is increased fivefold in response to low oxygen concentrations compared to normal oxygen conditions. In contrast ACE is undetectable following exposure to a low oxygen environment indicating a large drop in mRNA levels.

Real time PCR measurements have unexpectedly found increases in mRNA of ACE2 in patients with pre-eclampsia of 1.8 fold compared with biopsies from normal placentas.

This was completely unexpected. Brosnihan (2004) reported that plasma Ang(1-7) was suppressed in pre-eclamptic subjects compared with normal pregnancy. If a decrease in Ang(1-7) is observed then it would have been expected that ACE2 would be present in decreased concentrations in the body, as ACE2 produces Ang(1-7). Furthermore, as discussed above the presence of ACE2 in placenta itself was unexpected. Tipnis S.R., *et al.* (*J. Biol. Chem.* (2000), Vol. 275(No. 43), pages 33238-33243) studied ACE2 (which they called ACE H) expression in a number of tissues and failed to identify any expression in placenta as measured by Northern blotting.

Tipnis, *et al.* also report a 120 kDa soluble form of ACE2 which is able to cleave angiotensin I and angiotensin II, but not bradykinin or Hip-His-Leu, isolated from Chinese Hamster ovary cells. This is suggested as being a glycosylated protein lacking the transmembrane region of the protein. Deglycosylation with PNGase resulted in migration of the protein when subjected to SDS-PAGE at an apparent molecular weight of ca.85 kDa.

The Applicants have now identified a smaller soluble fragment of about 55 kDa. This has been found in blood, unlike the larger 85 kDa fragment which, whilst having been found in tissue cultured cells, has not been detected in blood or human body fluids (Warner F.J., *et al.*, *Cell. Mol. Life Sci.* (2004), pages 2704-2713). Warner, *et al.* do report that the ACE2 soluble form (the 85 kDa), having been isolated from urine in rats, however the paper indicates that the majority of animal studies have relied on indirect means of measuring ACE2 activity in plasma by measuring changes in levels of Ang II or Ang(1-7). Hence the finding of (1) the smaller (ca.55 kDa) soluble form of ACE2 and (2) the presence of the fragment in blood is totally unexpected.

The Applicants believe that the presence of increased ACE2 in pre-eclampsia is due to the need to increase the oxygen and blood supply to the foetus. The mother or foetus produces more ACE2 to dilate the blood vessels to the placenta.

The first aspect of the invention provides a method for screening for placental insufficiency, such as pre-eclampsia in a pregnant mammal such as a human or mouse comprising the steps of:

- (i) providing a sample of maternal or foetal bodily fluid or tissue;
- (ii) measuring the amount of Angiotensin-Converting Enzyme (ACE2) or fragment or a variant thereof, for example directly by detecting the protein, or indirectly by measuring the amount of mRNA encoding the ACE2, fragment or variant thereof in the sample; and
- (iii) comparing the amount with a known normal level of ACE2 or a fragment of variant thereof.

The normal level is a standard level of the protein, for example established by averaging levels in pregnant patients without pre-eclampsia.

Angiotensin-Converting Enzyme 2 (ACE2) is also known as ACE H. As discussed above, ACE2, when present as the full protein with a transmembrane region is capable of converting Ang I to a 9 amino acid peptide Ang(1-9), it is also capable of converting Ang II directly to Ang(1-7).

Figure 1 shows a summary of the interaction between ACE and ACE2 in the conversion of angiotensin I and angiotensin II.

The nucleotide sequence introduced amino acid sequence of cDNA encoding ACE H (ACE2) is disclosed in the article by Tipnis, *et al.* (2000 - *Supra*).

Appendix 1 shown the Genbank files for human and mouse ACE2. In the present application, the human ACE2 amino acid and nucleotide sequences (as shown in NM_021804.1 (GI:11225608)) are designated SEQ ID No. 1 and SEQ ID No. 2

(respectively) and the mouse ACE2 amino acid and nucleotide sequences (as shown in NM_027286.1 (GI:39930432)) are designated SEQ ID No. 3 and SEQ ID No. 4 (respectively). The amino acid and nucleotide sequences of the complete cds of human ACE2 (as shown in AB046569.1 (GI:13516971)) are designated SEQ ID No. 5 and SEQ ID No. 6, respectively.

The term “variant” preferably means a protein having the same activity as ACE2, that is having the ability to cleave angiotensin I and angiotensin II. However, it may also be a substantially inactive protein.

Preferably, the ACE2 is not capable of hydrolysing angiotensin(1-9), angiotensin(1-7), bradykinin, bradykinin(1-7) and/or neurotensin(1-14). Preferably, the ACE2 is capable of complete hydrolysis of angiotensin IV, des-Arg⁹ bradykinin, apelin-13, apelin-36, beta-casomorphin and/or dynorphin A 1-13.

Substrates and inhibitors of human soluble ACE2 are discussed in the article by Warner, *et al.* (*Cell. Mol. Life Sci.* (2004), Vol. 61, pages 2704-2713).

Preferably, the ACE2 activity is inhibited by EDTA, but not by captopril, lisinopril or enalaprilat. The ACE2 preferably functions as a carboxypeptidase.

The variants of ACE2 preferably have the same activity as the native ACE2, discussed above, but may be inactive. Preferably, the ACE2 variants have at least 80, 85, 90, 95 or 98% homology to one or more of the nucleic acid sequences shown in Appendix 1 and/or have at least 80, 85, 90, 95 or 98% identity to an ACE2 sequence shown in Appendix 1.

Preferably, the fragment of the ACE2 protein contains at least 20, 30, 40, 50 or 60 contiguous amino acids of a sequence shown in Appendix 1 or a variant thereof. Most preferably, the fragment has a molecular weight of approximately 55 kDa as determined by SDS-PAGE. Preferably, the fragment has a molecular weight of from 50 to 60 kDa, *e.g.* 50, 51, 52, 53, 54, 55, 56, 57, 58, 59 or 60 kDa.

An ACE2 variant is preferably encoded by a nucleic acid sequence or amino acid sequence which varies from an ACE2 sequence such as the sequence shown in Appendix 1 by the addition, deletion or substitution of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleotides or amino acids.

For example, there is redundancy in the genetic code which allows different nucleotide sequences to encode the same amino acids. The table below shows the mRNA triplets and the amino acids they encode:

	U		C		A		G	
U	UUU } Phe UUC } UUA } Leu UUG }		UCU } UCC } Ser UCA } UCG }		UAU } Tyr UAC } UAA* Stop UAG* Stop		UGU } Cys UGC } UGA* Stop UGG Trp	U C A G
C	CUU } CUC } Leu CUA } CUG }		CCU } CCC } Pro CCA } CCG }		CAU } His CAC } CAA } Gln CAG }		CGU } CGC } Arg CGA } CGG }	U C A G
A	AUU } AUC } Ile AUA } AUG** Met		ACU } ACC } Thr ACA } ACG }		AAU } Asn AAC } AAA } Lys AAG }		AGU } Ser AGC } AGA } Arg AGG }	U C A G
G	GUU } GUC } Val GUA } GUG** }		GCU } GCC } Ala GCA } GCG }		GAU } Asp GAC } GAA } Glu GAG }		GGU } GGC } Gly GGA } GGG }	U C A G

* Chain-terminating, or "nonsense" codons.

** Also used to specify the initiator formyl-Met-tRNA^{Met}.

The Val triplet GUG is therefore "ambiguous" in that it codes both valine and methionine.

It is known that variations in the nucleotide sequence encoding proteins or regulatory regions such as the promoter regions of genes encoding proteins may also affect the concentration of protein produced or the activity of the protein produced. Such variations, including single nucleotide polymorphisms (SNPs) are known to affect, for example, transcription levels of proteins or produce frame shift mutations or encode different amino acids. Such variations may result in the different levels of expression or activity observed. By variant, we also mean the upstream region encoding the promoter or other regulatory region, such as enhancer region, as well as the region encoding the ACE2 protein (including exon and introns).

Preferably, the variant and/or fragment are capable of being specifically bound by an antibody raised against ACE2, but not by an antibody specific for ACE.

The amount, such as concentration or activity of the ACE2, variant or fragment, may be determined as the concentration of the enzyme present, or alternatively by measuring the level of enzymatic activity. Alternatively, or additionally, the concentration of, for example, mRNA encoding the protein may be measured.

The presence of the protein may be detected by use of an antibody against the protein in an immunoassay. Such techniques for the detection of ACE2, *per se*, are known in the art.

Monoclonal or polyclonal antibodies against the protein may be produced using techniques well known in the art, for example using the method of Kohler and Milstein. The antibodies used may be, for example, antibodies of the classes IgG, IgM, IgA, IgD and IgE, binding fragments and hybrid derivatives of antibodies including, for example, Fab and F(ab')₂ fragments of antibodies. Such antibodies preferentially bind to the protein to allow the protein to be identified. Preferably, such antibodies or fragments have less than 10%, preferably less than 5% cross-reactivity with ACE.

Immunoassays use the preferential binding property of the antibodies to allow the identification of the protein. Immunoassay methods known in the art include competition assays, sandwich assays, agglomeration assays, precipitation assays, transistor bridge probe, particle sorting, light disturbing, light scattering and ultrasonic code immunoassays. Such immunoassays may use as labels, for example, radio isotopes, enzymes such as horseradish peroxidase, fluorogenic, chromogenic or chemiluminescent substances. Such assays themselves are well-known *per se* in the art, as indeed shown in, for example, WO 91/16633 and US 5,712,103 incorporated hearing by reference.

Preferably, the sample is selected from maternal blood, foetal blood, maternal urine, maternal faeces, maternal sputum, amniotic fluid and placental material such as a chorionic villus sample.

It is particularly expected to identify the presence of the soluble form (that is the 55 kDa fragment) of ACE2 in urine. Pre-eclampsia patients often have symptoms of renal failure. This results in the walls of the kidney becoming leaky to proteins. Hence, it is expected to identify the 55 kDa fragment within the urine of patients.

Inhibitors of ACE2, such as DX600 (see Warner, *et al. Supra*) may be labeled and used to detect the ACE2. Further generic ligands such as DNA or RNA nucleic acid 'aptamers' produced by the SELEX method to specifically interact with ACE2 would also make good detection agents (e.g. see: Hori T, Taguchi Y, Uesugi S and Kurihara Y. (2005), "The RNA ligands for mouse praline-rich RNA-binding protein (mouse Prpp) contain two consensus sequences in separate loop structure." *Nucleic Acids Res.* 12;33(1), pages 190-200).

Most preferably, the method provides the step of providing a sample of maternal blood or foetal cord blood, or maternal urine.

The measurement of mRNA expression may be determined by techniques well-known in the art, such as real-time PCR.

A further aspect of the invention provides an isolated fragment of ACE2 having an apparent molecular weight of 55 kDa as determined by SDS-PAGE. Preferably, this has one or more functions of ACE2 as defined above. Preferably 12% polyacrylamide gel (29:1 acrylamide to bisacrylamide) is used.

The identification of the new 55 kDa fragment is completely unexpected. It is expected that this fragment may be detected in other conditions or disorders having hypertension or hypotension as a symptom. Hence, the further aspect of the invention provides a method of screening for a disorder having hypertension or hypotension as a symptom, comprising:

- (i) obtaining a sample of bodily fluid or tissue;
- (ii) determining an amount of ACE2 55 kDa fragment present in the sample; and
- (iii) comparing the amount with a normal level of the ACE2 55 kDa fragment.

Preferably, the sample is blood or urine.

The identification that ACE2, a fragment of ACE2 or variants thereof, are markers for the treatment of placental insufficiency, such as pre-eclampsia, makes the proteins a suitable target for following the effect of a compound on the expression of the proteins, and therefore for identifying a drug candidate.

The invention therefore provides a drug candidate for the treatment of placental insufficiency, such as pre-eclampsia comprising:

- (i) providing a cell expressing ACE2, a fragment of ACE2 or a variant thereof;
- (ii) introducing a compound to the cell;

- (iii) determining the effect of the compound on the amount of ACE2, fragment or variant the cell.

The cell may be an isolated cell, a tissue culture cell or alternatively be found within the tissue or body of an animal, such as a mouse or other rodent model. Alternatively, the cell may be in a patient, for example in a patient taking part in a drug trial.

Preferably, the ACE2 fragment has an apparent molecular weight of 55 kDa and is as defined according to the invention.

The invention also provides a method of determining a drug candidate for the treatment of a disorder involving hypertension or hypotension, comprising:

- (i) providing a cell expressing an ACE2 fragment;
- (ii) introducing a compound to the cell;
- (iii) determining the effect of the compound on the ACE2 fragment produced by the cell.

As ACE2 is linked to the regulation of blood pressure, it is expected that ACE2, or fragments or variants, are likely to be suitable for use in the manufacture of medicaments to treat placental insufficiency, such as pre-eclampsia. Preferably, the fragment is a 55 kDa fragment as defined above.

Additionally, the invention provides an ACE2 fragment as defined above, for use in the manufacture of a medicament to treat a hypertensive disorder.

Methods of treating a placental insufficiency and/or a hypertensive disorder, comprising administering a pharmaceutically effective amount of ACE2, or a fragment or variant

thereof, is also provided. Preferably, the fragment is a 55 kDa fragment of ACE2 as defined above.

Assay kits for use in a method according to the invention comprising means for measuring the amount of ACE2, fragment or variant thereof, and instructions for using the assay kit are also provided.

Preferred ACE2 primers are:

(forward) 5' –GATGCCTCCCTGCTCATTG-3' (SEQ ID No. 7) and

(reverse) 5' –AGAACTTCTCGGCCTCCTTG3' (SEQ ID No. 8).

The invention will now be described by way of example only with reference to the following figures:

Figure 1 shows a schematic diagram indicating the interaction between ACE and ACE2 in the regulation of blood pressure.

Figure 2 shows ACE2 Western blot. Lane 1, purified recombinant ACE2 of full length. Lanes 2, 3: placental protein extract at two, fivefold different, loadings. Lanes 4, 6 protein extract of whole foetal chord blood at two, fivefold different, loadings. Lanes 7, 8 protein extract of whole adult blood at two, fivefold different, loadings. Lane 5, size markers. The arrows indicates the control protein (A) present abundantly in lane 1 and less abundantly in lanes 4, 6, 7, 8 and the 55 KDa species (B) present abundantly in lanes 2, 3, 4, 6, 7, 8. The control protein is non-glycosylated commercially available recombinant ACE2 protein.

Figure 3 shows immunocytochemistry localisation of ACE2 in human placenta sections. The ACE2 (blue stain in the original image) appears mainly in the maternal blood circulating in the intervillous spaces. Staining is also evident in the foetal vessel lumens

and endothelium. The erythrocytes appear red indicating that the circulating ACE2 is soluble in the serum rather than associated with cells.

Figure 4 shows a dot blot analysis of serial-diluted urine samples probed with anti-ACE2 antibody. Each row contains three dots of sequentially diluted urine from one sample (from left to right: neat, 1 in 3 dilution, 1 in 9 dilution). Rows are ordered with those displaying the strongest reactivity towards the top and those with no reactivity at any dilution at the bottom. Sample reference numbers are to the right of each row. N: normal subjects, PET: patients with pre-eclampsia, ?: unknown status. The samples derived from pre-eclamptic patients are clustered towards the top indicating an association of pre-eclampsia with anti-ACE2 antibody reactivity.

Mouse monoclonal anti-human ACE2 IgG affinity purified antibody was purchased from Alpha Diagnostic International through autogenbioclear.com. Western blot analysis was performed by standard methods. Briefly, human adult or foetal chord blood or placental extracts were prepared in SDS denaturing buffers and electrophoresed under denaturing conditions before being electro transferred to nitrocellulose membranes along with suitable molecular weight markers. The membranes were treated with 2% dried skimmed milk to block non specific interactions before probing with ACE2 antibody diluted 1:2000. Membranes were washed in buffer containing 0.1% Triton X detergent before the position of the bound antibody were visualised using chemiluminescence with a commercial kit (ECLTM Western Blot Analysis System, Cat RPN2109, Amersham Biosciences, Buckinghamshire, UK).

The same ACE2 antibody was used for immuno histochemical localisation of ACE2 in microscope slide-mounted sections of human placentas. The paraffin embedded specimens were dewaxed and hydrated by successive washes in xylene and decreasing concentrations of ethanol in water. The slides were then reacted with the ACE2 antibody diluted at 1:200 in an appropriate blocking reagents before washes in buffer containing Tween 20 detergent to remove loosely bound antibody. Bound antibody was then

visualised by reaction with an alkaline phosphatase-conjugated anti-mouse secondary antibody followed by colour development using commercially available NBT reagents (Promega corporation USA).

Real-time PCR quantitations of ACE2 mRNA levels were determined from RNA samples made using Trizol reagent (Invitrogen) from flash-frozen fresh placenta biopsies using commercial reagents (Applied Biosystems SYBR green (TM) system). The following primers were used:

(forward) 5' –GATGCCTCCCTGCTCATTTG-3' (SEQ ID No. 7) and

(reverse) 5' –AGAACTTCTCGGCCTCCTTG-3' (SEQ ID No. 8).

Other primers may also be made by the skilled person using the known ACE2 protein sequence as basis.

Dot blot analysis was used to determine the relative amounts of ACE2 in urine of normal subjects and pre-eclamptic patients. Urine samples were 3-fold serially diluted with PBS. The samples were transferred to nitrocellulose membrane using a vacuum manifold. The membranes were treated as per standard dot blot and western blot procedures and then probed with anti-ACE2 antibody (mouse monoclonal anti-human ACE2 IgG affinity purified antibody, as described above). The bound antibody was visualised using chemiluminescence with a commercial kit (ECLTM Western Blot Analysis System, Cat RPN2109, Amersham Biosciences, Buckinghamshire, UK).

The inventors have discovered that ACE2 is present in placental tissue in a truncated soluble form with a molecular weight around 55 kDa compared to a predicted molecular weight of around 105 KDa for the membrane bound native form described in the literature. The evidence comes from western blot analysis (Figure 2) and from immunocytochemistry analysis of placental biopsy (Figure 3). The soluble ACE2 species is also detectable by western blot in adult and foetal (chord) blood. 12% PAGE gels were preferably used (29:1 acrylamide:bisacrylamide)

Studies using explanted normal human placenta biopsies, show that the levels of ACE2 mRNA are increased 5-fold in response to low oxygen conditions compared to normal oxygen conditions (determined by real time PCR). Similar experiments demonstrate that mRNA for ACE is undetectable following exposure to a low oxygen environment indicating a large drop in mRNA levels.

Real time PCR measurements of ACE2 mRNA levels in placental biopsies derived from patients with pre-eclampsia, were found to be increased 1.8-fold compared to the levels in biopsies from normal placentas.

Dot blot analysis shows a good correlation between the presence of ACE2 in urine and pre-eclampsia.

APPENDIX 1 Genbank files for human and mouse ACE2

LOCUS NM_021804 3405 bp mRNA linear PRI 26-OCT-2004
 DEFINITION Homo sapiens angiotensin I converting enzyme (peptidyl-dipeptidase A) 2 (ACE2), mRNA.
 ACCESSION NM_021804
 VERSION NM_021804.1 GI:11225608
 KEYWORDS .
 SOURCE Homo sapiens (human)
 ORGANISM Homo sapiens
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 REFERENCE 1 (bases 1 to 3405)
 AUTHORS Douglas,G.C., O'Bryan,M.K., Hedger,M.P., Lee,D.K., Yarski,M.A., Smith,A.I. and Lew,R.A.
 TITLE The novel angiotensin-converting enzyme (ACE) homolog, ACE2, is selectively expressed by adult Leydig cells of the testis
 JOURNAL Endocrinology 145 (10), 4703-4711 (2004)
 PUBMED 15231706
 REFERENCE 2 (bases 1 to 3405)
 AUTHORS Turner,A.J., Hiscox,J.A. and Hooper,N.M.
 TITLE ACE2: from vasopeptidase to SARS virus receptor
 JOURNAL Trends Pharmacol. Sci. 25 (6), 291-294 (2004)
 PUBMED 15165741
 REMARK GenerIF: ACE2 also serves as the cellular entry point for the severe acute respiratory syndrome virus and the enzyme is therefore a prime target for pharmacological intervention on several disease fronts--REVIEW
 REFERENCE 3 (bases 1 to 3405)
 AUTHORS Towler,P., Staker,B., Prasad,S.G., Menon,S., Tang,J., Parsons,T., Ryan,D., Fisher,M., Williams,D., Dales,N.A., Patane,M.A. and Pantoliano,M.W.
 TITLE ACE2 X-ray structures reveal a large hinge-bending motion important for inhibitor binding and catalysis
 JOURNAL J. Biol. Chem. 279 (17), 17996-18007 (2004)
 PUBMED 14754895
 REMARK GenerIF: large hinge-bending motion is important for inhibitor binding and catalysis in ACE2
 REFERENCE 4 (bases 1 to 3405)
 AUTHORS Wong,S.K., Li,W., Moore,M.J., Choe,H. and Farzan,M.
 TITLE A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2
 JOURNAL J. Biol. Chem. 279 (5), 3197-3201 (2004)
 PUBMED 14670965
 REMARK GenerIF: ACE2 binds to a 193-amino acid fragment of the SARS coronavirus S protein
 REFERENCE 5 (bases 1 to 3405)
 AUTHORS Li,W., Moore,M.J., Vasilieva,N., Sui,J., Wong,S.K., Berne,M.A., Somasundaran,M., Sullivan,J.L., Luzuriaga,K., Greenough,T.C., Choe,H. and Farzan,M.
 TITLE Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus
 JOURNAL Nature 426 (6965), 450-454 (2003)
 PUBMED 14647384
 REMARK GenerIF: ACE2 is a functional receptor for the coronavirus that causes severe acute respiratory syndrome (SARS-CoV).
 GenerIF: a metallopeptidase, angiotensin-converting enzyme 2 (ACE2), isolated from SARS coronavirus (SARS-CoV)-permissive Vero E6 cells, that efficiently binds the S1 domain of the SARS-CoV S protein; ACE2 is a functional receptor for SARS-CoV
 REFERENCE 6 (bases 1 to 3405)

- AUTHORS Clark,H.F., Gurney,A.L., Abaya,E., Baker,K., Baldwin,D., Brush,J., Chen,J., Chow,B., Chui,C., Crowley,C., Currell,B., Deuel,B., Dowd,P., Eaton,D., Foster,J., Grimaldi,C., Gu,Q., Hass,P.E., Heldens,S., Huang,A., Kim,H.S., Klimowski,L., Jin,Y., Johnson,S., Lee,J., Lewis,L., Liao,D., Mark,M., Robbie,E., Sanchez,C., Schoenfeld,J., Seshagiri,S., Simmons,L., Singh,J., Smith,V., Stinson,J., Vagts,A., Vandlen,R., Watanabe,C., Wieand,D., Woods,K., Xie,M.H., Yansura,D., Yi,S., Yu,G., Yuan,J., Zhang,M., Zhang,Z., Goddard,A., Wood,W.I., Godowski,P. and Gray,A.
- TITLE The secreted protein discovery initiative (SPDI), a large-scale effort to identify novel human secreted and transmembrane proteins: a bioinformatics assessment
- JOURNAL Genome Res. 13 (10), 2265-2270 (2003)
- PUBMED 12975309
- REFERENCE 7 (bases 1 to 3405)
- AUTHORS Donoghue,M., Wakimoto,H., Maguire,C.T., Acton,S., Hales,P., Stagliano,N., Fairchild-Huntress,V., Xu,J., Lorenz,J.N., Kadambi,V., Berul,C.I. and Breitbart,R.E.
- TITLE Heart block, ventricular tachycardia, and sudden death in ACE2 transgenic mice with downregulated connexins
- JOURNAL J. Mol. Cell. Cardiol. 35 (9), 1043-1053 (2003)
- PUBMED 12967627
- REMARK GenerIF: We tested the hypothesis that cardiac ACE2 activity contributes to features of ventricular remodeling associated with the renin-angiotensin system
- REFERENCE 8 (bases 1 to 3405)
- AUTHORS Harmer,D., Gilbert,M., Borman,R. and Clark,K.L.
- TITLE Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme
- JOURNAL FEBS Lett. 532 (1-2), 107-110 (2002)
- PUBMED 12459472
- REMARK GenerIF: tissue distribution of ACE 2 mRNA by RT-PCR
- REFERENCE 9 (bases 1 to 3405)
- AUTHORS Crackower,M.A., Sarao,R., Oudit,G.Y., Yagil,C., Kozieradzki,I., Scanga,S.E., Oliveira-dos-Santos,A.J., da Costa,J., Zhang,L., Pei,Y., Scholey,J., Ferrario,C.M., Manoukian,A.S., Chappell,M.C., Backx,P.H., Yagil,Y. and Penninger,J.M.
- TITLE Angiotensin-converting enzyme 2 is an essential regulator of heart function
- JOURNAL Nature 417 (6891), 822-828 (2002)
- PUBMED 12075344
- REFERENCE 10 (bases 1 to 3405)
- AUTHORS Vickers,C., Hales,P., Kaushik,V., Dick,L., Gavin,J., Tang,J., Godbout,K., Parsons,T., Baronas,E., Hsieh,F., Acton,S., Patane,M., Nichols,A. and Tummino,P.
- TITLE Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase
- JOURNAL J. Biol. Chem. 277 (17), 14838-14843 (2002)
- PUBMED 11815627
- REMARK GenerIF: role in hydrolyzing biological peptides
- REFERENCE 11 (bases 1 to 3405)
- AUTHORS Tipnis,S.R., Hooper,N.M., Hyde,R., Karran,E., Christie,G. and Turner,A.J.
- TITLE A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase
- JOURNAL J. Biol. Chem. 275 (43), 33238-33243 (2000)
- PUBMED 10924499
- REFERENCE 12 (bases 1 to 3405)
- AUTHORS Donoghue,M., Hsieh,F., Baronas,E., Godbout,K., Gosselin,M., Stagliano,N., Donovan,M., Woolf,B., Robison,K., Jeyaseelan,R., Breitbart,R.E. and Acton,S.
- TITLE A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9

JOURNAL Circ. Res. 87 (5), E1-E9 (2000)
 PUBMED 10969042
 COMMENT REVIEWED REFSEQ: This record has been curated by NCBI staff. The reference sequence was derived from AF241254.1.

Summary: The protein encoded by this gene belongs to the angiotensin-converting enzyme family of dipeptidyl carboxydipeptidases and has considerable homology to human angiotensin 1 converting enzyme. This protein catalyzes the cleavage of angiotensin I into angiotensin 1-9. The organ- and cell-specific expression of this gene suggests that it may play a role in the regulation of cardiovascular and renal function, as well as fertility.

COMPLETENESS: complete on the 3' end.

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 Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
 REFERENCE 1
 AUTHORS Komatsu,T., Suzuki,Y., Imai,J., Sugano,S., Hida,M., Tanigami,A., Muroi,S., Yamada,Y. and Hanaoka,K.
 TITLE Molecular cloning, mRNA expression and chromosomal localization of mouse angiotensin-converting enzyme-related carboxypeptidase (mACE2)
 JOURNAL DNA Seq. 13 (4), 217-220 (2002)
 PUBMED 12487024
 REMARK GeneRIF: ACE2 gene is expressed mainly in the kidney and the lungs.
 REFERENCE 2 (bases 1 to 2760)
 AUTHORS Crackower,M.A., Sarao,R., Oudit,G.Y., Yagil,C., Kozieradzki,I., Scanga,S.E., Oliveira-dos-Santos,A.J., da Costa,J., Zhang,L., Pei,Y., Scholey,J., Ferrario,C.M., Manoukian,A.S., Chappell,M.C., Backx,P.H., Yagil,Y. and Penninger,J.M.
 TITLE Angiotensin-converting enzyme 2 is an essential regulator of heart function
 JOURNAL Nature 417 (6891), 822-828 (2002)
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 COMMENT PROVISIONAL REFSEQ: This record has not yet been subject to final NCBI review. The reference sequence was derived from AB053181.1. On Dec 18, 2003 this sequence version replaced gi:38086963.
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JOURNAL Unpublished
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AUTHORS Komatsu,T., Suzuki,Y. and Sugano,S.
TITLE Direct Submission
JOURNAL Submitted (25-JUL-2000) Takami Komatsu, the Institute of Medical
Science, Virology; 4-6-1, Minato-ku, Tokyo 108-8639, Japan
(E-mail:komatsu@ims.u-tokyo.ac.jp, Tel:81-3-5449-5283 (ex.75283),
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Claims

1. A method for screening for placental insufficiency, such as pre-eclampsia in a pregnant mammal such as a human or mouse comprising the steps of:
 - (i) providing a sample of maternal or foetal bodily fluid or tissue;
 - (ii) measuring the amount of Angiotensin-Converting Enzyme (ACE2) or a fragment or a variant thereof, for example directly by detecting the protein, or indirectly by measuring the amount of mRNA encoding the ACE2, fragment or variant thereof in the sample; and
 - (iii) comparing the amount with a known normal level of ACE2 or a fragment of variant thereof.
2. A method according to claim 1, wherein the ACE2 fragment has an apparent molecular weight of approximately 55 kDa as determined by SDS-PAGE.
3. A method according to claims 1 or 2, wherein the sample is selected from maternal blood, foetal blood, maternal urine, maternal faeces, maternal sputum, amniotic fluid and placental material such as a chorionic villus sample.
4. A method according to claim 2, comprising the steps of providing a sample of maternal blood or foetal chord blood or maternal urine and measuring the amount of 55 kDa ACE2 fragment present.
5. A method according to any preceding claim, wherein the amount of ACE2, fragment or variant thereof is determined by an immunoassay, enzymatic assay or real-time PCR.
6. An isolated fragment of ACE2 having an apparent molecular weight of 55 kDa as determined by SDS-PAGE.

7. A method of screening for a disorder having hypertension or hypotension as a symptom, comprising:

- (i) obtaining a sample of bodily fluid or tissue;
- (ii) determining an amount of ACE2 55 kDa fragment, as defined in claim 6, present in the sample; and
- (iii) comparing the amount with a normal level of the ACE2 55 kDa fragment

8. A method according to claim 7, wherein the sample is blood or urine.

9. A method of determining a drug candidate for the treatment of placental insufficiency, such as pre-eclampsia comprising:

- (i) providing a cell expressing ACE2, a fragment of ACE2 or a variant thereof;
- (ii) introducing a compound to the cell;
- (iii) determining the effect of the compound on the amount of ACE2, fragment or variant produced by the cell.

10. A method according to claim 9, wherein the ACE2 fragment has an apparent molecular weight of 55 kDa, as defined in claim 6.

11. A method of determining a drug candidate for the treatment of a disorder involving hypertension or hypotension, comprising:

- (i) providing a cell expressing an ACE2 fragment according to claim 6;

- (ii) introducing a compound to the cell;
 - (iii) determining the effect of the compound on the amount of ACE2 fragment produced by the cell.
12. ACE2, or a fragment or a variant thereof, for use in the manufacture of a medicament to treat a placental insufficiency, such as pre-eclampsia.
13. An ACE2 fragment as defined in claim 6 for use in a manufacture of a medicament to treat a hypertensive disorder.
14. A method of treating a placental insufficiency such as pre-eclampsia comprising administering a pharmaceutically effective amount of ACE2, or a fragment or a variant thereof.
15. A method of treating a hypertensive disorder comprising administering a pharmaceutically effective amount of a 55 kDa fragment of ACE2 as defined in claim 6.
16. An assay kit for use in a method according to claims 1 to 11 comprising means for measuring the amount of ACE2, fragment or variant thereof and instructions for using the assay kit.

1 / 4

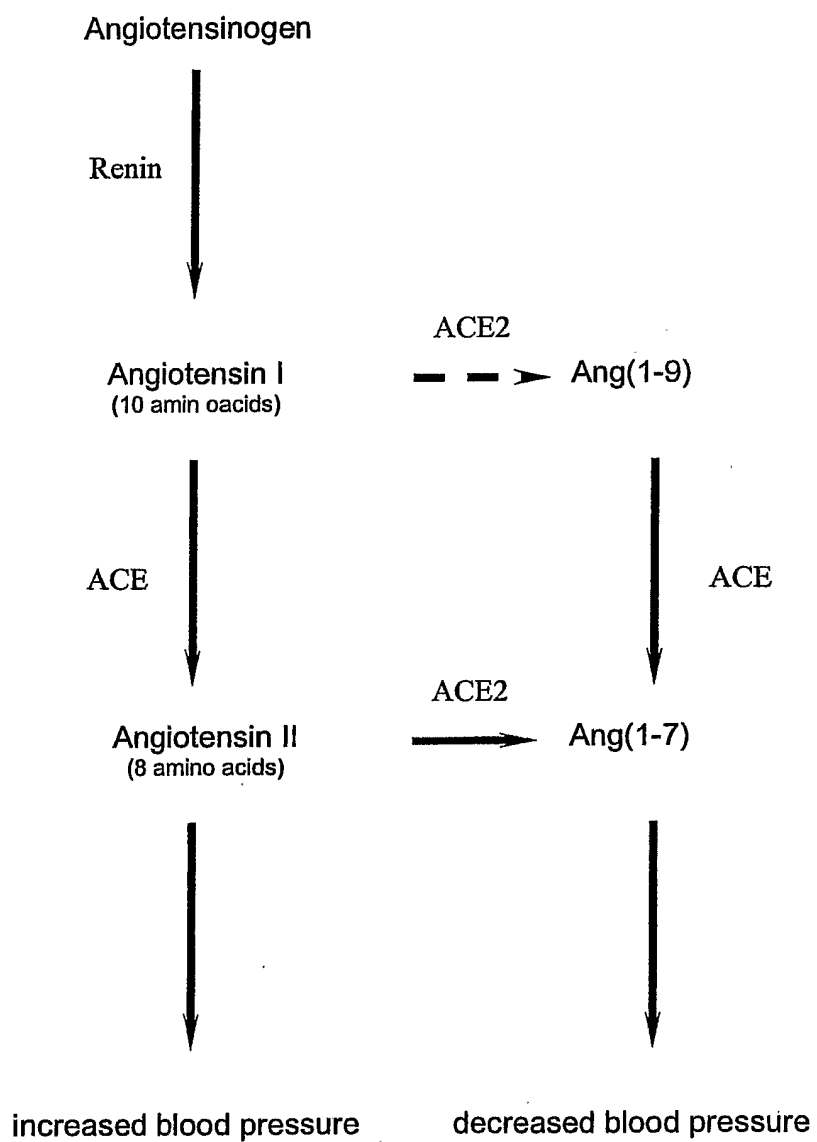


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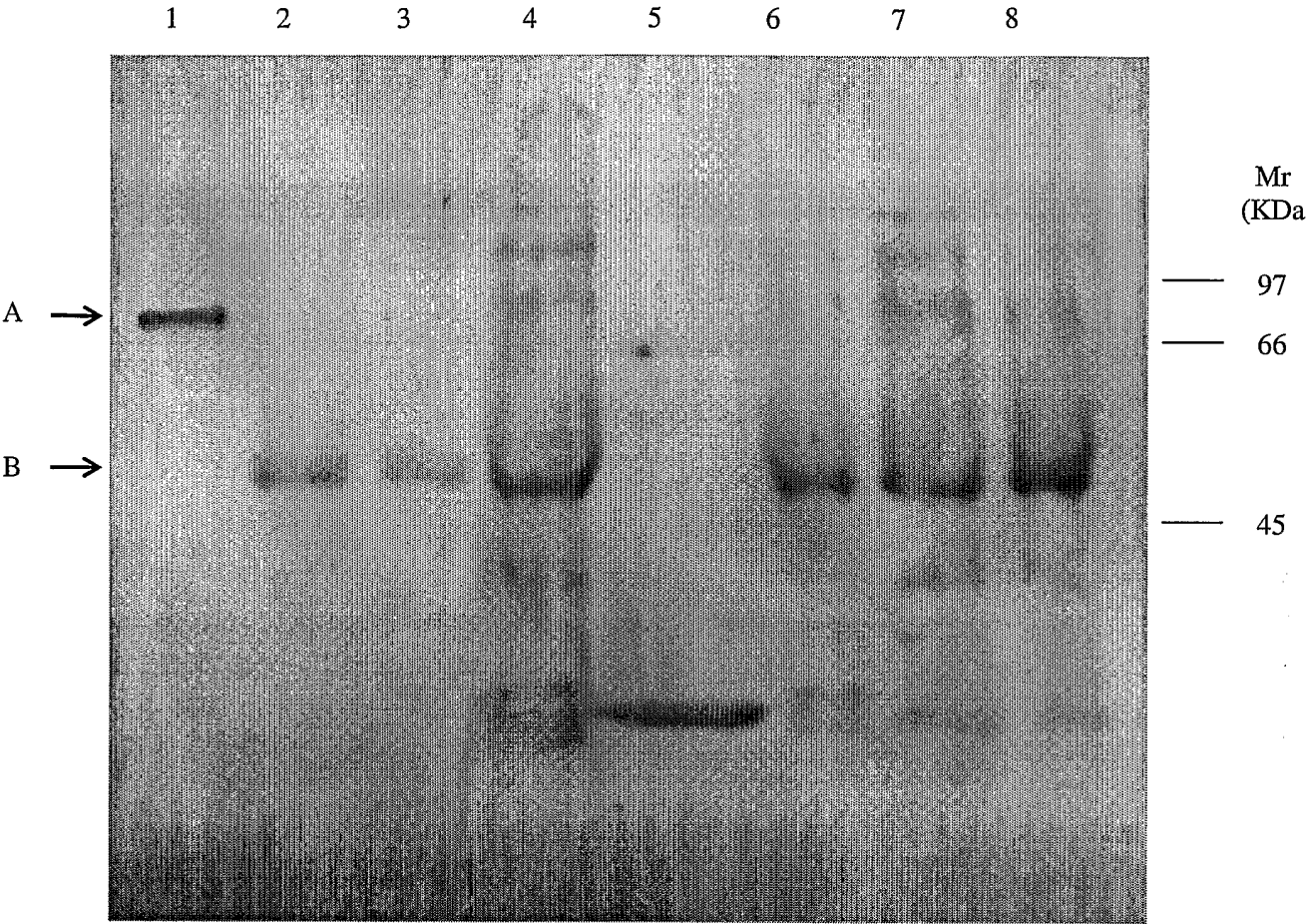


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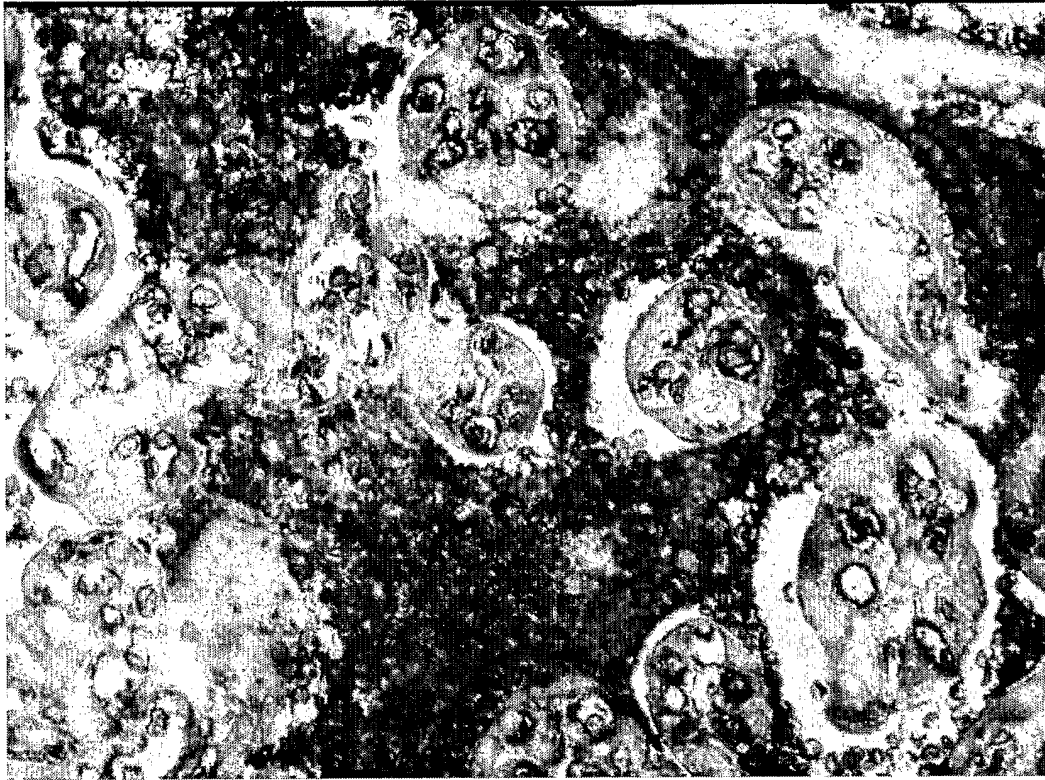


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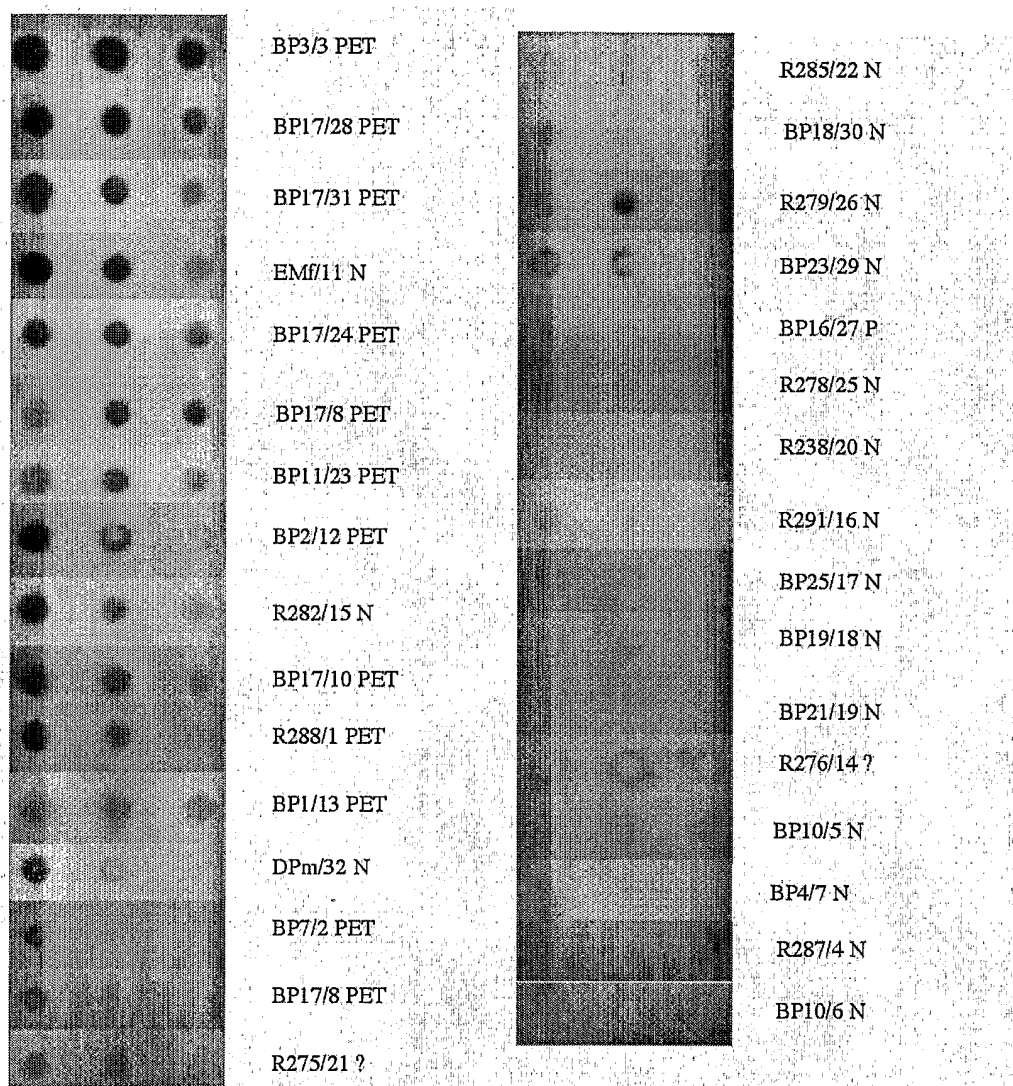


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His Ala Tyr Val Arg Arg Lys Leu Met Asp Thr Tyr Pro Ser Tyr Ile
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Ser Pro Thr Gly Cys Leu Pro Ala His Leu Leu Gly Asp Met Trp Gly
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Arg Phe Trp Thr Asn Leu Tyr Pro Leu Thr Val Pro Phe Ala Gln Lys
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Pro Asn Ile Asp Val Thr Asp Ala Met Met Asn Gln Gly Trp Asp Ala
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Glu Trp Ser Pro Tyr Ala Asp Gln Ser Ile Lys Val Arg Ile Ser Leu
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Lys Ser Ala Leu Gly Ala Asn Ala Tyr Glu Trp Thr Asn Asn Glu Met

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Pro	Pro	Val	Thr 740	Ile	Trp	Leu	Ile	Ile 745	Phe	Gly	Val	Val	Met 750	Ala	Leu
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Lys	Lys 770	Lys	Asn	Glu	Thr	Lys 775	Arg	Glu	Glu	Ser	Pro 780	Tyr	Asp	Ser	Thr
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Asn Tyr Asn Thr Asn Ile Thr Glu Glu Asn Val Gln Asn Met Asn Asn
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Ala Gly Asp Lys Trp Ser Ala Phe Leu Lys Glu Gln Ser Thr Leu Ala
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Gln Met Tyr Pro Leu Gln Glu Ile Gln Asn Leu Thr Val Lys Leu Gln
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Leu Gln Ala Leu Gln Gln Asn Gly Ser Ser Val Leu Ser Glu Asp Lys
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Ser Lys Arg Leu Asn Thr Ile Leu Asn Thr Met Ser Thr Ile Tyr Ser
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Thr Gly Lys Val Cys Asn Pro Asp Asn Pro Gln Glu Cys Leu Leu Leu
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Glu Pro Gly Leu Asn Glu Ile Met Ala Asn Ser Leu Asp Tyr Asn Glu
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Arg Leu Trp Ala Trp Glu Ser Trp Arg Ser Glu Val Gly Lys Gln Leu
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Arg Pro Leu Tyr Glu Glu Tyr Val Val Leu Lys Asn Glu Met Ala Arg
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Val Asn Gly Val Asp Gly Tyr Asp Tyr Ser Arg Gly Gln Leu Ile Glu
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His Ala Tyr Val Arg Ala Lys Leu Met Asn Ala Tyr Pro Ser Tyr Ile
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Ser Pro Ile Gly Cys Leu Pro Ala His Leu Leu Gly Asp Met Trp Gly
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Arg Phe Trp Thr Asn Leu Tyr Ser Leu Thr Val Pro Phe Gly Gln Lys
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Pro Asn Ile Asp Val Thr Asp Ala Met Val Asp Gln Ala Trp Asp Ala
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Pro Asn Met Thr Gln Gly Phe Trp Glu Asn Ser Met Leu Thr Asp Pro
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Gly Asn Val Gln Lys Ala Val Cys His Pro Thr Ala Trp Asp Leu Gly
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Lys Gly Asp Phe Arg Ile Leu Met Cys Thr Lys Val Thr Met Asp Asp
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Tyr Ala Ala Gln Pro Phe Leu Leu Arg Asn Gly Ala Asn Glu Gly Phe
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His Leu Lys Ser Ile Gly Leu Leu Ser Pro Asp Phe Gln Glu Asp Asn
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Glu Thr Glu Ile Asn Phe Leu Leu Lys Gln Ala Leu Thr Ile Val Gly
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775

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